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**THE EFFECTS OF SELECTIVE INHIBITORS OF N-GLYCOSYLATION AND  
ENDOPLASMIC RETICULUM STRESS INDUCERS ON NEUROBLASTOMA  
CELL PROLIFERATION AND APOPTOSIS**

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## DECLARATION

I, **Ebtehal Elkamel M. Eshiak**, declare that “**The Effects of Selective Inhibitors of N-Glycosylation and Endoplasmic Reticulum Stress Inducers on Neuroblastoma Cell Proliferation and Apoptosis**” is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.

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# DEDICATION

*This study is dedicated to my family.*



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## ABSTRACT

Among all childhood cancers diagnosed, neuroblastoma (NB) is the most prevalent during infancy. In patients younger than 15 years, it accounts for more than 7% of malignancies and about 15% of all paediatric mortalities. A family history of NB is considered a genetic predisposition and risk factor. In 1-2% of inherited cases, the molecular causative factors linked to the disease are germline mutations, including anaplastic lymphoma kinase (*ALK*, gain of function), *PHOX2B* (loss of function) and *MYCN* (gain of function). NB differs from most solid tumours because of its heterogeneity in both pathobiologic and clinical behaviour, ranging from spontaneous regression to highly-aggressive metastatic disease resistant to conventional and investigational anticancer drugs. Hence, NB embodies a spectrum of disease.

Moreover, disease progression (uncontrolled cellular proliferation), a recognizable signature of NB, may be set off by inadequate targeting of resistant subpopulations (e.g., side-population cells or cancer stem cells/tumour-initiating cells) in the tumour microenvironment (TME). The complex molecular heterogeneity responsible for the disparate responses to treatment of NB provides a platform for discovering molecules and pathways for specific biologically-targeted interventions. In spite of contemporary improvements in intensive multimodal therapeutic approaches to NB (surgery, local radiation and differentiation therapy with 13-*cis*-retinoic acid, and myeloablative cytotoxic therapy with autologous haematopoietic stem cell transplantation), the overall survival of high risk patients has persistently been less than 40%.

Since NB is a heterogeneous paediatric tumour that arises from the embryonic neural crest, it is not surprising that cancer stem cells (CSCs) or tumour initiating cells (TICs) have been implicated in tumour relapse and resistance to chemotherapy in high-risk neuroblastoma (HR-NB) patients. In 40-50% of the HR-NB cases, amplification of the *MYCN* gene remains the major key predictor of poor clinical outcomes. HR-NB patients with *MYCN* amplification generally show the development of resistance to induction chemotherapy. Thus, novel effective

therapies are urgently needed to improve clinical outcomes of HR-NB patients. Remarkably, coordinated expression of the *MYC* gene and those that encode for the ABC multidrug resistance transporters has been confirmed in a larger number of NB patient samples, experimental NB models and NB cell lines, but more work is needed to clarify this poorly understood prosurvival signature. Earlier work has found that high levels of ABCB1 (P-glycoprotein/P-gp/MDR1) RNA are frequently associated with resistance to chemotherapy in NB. Recent studies have unequivocally provided evidence that *MYCN* enhances *P-gp/MDR1* gene expression in the human metastatic NB IGR-N-91 model.

CSCs in the heterogeneous NB TME exhibit high resistance to standard therapy, a cancer hallmark that correlates with overexpression of some members of the ABC family of MDR transporters, increased expression of anti-apoptotic molecules, increased DNA repair capacity and activation of specific stem cell survival signals. In recent years, the NB glycome has emerged as an important research paradigm that aims to show the significant impact of NB glycans in the TME on tumour progression, MDR and patient clinical responses to anticancer therapy. The role of protein N-glycosylation in neural transmission is widely recognized, but less well understood. Glycosylation is the multifaceted post-translational modification of proteins that takes place in the endoplasmic reticulum (ER) and offers a landscape for developing novel targeted approaches to CSC eradication and anticancer chemotherapy.

Altered glycans on tumour- and host-cell surfaces and constituents of the extracellular matrix in the TME mediate key events in cancer pathogenesis, progression and survival. Glycan-based therapeutic rationales have been applied to cancers of the liver, pancreas, breast, lung, gastrointestinal system, melanomas, and lymphomas, but rarely to NBs. The success of anti-disialoganglioside (GD2, a glycolipid antigen) antibodies support glycan-based therapies for NB and also implies a proof-of-concept for protein glycosylation-based therapies for NB. N-glycosylation determines protein conformation, stability and function, degradation of misfolded proteins, programmed cell death (apoptosis), cell survival and immunity.

Any disruption of this function of the ER to coordinate and maintain proper protein folding can lead to the ER stress (ERS) response. Prolonged ERS triggers the unfolded response (UPR) and eventually apoptosis. However, cancer cells and their prosurvival TME usually adapt through various mechanisms, including epithelial-mesenchymal transition (EMT), to evade ERS-induced apoptosis, and so continue to proliferate disproportionately, leading to relapse. P-gp and many other ABC drug transporters are associated with resistance to chemotherapy in NB. P-gp protects drug-resistant tumour cells from multiple forms of caspase-dependent apoptosis. As a glycoprotein, P-gp is also subject to protein N-glycosylation. It has been reported that inhibition of N-linked glycosylation of P-gp results in a reduced MDR phenotype. Inhibitors of N-glycosylation have been widely used to delineate the significance of glycosylation in MDR and the malignant phenotype.

Targeting of the ERS and UPR has been the subject of intense investigation in recent years. A fundamental tenet of the present study is that, transcriptionally, MYCN (a hallmark of NB) directly regulates the ABC transporters, and their upregulation correlates with poor prognosis. Most aggressive NBs exhibit MDR, attributable to p53 mutations and/or a loss of function induced during chemotherapy, which promote relapse. p53 is a stress protein activated in response to various stresses and, in different contexts, the activation of p53 can either keep cells alive (the survival pathway) or kill the cells (the death pathway). SK-N-BE(2) NB cells, selected for this study, also overexpress P-gp.

In the current study, selected glycosylation inhibitors and ERS inducers—tunicamycin (an antibiotic that blocks the rate-limiting step of protein N-glycosylation and induces ERS and apoptosis in cells), deoxynojirimycin (inhibits Golgi mannosidases and thus maturation of glycoproteins, causing ERS), celecoxib (a potent cyclooxygenase 2 inhibitor, known to impede sarcoplasmic/endoplasmic reticulum calcium ATPase/SERCA) and so triggering relentless  $\text{Ca}^{2+}$  dysregulation that can induce ERS-mediated apoptosis), swainsonine (a glycosidase inhibitor resulting in the accumulation of glycoproteins, hence causing ERS and initiating the

UPR and apoptosis),  $\beta$ -D-xyloside (disrupts glycoprotein assembly and reversibly inhibits cell proliferation by arresting cells in the G<sub>1</sub> phase of the cell cycle)—were tested for their potential to inhibit P-gp-mediated drug transport function and drug resistance as well as their efficacy to induce apoptosis in the SK-N-BE(2) NB cell line. This study focused on cell proliferation, viability, apoptosis and drug retention measured by inhibition of P-gp function. It was hypothesized that N-glycosylation inhibitors and ERS inducers will alter the expression of SK-N-BE(2) cancer cell signatures evaluated, namely, cell survival (proliferation, viability and apoptosis) and P-gp-mediated drug efflux function.

Based on the results of this study it can be accepted that, indeed, these investigative compounds altered the proliferation, viability, apoptosis and P-gp function in SK-N-BE(2) NB cells, but the conflicting and duality of effects imply that additional refinements are necessary in the conceptualization and execution of future studies. In the context of work that had already been done in this field, the present study adds to the existing body of knowledge and the dire need to unravel the mechanisms of MDR, including the role of N-glycosylation in P-gp-mediated drug efflux function in NB. Moreover, it emerged that a nobler perception of NB glycomics and the potential of glycosylation inhibitors may yield impressive translational therapeutic promise.

**Keywords:** childhood cancer, neuroblastoma, inhibitors of N-glycosylation, endoplasmic reticulum stress inducers, neuroblastoma drug resistance, cytotoxicity, P-glycoprotein-mediated drug efflux

## LIST OF ABBREVIATIONS

<b>ABMT</b>	Autologous Bone Marrow Transplantation
<b>ABC</b>	ATP-Binding Cassette
<b>ADCC</b>	Antibody Mediated Cell Cytotoxicity
<b>ALK</b>	Anaplastic Lymphoma Kinase
<b>AnnCy3</b>	Annexin-Cy3
<b>ANOVA</b>	Analysis of Variance
<b>ANS</b>	Autonomic Nervous System
<b>ASCT</b>	Autologous Stem-Cell Transplantation
<b>ATCC</b>	American Type Culture Collection
<b>ATRA</b>	All-Trans Retinoic Acid
<b>AURKA</b>	Aurora A Kinase
<b>BAC</b>	Bacitracin
<b>BDNF</b>	Brain-Derived Neurotropic Factor
<b>BFA</b>	Brefeldin A
<b>BMP</b>	Bone Morphogenetic Protein
<b>BMT</b>	Bone Marrow Transplantation
<b>BFA</b>	Brefeldin A
<b>Calcein-AM</b>	Calcein–Acetoxymethylester
<b>CAMs</b>	Cell Adhesion Molecules
<b>CBC</b>	Complete Blood Count
<b>CCG</b>	Children’s Cancer Group
<b>CCK-8</b>	Cell Counting Kit-8
<b>CD102</b>	Cluster of Differentiation 102
<b>6-CF</b>	6-Carboxyfluorescein
<b>6-CFDA</b>	6-Carboxyfluorescein Diacetate
<b>95%CI</b>	95% Confidence Interval
<b>COG</b>	Children’s Oncology Group
<b>COX</b>	Cyclooxygenase
<b>CRA</b>	13-Cis-Retinoic Acid (Isotretinoin)

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<b>CSCs</b>	Cancer Stem Cells
<b>CST</b>	Castanospermine
<b>CT</b>	Computed Tomography
<b>DBH</b>	Dopamine Beta-Hydroxylase Promoter
<b>DEVD</b>	Aspartic Acid, Glutamic Acid, Valine, Aspartic Acid
<b>2dGlc/2-DG</b>	2-Deoxyglucose
<b>DLTs</b>	Dose-Limiting Toxicities
<b>DMEM</b>	Dulbecco's Modified Eagle Medium
<b>DMSO</b>	Dimethylsulfoxide
<b>DNJ/DMJ</b>	Deoxynojirimycin/Deoxymannojirimycin
<b>Dol-PP</b>	Dolichol Pyrophosphate
<b>DON</b>	6-Diazo-5-Oxo-L-Norleucine
<b>GD2</b>	Diganglioside (Disialoganglioside)
<b>EC</b>	Epidural Compression
<b>ECM</b>	Extracellular Matrix
<b>EFS</b>	Event-Free Survival
<b>EMT</b>	Epithelial-to-Mesenchymal Transition
<b>ER</b>	Endoplasmic Reticulum
<b>ERAD</b>	Endoplasmic Reticulum-Associated Degradation
<b>ERQC</b>	Endoplasmic Reticulum Protein Quality Control System
<b>ERS</b>	Endoplasmic Reticulum Stress
<b>ERSAs</b>	Endoplasmic Reticulum Stress Aggravators
<b>FDA or USFDA</b>	Food and Drug Administration of the USA
<b>FDG</b>	18-Fluorodeoxy-Glucose (18F-FDG)
<b>FDG-PET</b>	Fluorodeoxyglucose Positron Emission Tomography
<b>FGF</b>	Fibroblast Growth Factor
<b>FISH</b>	Fluorescence In Situ Hybridization
<b>FKBP12</b>	FK-Binding Protein 12
<b>GAGs</b>	Glycosaminoglycans
<b>GATA</b>	GATA transcription factors are a family of transcription factors characterized by their ability to bind to the DNA sequence "GATA".
<b>GBPs</b>	Glycan-Binding Proteins

<b>GM-CSF</b>	Granulocyte Macrophage Colony-Stimulating Factor
<b>GN</b>	Ganglioneuroma
<b>GPI</b>	Glycosylphosphatidylinositol
<b>GRP/ GRPR</b>	Gastrin-Releasing Peptide/Gastrin-Releasing Peptide Receptor
<b>HAND2</b>	Heart- and Neural Crest Derivatives-Expressed Protein
<b>hESCs</b>	Human Embryonic Stem Cells
<b>HIF</b>	Hypoxia-Inducing Factor
<b>HIFBS</b>	Heat-Inactivated Foetal Bovine Serum
<b>HPR/4-HPR</b>	N-(4-Hydroxyphenyl) Retinamide
<b>HR-NB</b>	High-Risk Neuroblastoma
<b>HSCR</b>	Hirschsprung's Disease
<b>HSCT</b>	Haematopoietic Stem Cell Transplantation
<b>HSR</b>	Homogeneously Staining Region(s)
<b>hNCSCs</b>	Human Neural Crest Stem Cells
<b>HVA</b>	Homovanillic Acid
<b>IC<sub>50</sub></b>	The Half Maximal Inhibitory Concentration of a Drug
<b>ICAM-2</b>	Intercellular Adhesion Molecule-2
<b>IDRFs</b>	Image-Defined Risk Factors
<b>IGF2BP1</b>	Insulin-Like Growth Factor-2 Mrna-Binding Protein 1
<b>IMT</b>	Inflammatory Myofibroblastic Tumour
<b>INRG</b>	International Neuroblastoma Risk Group
<b>INRGSS</b>	International Neuroblastoma Risk Group Staging System
<b>INSS</b>	International Neuroblastoma Staging System
<b>INPC</b>	International Neuroblastoma Pathology Classification
<b>IUPAC</b>	International Union of Pure and Applied Chemistry
<b>IV</b>	Intravenous
<b>JNK</b>	c-Jun Amino N-Terminal Kinase
<b>LNN</b>	Large Nucleolar Neuroblastoma
<b>LOH</b>	Loss of Heterozygosity
<b>67LR</b>	67-kDa Laminin Receptor
<b>mAbs</b>	Monoclonal Antibodies
<b>MAPK</b>	Mitogen-Activated Protein Kinase



<b>MAT</b>	Myeloablative Therapy
<b>MASH1</b>	Mammalian Achaete Scute Homologue-1
<b>MDM2</b>	Mouse Double Minute 2 Homologue
<b>MDR</b>	Multidrug Resistance
<b>MEM</b>	Modified Eagles Medium
<b>MIBG</b>	Meta-Iodobenzylguanidine
<b>MKI</b>	Mitosis-Karyorrhexis Index
<b>MMPs</b>	Matrix Metalloproteases
<b>MRD</b>	Minimal Residual Disease
<b>MRI</b>	Magnetic Resonance Imaging
<b>MRP1</b>	Multidrug Resistance-Associated Protein-1
<b>MTDs</b>	Maximal-Tolerated Doses
<b>mTOR</b>	Mammalian Target of Rapamycin
<b>MSCs</b>	Mesenchymal Stromal Cells
<b>MYCL</b>	Myelocytomatosis Viral Oncogene
<b>MYCN</b>	The <i>v-myc</i> avian myelocytomatosis viral oncogene neuroblastoma-derived homolog. <i>MYCN</i> remains the best-characterized genetic marker of risk in neuroblastoma.
<b>NB(s)</b>	Neuroblastoma(s)
<b>NC</b>	Neural Crest
<b>NCAM</b>	Neural Cell Adhesion Molecule
<b>NGF</b>	Nerve Growth Factor
<b>NKT</b>	Natural Killer T Cells
<b>NMP</b>	Nucleophosmin
<b>NTRK</b>	Neurotrophic Tyrosine Receptor Kinase(S)
<b>NSAIDs</b>	Nonsteroidal Anti-Inflammatory Drugs
<b>NSCLC</b>	Non-Small-Cell Lung Carcinoma
<b>NT3</b>	Neurotrophin-3 Growth Factor
<b>ODC1</b>	Ornithine Decarboxylase 1
<b>OMS</b>	Opsoclonus-Myoclonus Syndrome
<b>OS</b>	Overall Survival
<b>PAH</b>	Pulmonary Arterial Hypertension
<b>PBS</b>	Phosphate Buffered Saline



<b>PBSCT</b>	Peripheral Blood Stem Cell Transplantation
<b>PCD</b>	Programmed Cell Death
<b>PET</b>	Positron Emission Tomography
<b>PFS</b>	Progression-Free Survival
<b>PI3K</b>	Phosphoinositide-3-Kinase
<b>Pgp</b>	P-glycoprotein
<b>PHOX2B</b>	Paired-Like Homeobox 2b
<b>PLC</b>	Phospholipid C
<b>pNTs</b>	Peripheral Neuroblastic Tumours
<b>POG</b>	Paediatric Oncology Group
<b>PPM1D</b>	Protein Phosphatase Magnesium-Dependent 1 Delta
<b>PS</b>	Phosphatidylserine
<b>PSA</b>	Polysialic Acid
<b>PTMs</b>	Post-Translational Modifications
<b>RA</b>	Retinoic Acid
<b>RARs/RXRs</b>	Retinoic Acid Receptors/Retinoic Acid X (Rexinoid) Receptors
<b>RAREs</b>	Retinoic Acid Response Elements
<b>RH</b>	Relative Humidity
<b>RTK</b>	Receptor Tyrosine Kinase(s)
<b>SEER</b>	Surveillance, Epidemiology, and End Results Programme
<b>SERCA</b>	Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase
<b>SNS</b>	Sympathetic Nervous System
<b>SPECT</b>	Single-Photon Emission Computed Tomography
<b>SWSN</b>	Swainsonine
<b>RET</b>	Rearranged During Transfection
<b>TAFs</b>	Tumour-Associated Fibroblasts
<b>TAMs</b>	Tumour-Associated Macrophages
<b>TGF<math>\beta</math></b>	Transforming Growth Factor Beta
<b>TH</b>	Tyrosine Hydroxylase
<b>TM</b>	Tunicamycin
<b>TME</b>	Tumour Microenvironment
<b>TICs</b>	Tumour-Initiating Cells

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<b>TRK/Trk</b>	Tyrosine Receptor Kinase
<b>TSG(s)</b>	Tumour Suppressor Gene(s)
<b>UPR</b>	Unfolded Protein Response
<b>VEGF</b>	Vascular Endothelial Growth Factor
<b>VIP</b>	Vasoactive Intestinal Peptide
<b>VMA</b>	Vanillylmandelic Acid
<b>Wnt</b>	Wingless/Integrated Proto-Oncogene
<b><math>\beta</math>-D-Xyl</b>	$\beta$ -D-Xyloside



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# CONTENTS

<b>DECLARATION</b> .....	<b>ii</b>
<b>DEDICATION</b> .....	<b>iii</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>iv</b>
<b>ABSTRACT</b> .....	<b>v</b>
<b>LIST OF ABBREVIATIONS</b> .....	<b>ix</b>
<b>CONTENTS</b> .....	<b>xv</b>
<b>LIST OF FIGURES</b> .....	<b>xix</b>
<b>LIST OF TABLES</b> .....	<b>xx</b>
<b>CHAPTER 1</b> .....	<b>1</b>
<i>INTRODUCTION AND LITERATURE REVIEW</i> .....	<i>1</i>
<b>SECTION A: NEUROBLASTOMA</b> .....	<b>1</b>
A1. Introduction.....	1
A2. What is Neuroblastoma? .....	2
A3. The Sympathetic Nervous System.....	3
A4. Epidemiology of Neuroblastoma: Incidence and Mortality Statistics .....	4
A5. Risk Factors for Neuroblastoma.....	7
A6. Staging of Neuroblastoma.....	9
A7. Prognostic Markers for Neuroblastoma.....	11
A8. Other Autonomic Nervous System Tumours in Children.....	12
A9. Detection, Diagnosis and Prognosis of Neuroblastoma.....	12
A9.1 Imaging and Laboratory Tests.....	12
A9.2 Histopathology of Neuroblastoma.....	24
A10. Clinical Presentation, Signs and Symptoms of Neuroblastoma.....	26
A10.1 Signs or Symptoms Caused by the Main Tumour .....	32
A10.1.1 Tumours in the Abdomen or Pelvis.....	32
A10.1.2 Tumours in the Chest or Neck.....	32
A10.2 Signs or Symptoms Caused by Metastatic Spread of the Cancer .....	33
A10.3 Signs or Symptoms Caused by Hormones Secreted by the Tumour .....	34
A11. Molecular Pathogenesis, Genetics and Genomics of Neuroblastoma.....	35
A11.1 Neural Development and Neuroblastoma.....	35
A11.2 EMT and MET Transitions in the Neural Crest .....	36
A11.3 Hallmarks of the Neuroblastoma Tumour Microenvironment .....	37
A11.4 Genetic Lesions, Transcriptional Networks and Oncogenic Drivers in Neuroblastoma.....	39
A11.4.1 Familial Genetic Lesions.....	41
A11.4.2 PHOX2B Germline Mutations .....	42
A11.4.3 Anaplastic Lymphoma Kinase .....	44
A11.4.4 Chromosome Gain and Oncogene Activation .....	45
A11.4.5 Amplification of MYCN and the 2p24 Locus .....	46
A11.4.6 Gain of Chromosome Arm 17q .....	49

A11.4.7 Amplification and Chromosome Gain of Other Loci .....	50
A12.4 Chromosome Loss and Tumour Suppressor Genes .....	51
A12.4.1 Loss of Heterozygosity of Chromosome 1p and CHD5, miR-34, KIF1B $\beta$ .....	51
A12.4.2 Loss of Heterozygosity of 11q and TSLC1 .....	52
A12.4.3 Loss of Heterozygosity of 14q .....	53
A13. Treatment and Management of Neuroblastoma.....	53
A13.1 Overall Therapeutic Landscape of Neuroblastoma .....	53
A13.1.1 Spontaneous Regression and Stage 4S Disease .....	54
A13.1.2 Surgery .....	56
A13.1.3 Chemotherapy .....	57
A13.1.4 Radiotherapy .....	60
A13.1.5 Haematopoietic / Peripheral Blood Stem Cell Transplantation .....	60
A13.1.6 Management of Minimal Residual Disease and Relapse.....	61
A13.1.7 Multidrug Resistance and Monitoring Response to Treatment .....	63
A13.1.8 Alleviating the Burden of Late Effects.....	66
A13.2 Current Research Milestones and Proposed Novel Therapies .....	66
A13.2.1 Differentiation and Retinoids .....	66
A13.2.2 mTOR Inhibitors .....	70
A13.2.3 Aurora A Kinase and MDM2 as MYCN Targets .....	73
A13.2.4 Tyrosine Receptor Kinase Neurotrophin Receptor Inhibitors .....	73
A13.2.5 Targeted Immunotherapy and Disialoganglioside .....	74
A13.2.6 Angiogenesis and VEGF Signalling Inhibitors .....	75
A13.2.7 The PI-3 Kinase-Akt-MDM2-Survivin Signalling Axis in High-Risk Neuroblastoma .....	76
A13.2.8 Gastrin-Releasing Peptide Receptors .....	77
A13.2.9 Anaplastic Lymphoma Kinase .....	77
A13.2.10 Future Therapeutic Perspective .....	78
<b>SECTION B: GLYCOBIOLOGY AND GLYCOMICS OF NEUROBLASTOMA.....</b>	<b>79</b>
B1. Orientation to Glycans.....	79
B2. Protein Glycosylation in Neuroblastoma.....	82
B2.1 General Principles of Glycosylation .....	82
B2.2 Gangliosides .....	82
B2.3 Intercellular Adhesion Molecule-2 .....	85
B2.4 Anaplastic Lymphoma Kinase.....	87
B2.5 Cell-Surface Mucin-Type O-Glycans .....	87
B2.6 Polysialic Acid .....	88
B2.7 Lectins (Glycan-Binding Proteins) .....	88
B2.8 Glycosyltransferases .....	90
B2.9 ATP-Binding Cassette Multidrug Transporters .....	91
B2.10 Inhibitors of N-Linked Glycosylation.....	93
<b>SECTION C: PROTEIN GLYCOSYLATION, ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE .....</b>	<b>98</b>
C1. Introduction .....	98
C2. Endoplasmic Reticulum Stress and the Unfolded Protein Response .....	99

---

C3. ER Stress and the UPR in Cancer.....	102
C4. Targeting ER Stress and the UPR.....	105
C5. The ERS and UPR in Perspective.....	114
<b>SECTION D: RESEARCH CONTEXT.....</b>	<b>114</b>
D1. Problem Statement and Research Questions .....	114
D2. Purpose of the Study .....	117
D3. Aims of the Study.....	117
D4. Objectives of the Study .....	118
D5. Hypothesis.....	118
<b>SECTION E: SUMMARY.....</b>	<b>118</b>
<b>CHAPTER 2.....</b>	<b>119</b>
<b>RESEARCH METHODOLOGY.....</b>	<b>119</b>
2.1 Experimental Design .....	119
2.2 Drugs and Chemicals .....	119
2.3 Culture and Maintenance of SK-N-BE(2) Neuroblastoma Cells.....	120
2.4 Growth Curve Analysis of SK-N-BE (2) Neuroblastoma Cells .....	123
2.5 Cell Counting Kit-8 (CCK-8) Cell Viability Assay .....	123
2.6 Apotox-Glo™ Triplex Cytotoxicity, Cell Viability and Apoptosis Assays.....	124
2.6.1 Principle of the Apotox-Glo™ Triplex Assay.....	124
2.6.2 Assay Conditions for the ApoTox-Glo™ Triplex Assay .....	125
2.7 Measurement of P-Glycoprotein-Mediated Efflux Function.....	126
2.8 Annexin-V Cy3™ Apoptosis Assay .....	128
2.8.1 Principle of Annexin-V Cy3™ Apoptosis Assay.....	128
2.8.2 Assay Conditions for Annexin-V Cy3™ Apoptosis Assay.....	129
2.9 Statistical Analysis.....	130
<b>CHAPTER 3.....</b>	<b>131</b>
<b>RESULTS AND DISCUSSION.....</b>	<b>131</b>
3.1 Introduction.....	131
3.2 Morphology of SK-N-BE(2) Neuroblastoma Cells.....	132
3.3 Expression of P-Glycoprotein in SK-N-BE(2) Neuroblastoma Cells.....	132
3.4 Growth Curve Analysis of SK-N-BE(2) Neuroblastoma Cells .....	135
3.5 Cell Counting Kit-8 (CCK-8) Cell Viability Assays.....	136
3.6 Apotox-Glo™ Triplex Cell Cytotoxicity, Viability and Apoptosis Assays.....	139
3.7 Annexin-V Cy3™ Apoptosis Assays.....	146
3.8 Measurement of P-Glycoprotein-Mediated Efflux Function.....	152
3.9 Summary .....	154
<b>CHAPTER 4.....</b>	<b>156</b>
<b>CONCLUSION AND FUTURE DIRECTIONS .....</b>	<b>156</b>
4.1 Introduction.....	156
4.2 Research Hypothesis and Objectives of the Study .....	157
4.3 Context and Significance of the Study .....	157
4.3.1 Neuroblastoma Multidrug Resistance, Endoplasmic Reticulum Stress and Glycosylation.....	157

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4.3.2 Tunicamycin .....	161
4.3.3 Deoxynojirimycin .....	164
4.3.4 Celecoxib .....	164
4.3.5 Swainsonine .....	166
4.3.6 $\beta$ -D-Xyloside.....	166
4.4 Limitations of the Study.....	167
4.5 Conclusion and Future Directions.....	167
<b>REFERENCES.....</b>	<b>169</b>
<b>APPENDIX 1.....</b>	<b>212</b>
<i>COPYRIGHT CLEARANCE: WILEY GLOBAL PERMISSIONS.....</i>	<i>212</i>
<b>APPENDIX 2.....</b>	<b>213</b>
<i>COPYRIGHT CLEARANCE: CANCER, JOHN WILEY AND SONS.....</i>	<i>213</i>
<b>APPENDIX 3.....</b>	<b>214</b>
<i>COPYRIGHT CLEARANCE: SEMINARS IN CANCER BIOLOGY, ELSEVIER .....</i>	<i>214</i>
<b>APPENDIX 4.....</b>	<b>215</b>
<i>COPYRIGHT CLEARANCE: ANNUAL REVIEW OF MEDICINE.....</i>	<i>215</i>
<b>APPENDIX 5.....</b>	<b>216</b>
<i>COPYRIGHT CLEARANCE: CANCER LETTERS .....</i>	<i>216</i>
<b>APPENDIX 6.....</b>	<b>217</b>
<i>COPYRIGHT CLEARANCE: GENOME MEDICINE.....</i>	<i>217</i>
<b>APPENDIX 7.....</b>	<b>218</b>
<i>COPYRIGHT CLEARANCE: CANCER LETTERS .....</i>	<i>218</i>
<b>APPENDIX 8.....</b>	<b>219</b>
<i>COPYRIGHT CLEARANCE: CANCER LETTERS .....</i>	<i>219</i>
<b>APPENDIX 9.....</b>	<b>220</b>
<i>COPYRIGHT CLEARANCE: CURRENT OPINION IN CELL BIOLOGY.....</i>	<i>220</i>
<b>APPENDIX 10.....</b>	<b>221</b>
<i>COPYRIGHT CLEARANCE: JOURNAL OF LEUKOCYTE BIOLOGY .....</i>	<i>221</i>
<b>APPENDIX 11.....</b>	<b>222</b>
<i>COPYRIGHT CLEARANCE: FRONTIERS IN ONCOLOGY.....</i>	<i>222</i>
<b>APPENDIX 12.....</b>	<b>223</b>
<i>COPYRIGHT CLEARANCE: ELSEVIER .....</i>	<i>223</i>

# LIST OF FIGURES

<b>Figure 1.1:</b> Organization of the nervous system.....	4
<b>Figure 1.2:</b> Structure and pathways of the sympathetic division of the nervous system .....	5
<b>Figure 1.3:</b> Rare and common genomic variants that predispose to neuroblastoma.....	10
<b>Figure 1.4:</b> Histology of peripheral neuroblastic tumours.....	29
<b>Figure 1.5:</b> Microscopic views of typical neuroblastoma histopathology .....	30
<b>Figure 1.6:</b> Neural crest programmed epithelial-to-mesenchymal transition .....	37
<b>Figure 1.7:</b> Clinicopathologic correlations of neuroblastoma .....	39
<b>Figure 1.8:</b> Contribution of the cells and ECM in the TME to the ten hallmarks of neuroblastoma .....	40
<b>Figure 1.9:</b> Pathways activated via communication between neuroblastoma and TME cells in the ECM .....	40
<b>Figure 1.10:</b> Chromosome regions and genes known to be involved in neuroblastoma oncogenesis .....	43
<b>Figure 1.11:</b> Mechanisms of MDR and the concept of MDR targeting based on collateral sensitivity.....	64
<b>Figure 1.12:</b> The mechanism of action of retinoids and rexinoids .....	68
<b>Figure 1.13:</b> Overview of the mTOR signalling pathway in cancer .....	71
<b>Figure 1.14:</b> Major human glycans .....	80
<b>Figure 1.15:</b> Theme and variation in the human glycome .....	81
<b>Figure 1.16:</b> Schematic representation of the major ganglioside biosynthesis pathways .....	84
<b>Figure 1.17:</b> Altered glycans and related pathophysiological events involved in NB progression.....	85
<b>Figure 1.18:</b> Neuroblastoma glycobiology impact on tumour growth and antitumour therapy.....	86
<b>Figure 1.19:</b> Glycosylation defining malignancy—invasive and metastatic phenotype of tumours.....	90
<b>Figure 1.20:</b> Endoplasmic reticulum protein folding function under normal physiological conditions.....	100
<b>Figure 1.21:</b> Core elements of the UPR signalling network.....	101
<b>Figure 1.22:</b> The three branches of the UPR.....	102
<b>Figure 1.23:</b> Endoplasmic reticulum protein folding function under ERS conditions.....	103
<b>Figure 1.24:</b> Involvement of UPR signaling during cell transformation and tumour growth.....	104
<b>Figure 1.25:</b> Tumour microenvironment and activation of ERS and UPR responses in cancer .....	105
<b>Figure 1.26:</b> Cellular impact of ERS aggravators that weigh on the yin vs yang balance .....	106
<b>Figure 1.27:</b> An overview of therapeutic ERS-based targeting of the main hallmarks of cancer .....	107
<b>Figure 2.1:</b> Experimental design: Assays and drugs used in this study .....	121
<b>Figure 3.1:</b> Morphology of SK-N-BE(2) neuroblastoma cells .....	132
<b>Figure 3.2:</b> Upregulation of P-glycoprotein expression in neuroblastoma cell lines.....	134
<b>Figure 3.3:</b> Growth curve analysis of SK-N-BE(2) neuroblastoma cells in culture .....	135
<b>Figure 3.4:</b> CCK-8 dose-response curves for test compounds assessed at varying concentrations .....	137
<b>Figure 3.5:</b> Effects of tunicamycin on SK-N-BE(2) cell cytotoxicity, viability and apoptosis .....	140
<b>Figure 3.6:</b> Effects of deoxynojirimycin on SK-N-BE(2) cell cytotoxicity, viability and apoptosis.....	141
<b>Figure 3.7:</b> Effects of celecoxib on SK-N-BE(2) cell cytotoxicity, viability and apoptosis.....	142
<b>Figure 3.8:</b> Effects of swainsonine on SK-N-BE(2) cell cytotoxicity, viability and apoptosis .....	143
<b>Figure 3.9:</b> Effects of $\beta$ -D-xyloside on SK-N-BE(2) cell cytotoxicity, viability and apoptosis .....	144
<b>Figure 3.10:</b> Fluorescence micrographs of the effects of tunicamycin on SK-N-BE(2) cell apoptosis .....	147
<b>Figure 3.11:</b> Fluorescence micrographs of the effects of deoxynojirimycin on SK-N-BE(2) cell apoptosis.....	148



<b>Figure 3.12:</b> Fluorescence micrographs of the effects of celecoxib on SK-N-BE(2) cell apoptosis.....	149
<b>Figure 3.13:</b> Fluorescence micrographs of the effects of swainsonine on SK-N-BE(2) cell apoptosis .....	150
<b>Figure 3.14:</b> Fluorescence micrographs of the effects of $\beta$ -D-xyloside on SK-N-BE(2) cell apoptosis.....	151
<b>Figure 3.15:</b> Effects of test compounds on P-glycoprotein function in SK-N-BE(2) cells.....	153

## LIST OF TABLES

<b>Table 1.1:</b> Epidemiological information on childhood and adolescent cancers 2016.....	6
<b>Table 1.2:</b> Trends in 5-year relative survival rates for children (birth to 14 years) by year of diagnosis .....	8
<b>Table 1.3:</b> The International Neuroblastoma Staging System (INSS).....	13
<b>Table 1.4:</b> The International Neuroblastoma Risk Group Staging System (INRGSS) .....	14
<b>Table 1.5:</b> Children's Oncology Group (COG) risk groups .....	15
<b>Table 1.6:</b> International Neuroblastoma Risk Group (INRG) classification.....	16
<b>Table 1.7:</b> Prognostic markers for neuroblastoma.....	17
<b>Table 1.8:</b> Survival of neuroblastoma patients by Children's Oncology Group (COG) risk group.....	18
<b>Table 1.9:</b> Other autonomic nervous system tumours in children .....	18
<b>Table 1.10:</b> Contemporary procedures and approaches used in confirming a diagnosis of neuroblastoma.....	19
<b>Table 1.11:</b> INPC classification of neuroblastic tumours .....	27
<b>Table 1.12:</b> Comparison of the INPC and the Shimada classification of neuroblastic tumours .....	28
<b>Table 1.13:</b> Specific classes and examples of N-glycosylation inhibitors .....	94
<b>Table 1.14:</b> Pharmacologic modulators commonly used in targeting ERS and UPR signalling.....	109
<b>Table 3.1:</b> Relative expression of known drug-resistance genes in neuroblastoma cell lines.....	133
<b>Table 3.2:</b> Regression analysis data and summary of dose-response parameters .....	138

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

### SECTION A: NEUROBLASTOMA

#### A1. Introduction

Neuroblastoma (NB) is the most common extracranial and deadly solid tumour in children and its origin has been clearly linked to the development of the sympathetic nervous system (SNS) because it originates from sympathetic precursor neuroblasts derived from the neural crest. Neuroblastoma represents 8-10% of all childhood tumours and accounts for approximately 15% of all cancer-related deaths in the paediatric population. The incidence of neuroblastoma is 10.2 cases per million children under 15 years of age, and nearly 500 new cases are reported annually. While 90% of cases are diagnosed before the age of 5, 30% of those are within the first year. The median age of diagnosis is 22 months. Rarely does it present in adolescence and adulthood, but outcomes are much poorer in this age group.

There does not appear to be an increased prevalence among races, but there is a slight predilection for males (1.2:1). With a family history noted in 1-2% of diagnoses, there are several reports of autosomal dominant patterns of inheritance. In such pedigrees, patients are frequently diagnosed at an earlier age (median age of 9 months) than those with sporadic disease and are more likely to have associated multiple primary cancers. Neuroblastoma has also been diagnosed in conjunction with other congenital conditions such as Hirschsprung's disease, congenital hypoventilation disorder and neurofibromatosis type 1. There was early interest in the co-occurrence of neuroblastoma and neurofibromatosis, as they are both disorders of neural crest cells. However, this may represent coincidence rather than a true association.<sup>1,2</sup>

Approximately half of newly diagnosed children with this tumour will present with metastatic disease or histologically aggressive large tumours that are at high risk for treatment failure. Neuroblastomas found in patients older than 1 year are usually aggressive and eventually kill the patients despite intensive therapy, whereas those in patients younger than 1 year often regress spontaneously or mature, resulting in a more favourable prognosis. Because of the unique biological features of NBs, the neoplasm shows a wide range of clinical hallmarks, including the highest rate of spontaneous regression of any human malignancy, a potential for undergoing both induced and spontaneous maturation and also aggressive clinical courses with poor survival outcomes. The biological characteristics of NB are complex aneusomies, aneuploidies and ploidy shifts acquired by the tumour cells, and some of these chromosomal changes are known to be associated with clinical behaviour. The most commonly observed aneusomies include gains of 17q and deletions or allelic losses of 1p and 11q.

Combinations of these and other less prevalent genetic changes are detected in different genetic and clinical subsets of NB, and shown to be associated with tumour phenotype. Notwithstanding intensive investigation to map the shortest region of overlaps at deleted segments of 1p and 11q, a consistently involved candidate tumour suppressor gene has not yet been identified. Similarly, although PPM1D has been reported to be the most likely target gene with a recurrent oncogenic role at the minimal common region of gains at 17q remains to be identified. For years, *MYCN* was the only oncogene known to be involved recurrently in approximately 22% of tumours, and the MYCN protein is overexpressed via high copy number gains of the gene in tumours with advanced stages and aggressive clinical behaviour. The sections that follow will describe the global disease landscape of neuroblastoma in greater detail.

## **A2. What is Neuroblastoma?**

Neuroblastoma (NB) is a paediatric cancer that originates from undifferentiated migratory neural crest (NC) progenitor or stem cells of the developing sympathetic nervous system

present in an embryo or foetus.<sup>3-6</sup> These early precursor nerve cells are called neuroblasts—the term *neuro* signifies nerves, while *blastoma* denotes a cancer that affects immature or developing cells. Thus, neuroblastoma is a solid tumour that stems from the developing sympathetic nervous system (SNS) and can be found anywhere along this system.<sup>7</sup> Neuroblastoma is prevalent in infants and young children—it rarely occurs in children older than 10 years.<sup>8</sup> About 1 out of 3 neuroblastomas start in the adrenal glands and 1 out of 4 begin in sympathetic nerve ganglia in the abdomen, whereas the rest start in sympathetic ganglia near the spine in the chest or neck, or in the pelvis.<sup>6</sup>

Since NBs are often widely disseminated and the tumours genetically heterogenous in terms of their growth and metastatic behaviour—some grow and spread quickly, while others grow slowly—it is challenging to pinpoint their origin and predict disease prognosis.<sup>9-11</sup> Sometimes in very young children, the cancer cells die for no reason and the tumour regresses spontaneously.<sup>12</sup> In other cases, the cells sometimes mature on their own into normal ganglion cells and stop dividing. This terminal differentiation makes the tumour a ganglioneuroma (GN).<sup>8</sup> In order to understand neuroblastoma, it is essential to reflect on how the SNS functions.<sup>13</sup> Therefore, the SNS is described briefly in the subsection that follows.

### **A3. The Sympathetic Nervous System**

The nervous system consists of the brain, spinal cord and the nerves that reach out from them to all areas of the body. The nervous system is essential for cognitive function, sensation, movement and many sensory and motor functions that we are hardly ever aware of, including heart rate, breathing, blood pressure, and digestion. This division of the nervous system is known as the autonomic nervous system (ANS). Figure 1.1 shows the organization of the nervous system.

The SNS is a subdivision of the ANS. The SNS includes nerve fibres that run along either side the spinal cord. Clusters of nerve cells called ganglia (plural of ganglion) occur at certain points

along the path of the nerve fibres. Nerve-like cells are found in the medulla (centre) of the adrenal glands located superiorly each kidney. These glands produce hormones (such as adrenaline (epinephrine) that help control heart rate, blood pressure, blood glucose and how the body reacts to stress. The main cells that make up the nervous system are called nerve cells or neurons. These cells interact with other types of cells in the body by releasing small amounts of chemical messengers (hormones). This is important, because neuroblastoma cells often release certain hormones that can cause symptoms of the tumour.

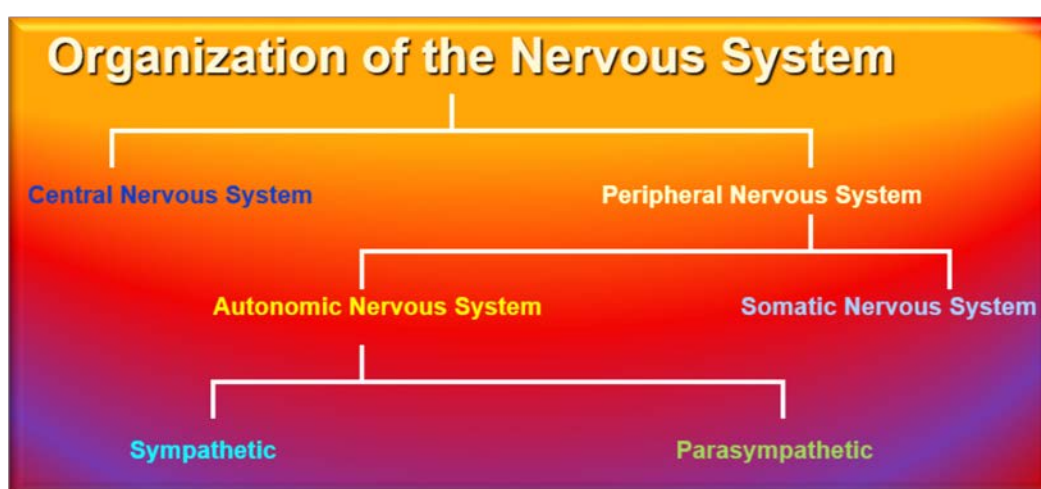
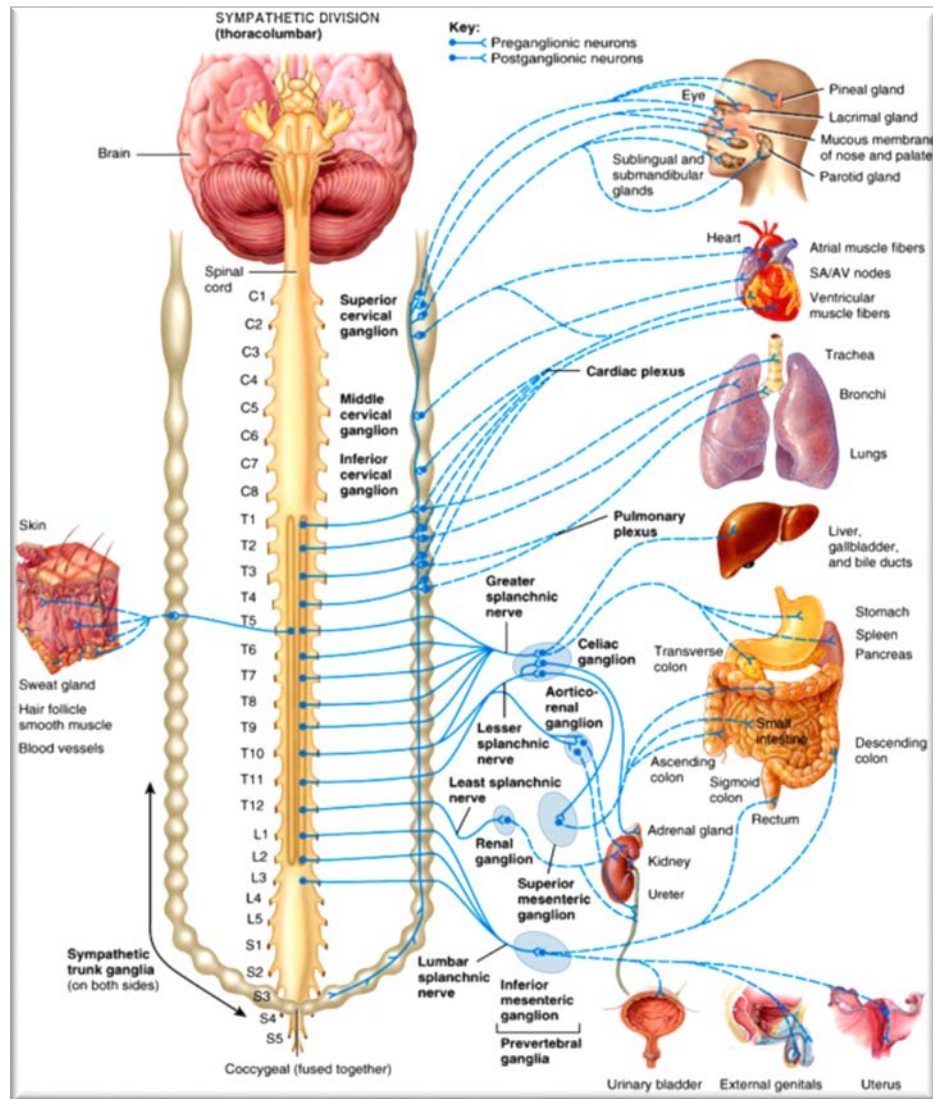


Figure 1.1: Organization of the nervous system

Figure 1.2 shows the greater structure of the SNS and thirty-one pairs of spinal nerves connected to the spinal cord, namely, 8 cervical nerve pairs (C1 through C8), 12 thoracic nerve pairs (T1 through T12), 5 lumbar nerve pairs (L1 through L5), 5 sacral nerve pairs (S1 through S5) and 1 coccygeal fused nerve pair.

#### **A4. Epidemiology of Neuroblastoma: Incidence and Mortality Statistics**

Neuroblastoma is by far the most common cancer in infants (less than 1 year old).<sup>14</sup> Neuroblastoma accounts for about 6% of all cancers in children, with about 700 new cases reported each year in the United States. This number has remained constant for many years. The average age of children when they are diagnosed is about 1 to 2 years.



**Source:**<sup>15</sup> Solid lines represent preganglionic axons; dashed lines represent postganglionic axons. Although the innervated structures are shown for only one side of the body for diagrammatic purposes, the sympathetic division actually innervates tissues and organs on both sides. Reproduced from Tortora GJ, Derrickson B. *Principles of Anatomy & Physiology*. 14 ed. Hoboken, NJ: John Wiley & Sons; 2014, with clearance from Wiley Global Permissions (Appendix 1)

**Figure 1.2:** Structure and pathways of the sympathetic division of the nervous system

In rare cases, NB is detected by ultrasound even before birth, but most cases (about 90%) are diagnosed by age 5. The malignancy is very rare in individuals over the age of 10 years. In about 2 of 3 cases, the disease has already spread to the lymph nodes or to other parts of the body when it is diagnosed. Table 1.1 summarizes a USA survey and epidemiological data on childhood and adolescent cancers retrieved from *Cancer Facts & Figures 2014—Special Section: Childhood and Adolescent Cancers* at [cancer.org/statistics](http://cancer.org/statistics). Table 1.2 outlines 5-year



survival rates for childhood cancers.<sup>16</sup> Cancer is the second most common cause of death among children aged 1 to 14 years in the United States, exceeded only by accidents. In 2016, an estimated 10,380 children (birth to 14 years) will be diagnosed with cancer.

**Table 1.1:** Epidemiological information on childhood and adolescent cancers 2016

<b>New Cases</b>	An estimated 10,380 new cases of childhood cancers (ages 0-14 years) are expected to occur in 2016.
<b>Incidence Trends</b>	Childhood cancer incidence rates have gradually increased by 0.6% per year since 1975, when population-based cancer registration began in the US.
<b>Deaths</b>	An estimated 1,250 cancer deaths are expected to occur among children in 2016. Cancer is the second leading cause of death in children aged 1-14 years, eclipsed only by accidents.
<b>Mortality Trends</b>	Childhood cancer death rates dropped by 66% from 1969 (6.5 per 100,000) to 2012 (2.2 per 100,000), mainly as a result of improved treatment and high rates of participation in clinical trials. From 2003 to 2012, the childhood cancer death rate declined by 1.3% per year.
<b>Survival</b>	Survival for all invasive childhood cancers combined has improved markedly over the last 30 years due to novel and improved treatment strategies. The five-year relative survival rate increased from 58% in the mid-1970s to 83% in most recent times (2005-2011). However, rates differ considerably according to cancer type, patient age and other variables (see also Tables 1.1 and 1.2).
Some paediatric cancer patients experience treatment-induced side effects long after treatment, including impairment of function of specific organs (e.g., cognitive defects) and secondary cancers. <sup>17</sup> The Children's Oncology Group (COG) has developed guidelines for screening and managing of late effect survivors of childhood cancer (see COG website at <a href="http://survivorshipguidelines.org">survivorshipguidelines.org</a> ). The <i>Childhood Cancer Survivor Study</i> has followed more than 14,000 long-term childhood cancer survivors and posted valuable information on <a href="http://ccss.stjude.org">ccss.stjude.org</a> .	

Approximately 1,250 children will die from the disease. Benign and borderline brain tumours are not included in the 2016 case estimates because the calculation method requires historical data and these tumours were not required to be reported until 2004. Leukaemia (76% of which are lymphoid leukemias) accounts for 30% of all childhood cancers (including benign brain tumours). Cancers of the brain and other nervous system are the second most common cancer type (26%), followed by soft tissue sarcomas (7%, almost one-half of which are rhabdomyosarcoma), neuroblastoma (6%), non-Hodgkin lymphomas, including Burkitt lymphoma (6%), renal (Wilms) tumours (5%), and Hodgkin lymphomas (3%).<sup>16</sup>

The 5-year survival rate refers to the percentage of children who live at least 5 years after their cancer is diagnosed. Many children may live much longer than 5 years (and many are even cured). In order to obtain 5-year survival rates, oncologists have to look at children who were treated at least 5 years ago. Improvements in treatment since then may result in a better outlook for children now being diagnosed with NB.<sup>8</sup>

## **A5. Risk Factors for Neuroblastoma**

Neuroblastoma is one of the most common childhood (age 0-14 years) cancers, being only surpassed in the paediatric age group by leukaemia and brain tumours.<sup>1-3,14,18</sup> A risk factor is any factor that affects an individual's chance of getting a disease such as cancer. Different cancers have different risk factors. Lifestyle-related risk factors such as body weight, physical activity, diet and smoking play a major role in many adult cancers. However, these factors usually take many years to influence cancer risk, and are not usually associated with childhood cancers, including NBs. Also, no environmental factors (such as exposures during the mother's pregnancy or in early childhood) are known to increase the child's chance of getting NB.

Neuroblastoma is a very heterogeneous disease with features ranging from spontaneous regression during the foetal period to disseminated metastasis at the time of diagnosis. Several risk factors have been identified to play a role in disease progression, including age at the time of initial presentation, tumour stage, histology and ploidy of tumour, and cytogenetic aberrations such *MYCN* amplification, loss of heterozygosity of 11q and gain of 17q.<sup>4,9,11,19-24</sup> According to the International Neuroblastoma Risk Group (INRG) task force report, age-range between 18 and 60 months is considered as a high risk group.<sup>25,26</sup>

Heredity is an important risk factor in about 1-2% of all NBs as children inherit an increased risk of developing NB from a parent. Children with the familial form of NB (those with an inherited tendency to develop this cancer) usually come from families with one or more members who had NB as infants.

**Table 1.2:** Trends in 5-year relative survival rates for children (birth to 14 years) by year of diagnosis

Childhood cancer	1975-1977	1978-1980	1981-1983	1984-1986	1987-1989	1990-1992	1993-1995	1996-1998	1999-2001	2002-2004	2005-2011
<b>All sites</b>	<b>58</b>	<b>62</b>	<b>67</b>	<b>68</b>	<b>72</b>	<b>76</b>	<b>77</b>	<b>79</b>	<b>81</b>	<b>83</b>	<b>83<sup>†</sup></b>
Acute lymphocytic leukaemia	57	66	71	72	78	83	84	87	89	92	91 <sup>†</sup>
Acute myeloid leukaemia	19	26	27 <sup>‡</sup>	31 <sup>‡</sup>	37 <sup>‡</sup>	42	41 <sup>‡</sup>	49	58	61	67 <sup>†</sup>
Bones and joints	50 <sup>‡</sup>	48	57 <sup>‡</sup>	57 <sup>‡</sup>	67 <sup>‡</sup>	67	74	70	70	78	77 <sup>†</sup>
Brain & other nervous system	57	58	57	62	64	64	71	75	74	75	74 <sup>†</sup>
Hodgkin lymphoma	81	87	88	90	87	97	95	96	94	98	98 <sup>†</sup>
<b>Neuroblastoma</b>	<b>53</b>	<b>57</b>	<b>55</b>	<b>52</b>	<b>63</b>	<b>76</b>	<b>67</b>	<b>66</b>	<b>72</b>	<b>73</b>	<b>74<sup>†</sup></b>
Non-Hodgkin lymphoma	43	53	67	70	71	77	81	83	90	85	88 <sup>†</sup>
Soft tissue	61	74	69	73	66	80	77	71	77	85	79 <sup>†</sup>
Wilms tumour	73	79	87	91	92	92	92	92	94	89	94 <sup>†</sup>

Data are for surveys conducted in the United States, 1975 to 2011.

\*Survival rates are adjusted for normal life expectancy and are based on follow-up of patients through 2012.

<sup>†</sup>The difference in rates between 1975 to 1977 and 2005 to 2011 is statistically significant ( $p < 0.05$ ).

<sup>‡</sup>The standard error of the survival rate is between 5 and 10 percentage points.

**Source**<sup>16</sup>

Many other large epidemiological studies focusing on the incidence, prognostic factors, and the treatment outcomes in their patients have been carried out in different parts of the world, including Turkey,<sup>27</sup> Norway,<sup>28</sup> The European Neuroblastoma Study Group,<sup>29</sup> The Surveillance, Epidemiology, and End Results Programme (SEER, <http://seer.cancer.gov>),<sup>30-32</sup> Iran,<sup>33,34</sup> Mexico<sup>35</sup> Australia, Europe, Japan, North America<sup>26</sup> and Italy.<sup>36</sup>



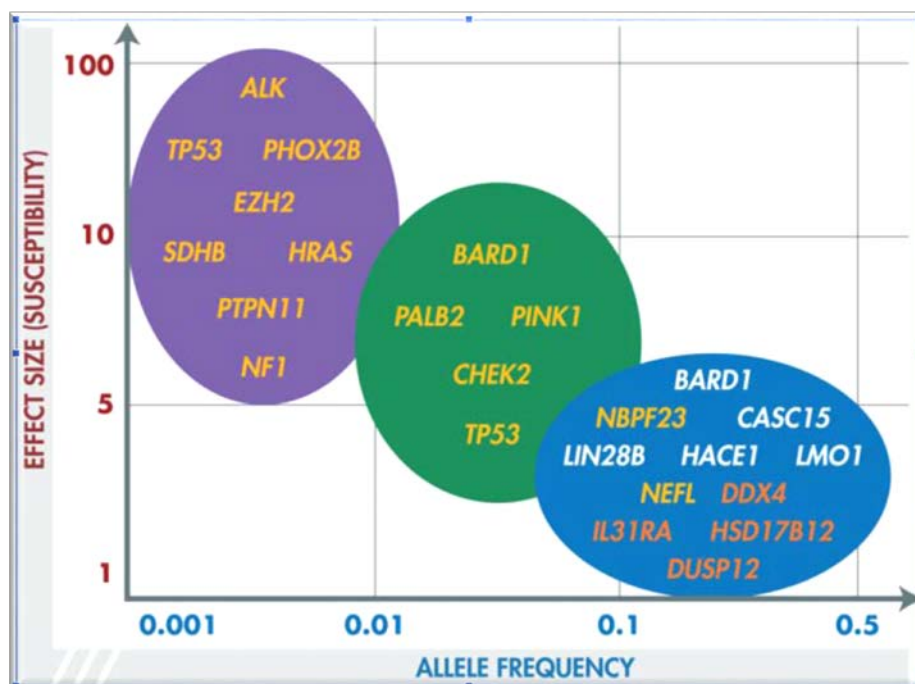
The average age at diagnosis of familial cases is younger than the age for sporadic (not inherited) cases. Children with familial NB sometimes develop 2 or more of these cancers in different organs (for example, in both adrenal glands or in more than one sympathetic ganglion). It's important to distinguish NBs that originate in more than one organ from those that have metastasized (spread) from one primary organ to secondary sites or organs (metastatic NBs). Frequently, tumours that have developed in several places at once implies a familial form. Both familial and sporadic NB can spread to other organs. A recent review encapsulates the current state of knowledge about NB genetics and genomics, highlighting the improved prognosis and potential therapeutic opportunities that have arisen from recent advances in understanding germline predisposition, recurrent segmental chromosomal alterations, somatic point mutations and translocations and clonal evolution in relapsed NB.<sup>37</sup> Figure 1.3 shows rare and common genomic variants that predispose to NB.

## A6. Staging of Neuroblastoma

The stages and risk groups for NB are complex and can be perplexing. A staging system is a standard approach used by a multidisciplinary cancer care team to classify the extent and burden of the cancer. Since the mid-1990s, most cancer centres have adopted the International Neuroblastoma Staging System (INSS) to stage NB.<sup>25</sup> This postsurgical staging system takes into account the results of surgery to remove the tumour. Neuroblastoma belongs to a group collectively known as peripheral neuroblastic tumours, which also includes intermixed ganglioneuroblastoma, ganglioneuroma, and nodular ganglioneuroblastoma.<sup>38-40</sup>

Neuroblastoma can further be divided based on the degree of neuroblastic differentiation (undifferentiated, poorly differentiated, and differentiating) and the mitosis-karyorrhexis index (MKI) (low, intermediate, or high).<sup>41</sup> Histologically, it has limited Schwannian cell production, is stroma-poor, and has abundant neuroblasts. The International Neuroblastoma Pathology Classification has been used to predict prognosis based on the histopathology of the tumour and age of the patient. This system takes into account the degree of cell differentiation, MKI,

and the presence of Schwann cells. Following these guidelines, the unfavorable group encompasses patients with any tumour over 60 months; undifferentiated tumours with a high MKI at any age; and undifferentiated or poorly differentiated tumours with intermediate or high MKI in children older than 18 months. In simplified form, the stages are summarized in Table 1.3.



**Source:**<sup>37</sup> Reproduced from Bosse KR, Maris JM. Advances in the translational genomics of neuroblastoma: From improving risk stratification and revealing novel biology to identifying actionable genomic alterations. *Cancer* 2016;122(1):20-33, Copyright, American Cancer Society, with permission from John Wiley and Sons. See Appendix 2 for copyright clearance.

**Figure 1.3:** Rare and common genomic variants that predispose to neuroblastoma

**Left:** In addition to anaplastic lymphoma kinase (ALK)-associated and paired-like homeobox 2B (PHOX2B)-associated familial neuroblastoma, neuroblastoma can also arise in the setting of genetic syndromes with underlying rat sarcoma oncogene-mitogen activated protein kinase (RAS-MAPK) pathway germline mutations, such as neurofibromin 1 (NF1) in neurofibromatosis type 1, 50 protein tyrosine phosphatase, nonreceptor type 11 (PTPN11) in Noonan syndrome,<sup>51,52</sup> and Harvey rat sarcoma viral oncogene homolog (HRAS) in Costello syndrome. Tumour protein 53 (TP53) mutations associated with Li Fraumeni syndrome, 53 enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) mutations associated with Weaver syndrome, and succinate dehydrogenase complex, Subunit B, iron sulphur (Ip) (SDHB) mutations in familial paraganglioma/pheochromocytoma (PGL/PCC) are also rarely associated with neuroblastoma genesis. **Middle:** Low-frequency alleles in multiple DNA damage-response genes (BRCA1-associated ring domain 1 [BARD1], checkpoint kinase 2 [CHEK2], partner and localizer of BRCA2 [PALB2], and TP53) with an intermediate effect size also contribute to neuroblastoma predisposition. PINK1 indicates phosphatase and tensin homolog-induced putative kinase 1. **Right:** More common alleles with a modest effect size discovered using a genome-wide association study approach also collectively contribute to neuroblastoma genesis and, at times, specifically to a high-risk (white) or low-risk (orange) neuroblastoma phenotype. CASC15 indicates cancer susceptibility candidate 15; DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; DUSP12, dual specificity phosphatase 12; HACE1, HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1; HSD17B12, hydroxysteroid (17b) dehydrogenase 12; IL31RA, interleukin 31 receptor A; LIN28B, lin-28 homologue B; LMO1, LIM domain only 1; NBPF23, neuroblastoma breakpoint family, member 23; NEFL, neurofilament, light polypeptide.

A risk-group staging system now coming into use is known as the International Neuroblastoma Risk Group Staging System (INRGSS).<sup>25</sup> It is analogous to the INSS, but it does not use the results of surgery to help define the stage. This allows oncologists to determine a stage before surgery, based on the results of imaging tests, usually a computed tomography (CT) or magnetic resonance imaging (MRI) scan, and a meta-iodobenzylguanidine (MIBG) scan, as well as examinations and biopsies. The stage can then be used to help predict how resectable the tumour is—that is how much of it can be removed with surgery. The INRGSS uses image-defined risk factors (IDRFs) which are seen on imaging tests that might mean the tumour will be harder to remove. This includes features like the tumour growing into a nearby vital organ or growing around important blood vessels. The INRGSS divides NBs into 4 stages (Table 1.4).

The Children's Oncology Group (COG) uses major prognostic factors (section A7) combined with the INSS stage of the disease, to place children into 3 different risk groups: low, intermediate, and high (Table 1.5). These risk groups are used to help predict how likely it is that a child can be cured. For example, a child in a low-risk group can often be cured with limited treatment, such as surgery alone. With children in higher risk groups, the chance of cure is not as great, so more intensive treatment is often needed. A newer risk group classification system, the International Neuroblastoma Risk Group (INRG) classification (Table 1.6), is now being studied and may soon replace the COG system above. This system is based on the newer INRGSS staging system, which includes the image-defined risk factors (IDRFs), as well as many of the prognostic factors discussed in section A7 below.

## **A7. Prognostic Markers for Neuroblastoma**

Prognostic markers are features that help predict whether the child's outlook for cure is better or worse than would be predicted by the stage alone. Markers used to help determine a child's prognosis are summarized in Table 1.7. Table 1.8 indicates the Children's Oncology Group (COG) survival outlook according to disease risk. Risk-based treatment approaches for NB

have been used for many years. However, the criteria employed to delimit risk in various institutional and cooperative groups were incongruent, limiting the evaluation of clinical trial results. To alleviate this drawback and boost collaborative research, homogenous pretreatment patient cohorts have been defined by the INRG classification system.

This treatment rationale has yielded improved outcomes, even though survival for high-risk patients remains poor, underscoring the dire need to develop more effective treatment strategies. Advances in our knowledge of the biology and genetic basis of NB have led to the development of targeted and potentially useful therapeutic modalities.<sup>10,42,43</sup> The collaborative ventures of institutions and international cooperative groups have refined risk classification and stratified treatment strategies, resulting in improved survival rates for NB patients.<sup>2,44,45</sup>

## **A8. Other Autonomic Nervous System Tumours in Children**

Not all childhood ANS tumours are malignant. Features of two of the more common ANS tumours are summarized in Table 1.9.

## **A9. Detection, Diagnosis and Prognosis of Neuroblastoma**

### **A9.1 Imaging and Laboratory Tests**

NBs are customarily suspected when a child presents with signs or symptoms, but a definite diagnosis is made after correlating physical examination with laboratory tests.<sup>44,46-52</sup> Table 1.10 outlines contemporary procedures used in confirming a diagnosis of NB.<sup>44,46-51,53-61</sup> Depending on the clinical presentation, the index of suspicion must be high. Initial diagnostic testing should include complete blood count (CBC), serum electrolytes, liver function tests and a chest radiograph, which may reveal calcifications or a posterior mediastinal mass. Complementary diagnostic findings will encompass increased levels of urine or serum catecholamines or catecholamine metabolites such as dopamine, vanillylmandelic acid (VMA) and homovanillic acid (HVMA).<sup>63,64</sup>

**Table 1.3:** The International Neuroblastoma Staging System (INSS)

Stage	Description
<b>1</b>	Localized tumour with complete gross excision with or without microscopic residual disease; ipsilateral and contralateral lymph node (LN) negative for tumour microscopically. The cancer is still in the area where it started. It is on one side of the body (right or left). All visible tumour has been removed completely by surgery (although looking at the tumour's edges under the microscope after surgery may show some cancer cells). Lymph nodes outside the tumour are free of cancer (although nodes enclosed within the tumour may contain neuroblastoma cells).
<b>2A</b>	Unilateral tumour with incomplete gross resection; ipsilateral and contralateral LN negative for tumour microscopically. The cancer is still in the area where it started and on one side of the body, but not all of the visible tumour could be removed by surgery. Lymph nodes outside the tumour are free of cancer (although nodes enclosed within the tumour may contain neuroblastoma cells).
<b>2B</b>	Unilateral tumour with or without complete gross excision with ipsilateral LN positive for tumour; contralateral LN negative microscopically. The cancer is on one side of the body, and may or may not have been removed completely by surgery. Nearby lymph nodes outside the tumour contain neuroblastoma cells, but the cancer has not spread to lymph nodes on the other side of the body or elsewhere.
<b>3</b>	The cancer has not spread to distant parts of the body, but one of the following is true of the cancer: <ul style="list-style-type: none"> <li>• Cannot be removed completely by surgery and it has crossed the midline (defined as the spine) to the other side of the body. It may or may not have spread to nearby lymph nodes.</li> <li>• Is still in the area where it started and is on one side of the body. It has spread to lymph nodes that are relatively nearby but on the other side of the body.</li> <li>• Is in the middle of the body and is growing toward both sides (either directly or by spreading to nearby lymph nodes) and cannot be removed completely by surgery.</li> </ul>
<b>4A</b>	Any primary tumour with dissemination to distant LN, bone, bone marrow, liver, skin, or other organs (except as defined for stage 4S). Tumour infiltrating across the midline with or without regional LN involvement, localized unilateral tumour with contralateral regional LN involvement, or midline tumour with bilateral extension by infiltration (unresectable) or by LN involvement. The cancer has spread to distant sites such as distant lymph nodes, bone, liver, skin, bone marrow, or other organs (but the child does not meet the criteria for stage 4S)
<b>4S</b>	Localized primary tumour (as defined for stage 1 or 2) with dissemination limited to skin, liver, or bone marrow (limited to infants <1 yr of age). Also called "special" neuroblastoma. The child is younger than 1 year. The cancer is on one side of the body. It might have spread to lymph nodes on the same side of the body but not to nodes on the other side. The neuroblastoma has spread to the liver, skin, and/or the bone marrow. However, no more than 10% of marrow cells are cancerous, and imaging tests such as a meta-iodobenzylguanidine (MIBG) scan do not show that the cancer has spread to the bones or the bone marrow.
<b>Recurrent</b>	While not formally part of the staging system, this term is used to describe cancer that has come back (recurred) after it has been treated. The cancer might come back in the area where it first started or in another part of the body.

**Table 1.4:** The International Neuroblastoma Risk Group Staging System (INRGSS)

Stage	Description
<b>L1</b>	Localized disease that does not involve vital structures and is confined to one body compartment, i.e., a tumour that has not spread from where it started and has not grown into vital structures as defined by the list of IDRFs. It is confined to one body compartment, such as the neck, chest, or abdomen.
<b>L2</b>	Localized disease with image-defined risk factors, i.e., a tumour that has not spread far from where it started (for example, it may have grown from the left side of the abdomen into the left side of the chest), but that has at least one IDRF.
<b>M</b>	Distant metastatic disease, i.e., a tumour that has spread (metastasized) to a distant part of the body (except tumours that are stage MS).
<b>MS</b>	Metastatic disease in children younger than 18 months with cancer spread only to skin, liver, and/or bone marrow. No more than 10% of marrow cells are cancerous, and an MIBG scan does not show spread to the bones and/or the bone marrow.
<b>IDRFs:</b> image-defined risk factors <b>MIBG:</b> meta-iodobenzylguanidine	



**Table 1.5:** Children’s Oncology Group (COG) risk groups

Risk group	Description
<b>Low risk</b>	<ul style="list-style-type: none"> <li>• All children who are Stage 1</li> <li>• Any child who is Stage 2A or 2B and younger than age 1</li> <li>• Any child who is Stage 2A or 2B, older than age 1, whose cancer has <i>no</i> extra copies of the <i>MYCN</i> gene</li> <li>• Any child who is Stage 4S (younger than age 1), whose cancer has favorable histology, is hyperdiploid (excess DNA) and has no extra copies of the <i>MYCN</i> gene</li> </ul>
<b>Intermediate risk</b>	<ul style="list-style-type: none"> <li>• Any child who is Stage 3, younger than age 1, whose cancer has no extra copies of the <i>MYCN</i> gene</li> <li>• Any child who is Stage 3, older than age 1, whose cancer has no extra copies of the <i>MYCN</i> gene and has favorable histology (appearance under the microscope)</li> <li>• Any child who is Stage 4, younger than age 1, whose cancer has no extra copies of the <i>MYCN</i> gene</li> <li>• Any child who is Stage 4S (younger than age 1), whose cancer has no extra copies of the <i>MYCN</i> gene and has normal DNA ploidy (number of chromosomes) and/or has unfavorable histology</li> </ul>
<b>High risk</b>	<ul style="list-style-type: none"> <li>• Any child who is Stage 2A or 2B, older than age 1, whose cancer has extra copies of the <i>MYCN</i> gene</li> <li>• Any child who is Stage 3, younger than age 1, whose cancer has extra copies of the <i>MYCN</i> gene</li> <li>• Any child who is Stage 3, older than age 1, whose cancer has extra copies of the <i>MYCN</i> gene</li> <li>• Any child who is Stage 3, older than 18 months of age, whose cancer has unfavorable histology</li> <li>• Any child who is Stage 4, whose cancer has extra copies of the <i>MYCN</i> gene regardless of age</li> <li>• Any child who is Stage 4 and older than 18 months</li> <li>• Any child who is Stage 4 and between 12 and 18 months old whose cancer has extra copies of the <i>MYCN</i> gene, unfavorable histology, and/or normal DNA ploidy (a DNA index of 1)</li> <li>• Any child who is Stage 4S (younger than age 1), whose cancer has extra copies of the <i>MYCN</i> gene</li> </ul>



**Table 1.6:** International Neuroblastoma Risk Group (INRG) classification

Classification
<ul style="list-style-type: none"> <li>• The child's age</li> <li>• Tumour histology</li> <li>• The presence or absence of <i>MYCN</i> gene amplification</li> <li>• Certain changes in chromosome 11 (known as an 11q aberration)</li> <li>• DNA ploidy (the total number of chromosomes in the tumour cells)</li> </ul>
<p>The INRG classification uses these factors to put children into 16 different pre-treatment groups (lettered A through R). Each of these pretreatment groups falls into 1 of 4 overall risk groups:</p> <ul style="list-style-type: none"> <li>• Very low risk</li> <li>• Low risk</li> <li>• Intermediate risk</li> <li>• High risk</li> </ul> <p>This system has not yet been widely adopted, but it is being researched in new treatment protocols.</p>

Elevated levels of non-specific biomarkers such as lactate dehydrogenase (>1500 U/ml),<sup>65</sup> ferritin (>142 ng/ml),<sup>66</sup> and neuron-specific enolase (>100 ng/ml) may be correlated with advanced stage neuroblastoma and/or relapse.<sup>54,65,67-71</sup> A computed tomography (CT) and functional single-photon emission computed tomography (SPECT) or positron emission tomography (PET) scan of the neck, chest and abdomen is the gold standard for diagnostic imaging as it can concurrently focus the tumour and determine the degree of disease progression.<sup>48,50,59</sup> Ultrasound may be used primarily to differentiate the tumour.<sup>48,72</sup> Magnetic resonance imaging (MRI) may be beneficial if there is concern for spinal extension, and imaging of the brain is only necessary in the setting of neurological symptoms.<sup>54,55,73-76</sup>

While not routinely used, a <sup>123/131</sup>I-radiolabelled meta-iodobenzylguanidine (MIBG) scan is valuable in both the detection of primary tumours and metastases since MIBG is a norepinephrine analogue that is selectively concentrated in sympathetic nervous tissue. MIBG has also proven exceptionally practical in surveillance of patient treatment responses and disease recurrence.<sup>54,55,57,76-80</sup> While MIBG is generally more sensitive for the detection of lesions, fluorodeoxyglucose positron emission tomography (FDG-PET) may be better at localizing soft tissue metastases.<sup>50,56,57</sup>



**Table 1.7:** Prognostic markers for neuroblastoma

Marker	Clinical Significance
<b>Age</b>	Younger children (under 12-18 months) are more likely to be cured than older children.
<b>Tumour histology</b>	Tumour histology is based on how the neuroblastoma cells look under the microscope. Tumours that contain more normal-looking cells and tissues tend to have a better prognosis and are said to have a favourable histology. Tumours whose cells and tissues look more abnormal under a microscope tend to have a poorer prognosis and are said to have an unfavourable histology.
<b>DNA ploidy</b>	The amount of DNA in each cell, known as ploidy or the DNA index, can be measured using special lab tests, such as flow cytometry or imaging cytometry. Neuroblastoma cells with about the same amount of DNA as normal cells (a DNA index of 1) are classified as diploid. Cells with increased amounts of DNA (a DNA index higher than 1) are termed hyperdiploid. In infants, hyperdiploid cells tend to be associated with earlier stages of disease, respond better to chemotherapy, and usually predict a more favourable prognosis (outcome) than diploid cells. Ploidy is not as useful a factor in older children.
<b>MYCN gene amplifications</b>	<i>MYCN</i> is an oncogene, a gene that helps regulate cell growth. Changes in oncogenes can make cells grow and divide too quickly, as with cancer cells. Neuroblastomas with too many copies (amplification) of the <i>MYCN</i> oncogene tend to grow quickly and are less likely to mature. Children whose neuroblastomas have this feature tend to have a worse prognosis than other children with neuroblastoma.
<b>Chromosome changes</b>	Tumour cells that are missing certain parts of chromosomes 1 or 11 (known as 1p deletions or 11q deletions) may predict a less favorable prognosis. It is thought that these chromosome parts, which are missing in many neuroblastomas, may contain important tumour suppressor genes (TSGs), but more studies are needed to verify this. Having an extra part of chromosome 17 (17q gain) is also linked with a worse prognosis. This probably means that there is an oncogene in this part of chromosome 17.
<b>Neurotrophin (nerve growth factor) receptors</b>	These are substances on the surface of normal nerve cells and on some neuroblastoma cells. They normally allow the cells to recognize neurotrophins—hormone-like chemicals that help the nerve cells mature. Neuroblastomas that have more of certain neurotrophin receptors, especially the nerve growth factor receptor called TrkA, may have a better prognosis.
<b>Serum markers</b>	Serum (blood) levels of certain substances can be used to help predict prognosis. Neuroblastoma cells release ferritin, an important regulator of the body's normal iron metabolism, into the blood. Patients with high ferritin levels tend to have a worse prognosis. Neuron-specific enolase (NSE) and lactate dehydrogenase (LDH) are synthesized by normal cells as well as by NB cells. Increased levels of NSE and LDH in the blood are often linked with a worse outlook in children with NB. A substance on the surface of many nerve cells known as ganglioside GD2 is often increased in the blood of NB patients. Although the usefulness of GD2 in predicting prognosis is unknown, it may turn out to be more important in treating NB.

**Table 1.8:** Survival of neuroblastoma patients by Children's Oncology Group (COG) risk group

Risk Group	5-Year Survival Rate
Low	Higher than 95%
Intermediate	90% to 95%
High	40% to 50%
Source: <i>Neuroblastoma Summary</i> , American Cancer Society ( <a href="http://www.cancer.org/">http://www.cancer.org/</a> )	

**Table 1.9:** Other autonomic nervous system tumours in children

Tumour	Features
Ganglioneuroma	A benign (non-cancerous) tumour made up of mature ganglion and nerve sheath cells.
Ganglioneuroblastoma	<p>A tumour that has both malignant and benign parts. It contains neuroblasts (immature nerve cells) that can grow and spread abnormally, similar to neuroblastoma, as well as areas of more mature tissue that are similar to ganglioneuroma.</p> <p>Ganglioneuromas are usually removed by surgery and looked at carefully under a microscope to be sure they do not have areas of malignant cells (which would give rise to a ganglioneuroblastoma). If the final diagnosis is ganglioneuroma, no other treatment is needed. If it's found to be a ganglioneuroblastoma, it's treated the same as a neuroblastoma.</p>
Source: <i>Neuroblastoma Summary</i> , American Cancer Society ( <a href="http://www.cancer.org/">http://www.cancer.org/</a> )	

**Table 1.10:** Contemporary procedures and approaches used in confirming a diagnosis of neuroblastoma

Procedure/Approach	Diagnostic Significance/Prognostic Value
<b>Medical history and physical examination</b>	If the child presents with signs or symptoms that might suggest neuroblastoma, a complete medical history, as well as a family history of any type of cancer, is indispensable. Possible signs of a neuroblastoma are an abnormal mass or swelling in the body, lumps or bumps under the skin or high blood pressure. Neuroblastomas that grow adjacent to the spinal cord can affect the movement and strength in the child's arms and legs, so particular attention has to be paid to these. Some signs that could be caused by neuroblastoma, such as fever and enlarged lymph nodes, are much more likely to be caused by an infection, so it is prudent to look for other signs of infection at first. If the history and examination imply a child might have a neuroblastoma (or another type of tumour), other specialized tests will be mandatory, including blood and urine tests, imaging tests, and biopsies. These tests are important because many of the symptoms and signs of neuroblastoma can also be caused by other diseases, such as infections, or even other types of cancer.
<b>Blood and urine catecholamine tests</b>	Sympathetic nerve cells normally release hormones called catecholamines, such as epinephrine (adrenaline) and norepinephrine (noradrenaline) into the blood. Eventually the body degrades these into metabolites which is secreted in urine. Neuroblastoma cells can also synthesize these hormones. In most cases, neuroblastoma cells make enough catecholamines to be detected by blood or urine tests. The 2 catecholamine metabolites most often measured are homovanillic acid (HVA) and vanillylmandelic acid (VMA).
<b>Other lab tests</b>	If neuroblastoma is suspected or has been found in a child, the doctor will order blood tests to check blood cell counts, liver and kidney function, and the balance of salts (electrolytes) in the body. A urinalysis (urine test) may also be done to further check kidney function.
<b>Imaging tests</b>	<p>Imaging tests use X-rays, magnetic fields, sound waves, or radioactive substances to create pictures of the inside of the body. Imaging tests can be performed for a number of rationales, including to help find out if a suspicious area might be cancerous, to learn how far cancer has spread, to help determine if treatment has been effective. Most children who have or might have neuroblastoma will have one or more of these tests. Children with neuroblastoma are often very young, so it can be hard to perform some of these tests.</p> <p><b>Ultrasound</b></p> <p>Ultrasound is often one of the first tests done in small children if a tumour is suspected, because it is fairly quick and easy, it does not use radiation, and it can often give the doctor a good view inside the body, especially in the abdomen (belly). This test uses sound waves to create pictures of organs or masses inside the body. For this test, the child lies on a table (or sits) while a small wand called a transducer is placed on the skin over the belly (which is first lubricated with gel).</p>

Continued/...

**Table 1.10:** Contemporary procedures and approaches used in confirming a diagnosis of neuroblastoma (continued)

Procedure/Approach	Diagnostic Significance/Prognostic Value
<b>Imaging tests (continued)</b>	<p data-bbox="862 475 1108 499"><b>Ultrasound (continued)</b></p> <p data-bbox="862 518 2033 738">The wand gives off sound waves and picks up the echoes as they bounce off organs. The echoes are converted by a computer into a black and white image on a screen. The test is not usually painful, but it might cause some discomfort if the transducer is pressed down hard on the belly. Ultrasound is used most often to look for tumours in the abdomen. It's not used to look in the chest because the ribs block the sound waves. Ultrasound can detect if kidneys have become swollen because the outflow of urine has been blocked by enlarged lymph nodes or a mass. It can also be used to help guide a biopsy needle into a suspected tumour to get a sample for testing. It is particularly useful in checking to see if tumours in the abdomen are shrinking. The pictures from ultrasound are not as detailed as those from some other tests, so even if a tumour is found, computed tomography (CT) or magnetic resonance imaging (MRI) scans (described below) might still be needed.</p> <p data-bbox="862 758 936 782"><b>X-rays</b></p> <p data-bbox="862 801 2033 991">The doctor may also order an X-ray of the chest or another part of the body as an early test if a child is having symptoms but it is not clear what might be causing them. But the images might not always be detailed enough to spot tumours. If neuroblastoma has already been diagnosed, X-rays can be useful to see if cancer has spread to certain bones. An X-ray of the head may be done to see if cancer has spread to the skull bones. A meta-iodobenzylguanidine (MIBG) scan or a bone scan (described below) is usually better for looking at the bones in the rest of the body, but X-rays may be used in infants, where these scans might not be possible. A standard chest X-ray may be done if doctors suspect that the tumour has invaded the lungs, but a CT or MRI scan of the chest can show the area in more detail.</p> <p data-bbox="862 1010 1299 1034"><b>Computed tomography (CT or CAT) scan</b></p> <p data-bbox="862 1053 2033 1329">CT scans are often used to look for neuroblastoma in the abdomen, pelvis and chest. The CT scan is an X-ray test that produces detailed cross-sectional images of parts of the body. Instead of taking one picture, like a regular X-ray, a CT scanner takes many pictures as it rotates around the child while s/he lies on a table. A computer then combines these pictures into images showing slices of the part of the body being studied. Unlike a regular X-ray, a CT scan creates detailed images of the soft tissues in the body. Before the test, the child may be asked to drink a contrast solution and/or get an intravenous (IV) injection of a contrast dye. This helps better outline structures in the body. The contrast may cause some flushing (a feeling of warmth, especially in the face). Some people are allergic and get hives. Rarely, more serious reactions like laboured breathing or low blood pressure can occur. The doctor needs to ascertain if the child has any allergies or has ever had a reaction to any contrast material used for X-rays. CT scans take longer than regular X-rays. Younger children may be sedated before the test to reduce movement and help make sure the pictures come out well.</p>

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**Table 1.10:** Contemporary procedures and approaches used in confirming a diagnosis of neuroblastoma (continued)

Procedure/Approach	Diagnostic Significance/Prognostic Value
<b>Imaging tests (continued)</b>	<p><b>CT-guided needle biopsy</b></p> <p>CT scans can also be used to help guide a biopsy needle into a tumour. For this procedure, the child lies on the CT scanning table while a radiologist advances a biopsy needle through the skin and toward the mass. CT scans are repeated until the needle is within the mass. A biopsy sample is then removed and looked at under a microscope. In children, this procedure is always done under general anaesthesia.</p> <p><b>Magnetic resonance imaging (MRI) scan</b></p> <p>MRI scans provide detailed images of soft tissues in the body. These scans are very helpful in looking at the brain and spinal cord. They may be slightly better than CT scans for seeing the extent of a neuroblastoma tumour, especially around the spine, but this test can be harder to do in small children. MRI scans use radio waves and strong magnets to create the images instead of x-rays, so there is no radiation. A contrast material called gadolinium may be injected into a vein before the scan to better see details, but this is needed less often than with a CT scan. It usually does not cause allergic reactions, but it can cause other problems in children with kidney disease, so doctors are careful when they use it. MRI scans take longer than CT scans, often up to an hour. For most MRI machines, the child has to lie inside a narrow tube, which is confining and can be distressing. Newer, more open MRI machines may be an option in some cases, but they still require the child to stay still for long periods of time. The MRI machine also makes loud buzzing and clicking noises that may be disturbing. Younger children are often given medicine to help keep them calm or even asleep during the test.</p> <p><b>Meta-iodobenzylguanidine (MIBG) scan</b></p> <p>This scan uses a form of the chemical meta-iodobenzylguanidine (MIBG) that contains a small amount of radioactive iodine. MIBG is similar to norepinephrine, a hormone produced by sympathetic nerve cells. It is injected into a vein and travels through the blood, and in most patients it will attach to neuroblastoma cells anywhere in the body. Several hours or days later, the body is scanned with a special camera to look for areas that incorporated the radioactivity. This helps doctors tell where the neuroblastoma is and whether it has spread to the bones and/or other parts of the body. This test is preferred by many doctors as a standard test in children with neuroblastoma. It can be repeated after treatment to see if it has been effective. It is also good to know if the tumour takes up the MIBG because in some cases, this radioactive molecule can be used at higher doses to treat the neuroblastoma.</p>

Continued/...

**Table 1.10:** Contemporary procedures and approaches used in confirming a diagnosis of neuroblastoma (continued)

Procedure/Approach	Diagnostic Significance/Prognostic Value
<b>Imaging tests (continued)</b>	<p><b>Positron emission tomography (PET) scan</b></p> <p>For a PET scan, a radioactive substance, usually a glucose analogue, known as fluorine-18-fluorodeoxy-glucose (18F-FDG) is injected into the blood. The amount of radioactivity used is very low and will pass out of the body within a day or so. Because cancer cells in the body are growing quickly, they absorb large amounts of the radioactive sugar. After about an hour, your child will be moved onto a table in the PET scanner. He or she will lie on the table for about 30 minutes while a special camera creates a picture of areas of radioactivity in the body. Younger children may be given medicine to help keep them calm or even asleep during the test. The picture from a PET scan is not as detailed as a CT or MRI scan, but it can provide helpful information about the whole body. Some newer machines can do a PET and CT scan at the same time (PET/CT scan). This lets the doctor compare areas of higher radioactivity on the PET scan with the more detailed appearance of that area on the CT scan.</p> <p><b>Bone scan</b></p> <p>A bone scan can help show if a cancer has spread to the bones, and can provide a picture of the entire skeleton at once. Neuroblastoma often causes bone damage, which a bone scan can find. This test used to be done routinely, but in some centers it has been replaced by use of MIBG or PET scans. For this test, a small amount of low-level radioactive material (technetium-99) is injected into a vein. (The amount of radioactivity used is very low and will pass out of the body within a day or so.) The substance settles in areas of damaged bone throughout the skeleton over the course of a couple of hours. Your child then lies on a table for about 30 minutes while a special camera detects the radioactivity and creates a picture of the skeleton. Younger children may be given medicine to help keep them calm or even asleep during the test. Areas of active bone changes attract the radioactivity and appear as “hot spots” on the skeleton. These areas may suggest cancer, but other bone diseases can also cause the same pattern. To help tell these apart, other imaging tests such as plain x-rays or MRI scans, or even a bone biopsy might be needed.</p>
<b>Biopsies</b>	<p>Examinations and tests might strongly suggest a child has neuroblastoma, but a biopsy (removing some of the tumour for viewing under a microscope and other lab testing) is often done to be sure. During a biopsy, the doctor removes a sample of the tumour mass. In adults, biopsies are sometimes done using local anesthetic (numbing medicine), but in children they are more often done while the child is under general anaesthesia. There are 2 main types of biopsies:</p> <p><b>Incisional (open or surgical) biopsy</b></p> <p>This type of biopsy is done by removing a piece of the tumour through an incision (cut) in the skin. For tumours deep in the body this may be done laparoscopically using long, thin surgical tools inserted through small cuts in the skin.</p>

Continued/...

**Table 1.10:** Contemporary procedures and approaches used in confirming a diagnosis of neuroblastoma (continued)

Procedure/Approach	Diagnostic Significance/Prognostic Value
<b>Biopsies (continued)</b>	<p data-bbox="862 416 1093 440"><b>Needle (closed) biopsy</b></p> <p data-bbox="862 461 2033 651">For this type of biopsy, a thin, hollow needle is placed through the skin and into the tumour to remove a small sample. If the tumour is deep within the body, CT scans or ultrasound can be used to help guide the needle into the tumour. The biopsy samples are sent to a lab, where they are viewed under a microscope by a pathologist (a doctor with special training in identifying cancer cells). Some neuroblastomas are easily recognized when looked at by experienced doctors. But some may be hard to tell apart from other types of child cancers. In these cases, special lab tests must be done to show the tumour is a neuroblastoma. Other lab tests may also be done on neuroblastoma samples to help determine how quickly the tumour is likely to grow.</p> <p data-bbox="862 671 1234 695"><b>Bone marrow aspiration and biopsy</b></p> <p data-bbox="862 716 2033 1018">Neuroblastoma often spreads to the bone marrow (the soft inner parts of certain bones). If blood or urine levels of catecholamines are increased, then finding cancer cells in a bone marrow sample is enough to diagnose neuroblastoma (without getting a biopsy of the main tumour). If neuroblastoma has already been diagnosed by a biopsy done elsewhere in the body, bone marrow tests are done to help determine the extent of the disease. A bone marrow aspiration and biopsy are usually done at the same time. In most cases the samples are taken from the back of both of the pelvic (hip) bones. Even when the area is numbed with local anesthetic, these tests can be painful, so in most cases the child is also given other medicines to reduce pain or even be asleep during the procedure. For a bone marrow aspiration, a thin, hollow needle is inserted into the bone and a syringe is used to suck out a small amount of liquid bone marrow. A bone marrow biopsy is usually done just after the aspiration. A small piece of bone and marrow is removed with a slightly larger needle that is pushed down into the bone. Once the biopsy is done, pressure is applied to the site to help stop any bleeding. Samples from the bone marrow are sent to a lab, where they are looked at and tested for the presence of cancer cells.</p>
<p data-bbox="203 1050 2033 1150"><b>Source:</b> <i>Neuroblastoma Summary</i>, American Cancer Society (<a href="http://www.cancer.org/">http://www.cancer.org/</a>). For detailed references, see text. Examples of more general references have been published previously.<sup>47,52,62</sup> See also PDQ Screening and Prevention Editorial Board. Neuroblastoma Screening (PDQ®): Health Professional Version. 2014 Feb 6. In: PDQ Cancer Information Summaries [Internet]. Bethesda (MD): National Cancer Institute (US); 2002-. Available from: <a href="http://europepmc.org/books/NBK66025">http://europepmc.org/books/NBK66025</a> and PDQ Pediatric Treatment Editorial Board. Neuroblastoma Treatment (PDQ®): Health Professional Version. 2016 Jan 14. In: PDQ Cancer Information Summaries [Internet]. Bethesda (MD): National Cancer Institute (US); 2002-. Available from: <a href="http://europepmc.org/books/NBK65747">http://europepmc.org/books/NBK65747</a>.</p>	



Despite advances in diagnostic medicine, the diagnosis of neuroblastoma can only be confirmed pathologically with tissue obtained from the tumour or bone marrow. Specimens can be obtained either during resection of the primary tumour or as an open biopsy for unresectable disease. Bilateral posterior iliac crest marrow aspirates are required to exclude metastatic disease. Molecular studies, such as fluorescence in situ hybridization (FISH), can be performed on tissue samples to note ploidy and other chromosomal aberrations.<sup>81,82</sup> Recently, expression of the insulin-like growth factor-2 mRNA-binding protein 1 (*IGF2BP1*) gene has been found to be associated with more advanced tumours and decreased patient survival in neuroblastoma, suggesting its prognostic value.<sup>83,84</sup>

## A9.2 Histopathology of Neuroblastoma

Neuroblastoma, ganglioneuroblastoma and ganglioneuroma are classified as peripheral neuroblastic tumours (pNTs) which constitute a clinically and genomically complex disease. The pNTs represent significant disease models for analyzing the biologic and prognostic relationships between molecular/genomic alterations and accompanying morphological appearances. The International Neuroblastoma Pathology Classification (INPC) is particularly useful for patient stratification and protocol assignment in clinical trials of the Children's Oncology Group.<sup>85-91</sup> Table 1.11 summarizes the INPC classification of neuroblastic tumours and Table 1.12 shows a comparison between the categories and subtypes used in the INPC and the original Shimada classification.<sup>92,93</sup>

Joshi and co-authors<sup>94</sup> advocated minor modifications to the terminology of the Shimada classification to include “borderline” ganglioneuroblastoma for the “stroma-rich, well-differentiated” subtype which is now called “ganglioneuroma (Schwannian stroma-dominant), maturing” subtype in the INPC. Morphologic confirmation of the Schwannian stroma-poor, stroma-rich, and stroma-dominant categories, as well as among subtypes in each category, may be challenging since the subtypes may express stages of a biologic and morphologic continuum. Likewise, macroscopic categorizing a nodular lesion of the composite tumour may

be arduous.<sup>90,91,93</sup> The INPC further differentiates between ‘favourable’ and ‘unfavourable’ histology groups<sup>95</sup> based on the age-linked morphological changes<sup>96</sup> (cut-offs of 18 and 60 months at diagnosis) by three major biologic/molecular mechanisms:

1. **Cross-talk between neuroblastic cells and Schwann cells** essential for tumour maturation; three categories, i.e., neuroblastoma (Schwannian stroma-poor), ganglioneuroblastoma-intermixed (Schwannian stroma-rich), and ganglioneuroma (Schwannian stroma-dominant), are defined;
2. **High-affinity nerve growth factor (NGF; trkA/II)** expression critical for neuroblastic differentiation; 3 subtypes, i.e., undifferentiated, poorly differentiated, and differentiating, are defined in the neuroblastoma category; and
3. **MYCN amplification** as the powerful driving force for preventing neuroblastic differentiation and promoting mitotic and karyorrhectic activities; 3 classes of MKI (mitosis-karyorrhexis index), i.e., low <100/5000 cells, intermediate 100–200/5000 cells, and high >200/5000 cells, are defined in the neuroblastoma category. The INPC also includes the fourth category – ganglioneuroblastoma, nodular (composite, Schwannian stroma-dominant/stroma-rich and stroma-poor).<sup>85,97</sup>

A new subtype of large nucleolar neuroblastoma (LNN) in the NB category have a characteristic nucleus containing large and prominent nucleoli, but do not show cytoplasmic enlargement/maturation. LCN can well be included in this group of LNN, as a large cell variant of undifferentiated/poorly differentiated NB.<sup>98</sup> Representative images of the histology of peripheral neuroblastic tumours are shown in Figures 1.4 and 1.5. Neuroblastoma predominantly comprises neuroblasts at different stages of differentiation and a varying amount of Schwannian-like stroma. The proportion of both cell types fluctuates according to the degree of tumour maturation and a correlation exists between the degree of differentiation of the neuroblastic subtype, the proportion of the Schwannian-like stroma and disease

prognosis. Undifferentiated stroma-poor NB is the most malignant and the stroma rich ganglioneuroma (GN) is a benign form. The relationship between Schwannian-like stromal cells and neuroblastic cells needs further clarification.

The amount of Schwannian-like stroma in the tumour is associated with better prognosis since it is thought that Schwannian-like cells may downregulate tumour growth signal transduction pathways by secreting antiproliferative and/or antiangiogenic factors. In this regard, experimental evidence shows that when co-cultured *in vitro*, neuroblasts derived from neuroblastoma tumours can enhance the proliferation of Schwann cells. Schwannian-like stromal cells in neuroblastic tumours are likely to be reactive in nature and may have been recruited from normal tissue.<sup>95,101</sup>

### **A10. Clinical Presentation, Signs and Symptoms of Neuroblastoma**

The signs and symptoms of neuroblastoma vary widely, depending on the size of the tumour, where it is, how far it has spread, and if the tumour cells secrete hormones.<sup>62</sup> Many of the signs and symptoms below are more likely to be caused by factors other than neuroblastoma. For example, a neuroblastoma may arise from sympathetic nervous tissue anywhere in the body, but most often develops in the abdomen. The presentation depends on the local effects of the solid tumour and any metastases. An abdominal mass in a child may also be due to Wilms' tumour (also known as nephroblastoma).<sup>73</sup> This neoplasm may present with renal signs and symptoms, such as hypertension,<sup>102</sup> haematuria and abdominal pain.<sup>11,103-107</sup>

Multiple factors play a role in a patient's clinical presentation since it depends largely on tumour location, size, degree of invasion, effects from catecholamine secretion,<sup>108</sup> and symptoms due to paraneoplastic syndromes.<sup>109,110</sup> Nearly 65% of tumours arise in the abdomen with half of those localized to the medulla of the adrenal gland.<sup>59,111-113</sup> However, they can occur in the neck (5%), chest (20%), or pelvis (5%), and 1% of patients have no detectable primary tumours.<sup>114</sup>

**Table 1.11:** INPC classification of neuroblastic tumours

<b>International Neuroblastoma Pathology Classification</b>				
<b>Category</b>	<b>Favorable Histology</b>		<b>Unfavorable Histology</b>	
Neuroblastoma (Schwannian stroma-poor)	Grade of Differentiation	MKI	Grade of Differentiation	MKI
Age <18 months	poorly differentiate differentiating	low or intermediate low or intermediate	undifferentiated poorly differentiate differentiating	any high high
Age 18 – 60 months	differentiating	low	undifferentiating poorly differentiated differentiating	any any intermediate or high
Age ≥60 months	–	–	any	any
Ganglioneuroblastoma Intermixed (Schwannian stroma-rich)	all cases		–	–
Ganglioneuroma (Schwannian stroma-dominant)	all cases		–	–
Ganglioneuroblastoma, Nodular (composite, Schwannian stroma-rich/ stroma-dominant and stroma-poor)	favorable subset		unfavorable subset	

*MKI: Mitosis-Karyorrhexis Index, Low <100/5,000 cells, Intermediate 100-200/5,000 cells, High >200/5,000 cells*  
*Ganglioneuroblastoma, Nodular: Distinction of Favorable and Unfavorable subsets by the same criteria of age-appropriate evaluation of Grade of Differentiation and MKI as used for the Neuroblastoma category.*

Source:<sup>86</sup>

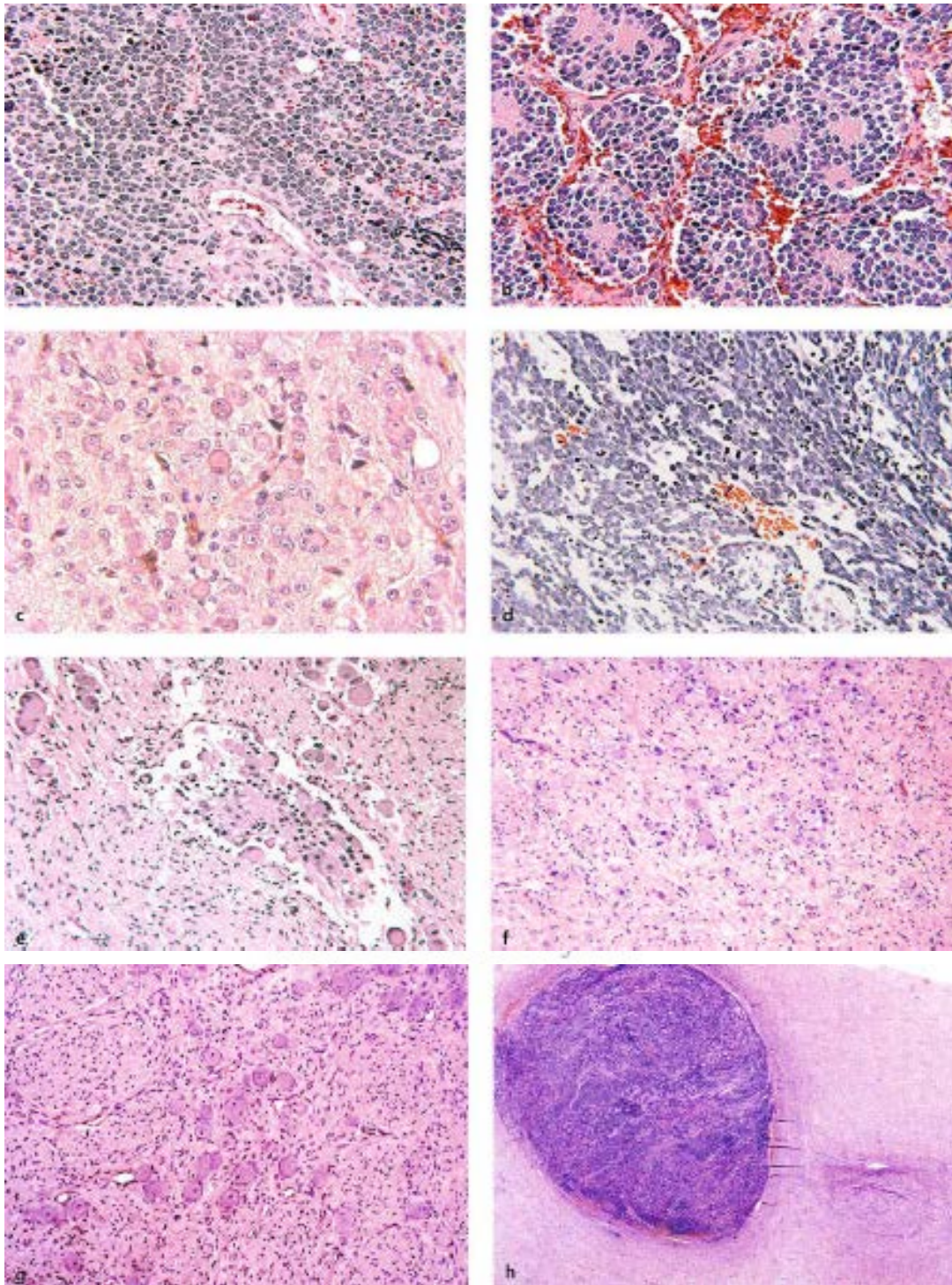
**Table 1.12:** Comparison of the INPC and the Shimada classification of neuroblastic tumours

International neuroblastoma pathology classification		
Schwannian (ganglioneuromatous) development	Category and subtype	Shimada classification
None to minimal	Neuroblastoma (Schwannian stroma-poor)	Stroma-poor
	Undifferentiated Poorly differentiated	Undifferentiated
None to minimal to <50% of the tumor tissue	Differentiating	Differentiating
	Ganglioneuroblastoma, intermixed (Schwannian stroma-rich) Ganglioneuroma, (Schwannian stroma-dominant)	Stroma-rich Intermixed
Dominant	Maturing <sup>a</sup>	Stroma-rich Well differentiated
	Mature	Ganglioneuroma
Proportion of Schwannian stroma-rich/stroma dominant and stroma-poor (nodular) areas, variable	Ganglioneuroblastoma, nodular (Composite Schwannian stroma-rich/stroma-dominant and stroma-poor)	Stroma-rich Nodular

<sup>a</sup> This subtype is termed "borderline" ganglioneuroblastoma, according to the Joshi classification.

Source:<sup>90</sup>

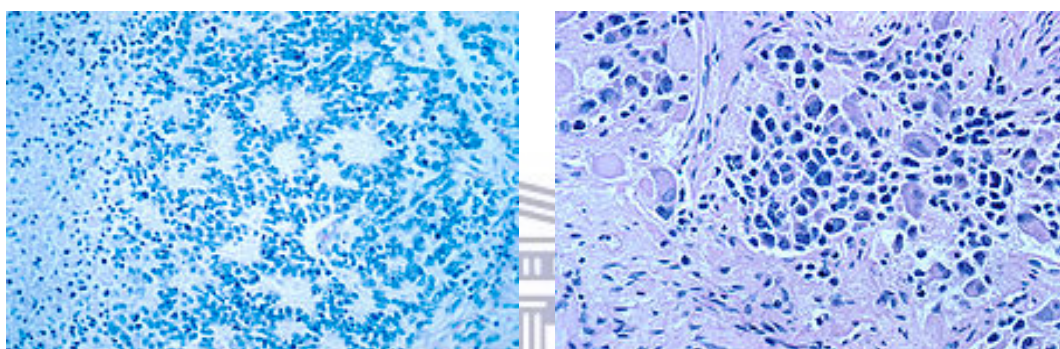
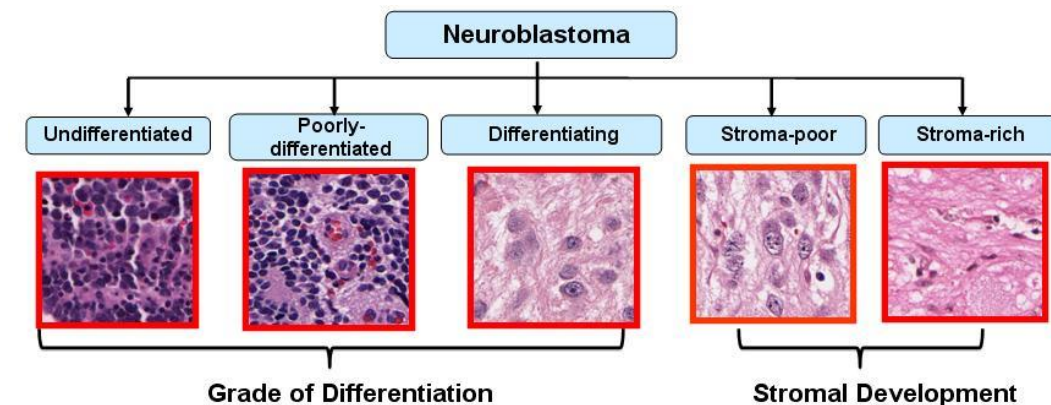




**Source:**<sup>91</sup> a Neuroblastoma (Schwannian stroma-poor), undifferentiated subtype. b Neuroblastoma (Schwannian stroma-poor), poorly differentiated subtype. c Neuroblastoma (Schwannian stroma-poor), differentiating subtype. d Neuroblastoma (Schwannian stroma-poor) with a high mitosis–karyorrhexis index. e Ganglioneuroblastoma, intermixed (Schwannian stroma-rich). f Ganglioneuroma (Schwannian stroma-dominant), maturing subtype. g Ganglioneuroma (Schwannian stroma-dominant), mature subtype. h Ganglioneuroblastoma, nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor).

**Figure 1.4:** Histology of peripheral neuroblastic tumours





**Neuroblastoma with rosette formation (x40)**

**Stroma-rich ganglioneuroblastoma (x40)**

**Sources:** <https://visualsonline.cancer.gov/details.cfm?imageid=2593>; On microscopy, the tumour cells are typically described as small, round and blue, and rosette patterns (Homer-Wright rosettes) may be seen. Homer-Wright rosettes are tumour cells around the neuropil, not to be confused with pseudorosettes, which are tumour cells around a blood vessel. Two typical true rosettes in retinoblastoma occur in the form of Flexner-Wintersteiner and Homer-Wright rosettes (<http://www.pathologystudent.com/?p=5400>; <https://en.wikipedia.org/wiki/Neuroblastoma#Histology>).<sup>99,100</sup>

**Figure 1.5:** Microscopic views of typical neuroblastoma histopathology

Many patients are asymptomatic, yet some may present with constitutional symptoms (malaise, fevers, and weight loss), an enlarging mass, pain, abdominal distension, lymphadenopathy, or respiratory distress secondary to compression or hepatomegaly. Pelvic masses may cause constipation or difficulty urinating, while thoracic involvement can cause dysphagia, dyspnea, or rarely, thoracic outlet syndrome. For cervical tumours, a patient develops Horner's syndrome,<sup>52</sup> and in up to 15% of patients, epidural extension may result in neurological deficits such as progressive paralysis.<sup>1,11,37,46,115-117</sup>



At the time of diagnosis, 50% of patients present with localized disease while 35% already have regional lymph node spread. Metastasis can occur by haematogenous and/or lymphatic route, seeding bone marrow,<sup>118</sup> liver, and bone. NBs originating from cells of the primitive neural crest eventually populate the sympathetic ganglia and the inner adrenal gland. In approximately one-half of cases, the primary tumour arises at the level of the paravertebral ganglia and may infiltrate the adjacent intervertebral foramina and compress the intraspinal structures. Although modern imaging studies document the infiltration of the intervertebral foramina by the tumour in at least one-third of neuroblastomas, only 5–7% of the cases develop symptoms related to epidural compression (EC).<sup>119</sup>

In these instances, various neurological deficits can ensue which may progressively worsen, and could end in paraplegia.<sup>74</sup> The signs of EC are difficult to detect in an early phase, especially among the youngest children, and this may account for the frequent delay in diagnosis and thus lead to development of permanent neurological impairment. Several studies have analyzed the outcome of various types of treatment for EC. Neurosurgical decompression, chemotherapy and radiation therapy have all proven to be effective in relieving the symptoms.

Although the occurrence of short-term sequelae were reported in some publications, only one study addressed the issue of the authors found that the majority of children actually did recover normal neurological function, but they developed an excess of spinal deformities, in particular when treatment included laminectomy.<sup>119-121</sup> Commonly, the orbits are involved, which manifests as periorbital swelling and proptosis (“raccoon eyes”).<sup>64</sup>

When dissemination occurs to the skin, patients develop blue subcutaneous nodules known as blueberry muffin syndrome. Surprisingly, this is associated with a favourable prognosis with likely spontaneous tumour regression. Because of its neuroendocrine properties, neuroblastoma has the potential to secrete catecholamines, which results in early-onset hypertension and tachycardia.<sup>108,122</sup> Patients may also experience paraneoplastic

syndromes.<sup>110,122</sup> Examples include intractable diarrhoea with electrolyte disturbances due to release of vasoactive intestinal peptide (VIP),<sup>123</sup> encephalomyelitis, or sensory neuropathy. There have been reports of the development of opsoclonus-myoclonus syndrome (OMS),<sup>115,124</sup> which occurs when antibodies cross-react with cerebellar tissue.<sup>1,125-127</sup> The characteristic symptoms and signs of OMS include rapid, conjugate eye nystagmus with involuntary spasms of the limbs. Interestingly, the patients with intractable diarrhoea due to VIP secretion or OMS generally tend to present with less aggressive neuroblastomas. Thus far, symptomatic paraneoplastic syndromes are rarely diagnosed (prevalence < 0.01% of all cancer), but they may indicate early signs of disease relapse.<sup>1,128</sup>

## **A10.1 Signs or Symptoms Caused by the Main Tumour**

### **A10.1.1 Tumours in the Abdomen or Pelvis**

One of the most common signs of a neuroblastoma is a large lump or swelling in the child's abdomen.<sup>129</sup> The child might not want to eat (which can lead to weight loss). If the child is old enough, s/he may complain of feeling full or having belly pain. But the lump itself is usually not painful to the touch. Sometimes, a tumour in the abdomen or pelvis can affect other parts of the body. For example, tumours that press against or grow into the blood and lymph vessels in the abdomen or pelvis can stop fluids from getting back to the heart. This can sometimes lead to swelling in the legs and, in boys, the scrotum. In some cases, the pressure from a growing tumour can affect the child's bladder or bowel, which can cause problems urinating or having bowel movements.

### **A10.1.2 Tumours in the Chest or Neck**

Tumours in the neck can often be seen or felt as a hard, painless lump. If the tumour is in the chest, it might press on the superior vena cava. This can cause swelling in the face, neck, arms, and upper chest (sometimes with a bluish-red skin colour). It can also cause headaches, dizziness, and a change in consciousness if it affects the brain. The tumour might also press on

the throat or windpipe, which can cause coughing and troubled breathing (dyspnoea) or swallowing. Neuroblastomas that press on certain nerves in the chest or neck can sometimes cause other symptoms, such as a drooping eyelid and a small pupil (the black area in the centre of the eye). Pressure on other nerves near the spine might affect the child's ability to feel or move their arms or legs.

### **A10.2 Signs or Symptoms Caused by Metastatic Spread of the Cancer**

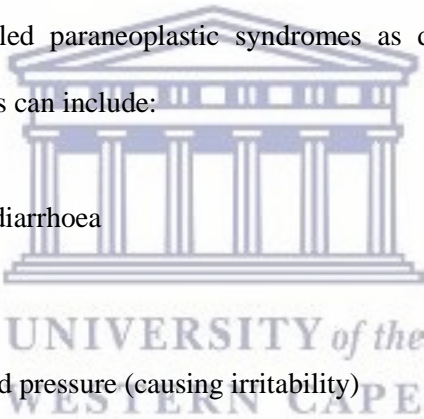
About 2 out of 3 neuroblastomas have already spread to the lymph nodes or other parts of the body by the time they are found. Lymph nodes are bean-sized collections of immune cells found throughout the body. Cancer that has spread to the lymph nodes can cause them to swell. These nodes can sometimes be felt as lumps under the skin, especially in the neck, above the collarbone, under the arm, or in the groin. Enlarged lymph nodes in children are much more likely to be a sign of infection rather than cancer, but they should be checked by a doctor. Neuroblastoma often spreads to bones. A child who can talk may complain of bone pain. The pain may be so bad that the child limps or refuses to walk. If it spreads to the bones in the spine, tumours can press on the spinal cord and cause weakness, numbness, or paralysis in the arms or legs.

Cancer that has spread to the bones and around the eyes is common and can lead to bruising around the eyes or cause an eyeball to stick out slightly. The cancer can also spread to other bones in the skull, causing bumps under the scalp. If the cancer spreads to the bone marrow, the child may not have enough red blood cells, white blood cells, or platelets. These shortages of blood cells can result in tiredness, irritability, weakness, frequent infections, and excessive bruising or bleeding from small cuts or scrapes. Rarely, large tumours can start to break down, leading to a loss of clotting factors in the blood. This can result in a high risk of serious bleeding, which is known as a consumption coagulopathy and can be life threatening. A special widespread form of neuroblastoma (known as stage 4S)<sup>97</sup> occurs only during the first few months of life. In this special form, the neuroblastoma has spread to the liver, to the skin, and/or

to the bone marrow (in small amounts). Blue or purple bumps that look like small blueberries may be a sign of spread to the skin. The liver can become enlarged and can be felt as a mass on the right side of the belly. Sometimes it can grow large enough to push up on the lungs, which can make it hard for the child to breathe. Despite the fact that the cancer is already widespread when it is found, stage 4S neuroblastoma is very treatable, and often shrinks or spontaneously regresses. Almost all children with this form of neuroblastoma can be cured.<sup>12,97,130</sup>

### **A10.3 Signs or Symptoms Caused by Hormones Secreted by the Tumour**

Neuroblastomas sometimes release hormones that can cause problems with tissues and organs in other parts of the body, even though the cancer has not spread to those tissues or organs. These problems are called paraneoplastic syndromes as described above. Symptoms of paraneoplastic syndromes can include:

- 
- ❧ Constant diarrhoea
  - ❧ Fever
  - ❧ High blood pressure (causing irritability)
  - ❧ Rapid heartbeat
  - ❧ Reddening (flushing) of the skin
  - ❧ Sweating

An uncommon set of symptoms is called the opsoclonus-myoclonus-ataxia syndrome (OMS) or “dancing eyes, dancing feet.”<sup>52,64</sup> The child has irregular, rapid eye movements (opsoclonus), twitch-like muscle spasms (myoclonus), and appears uncoordinated when standing or walking (ataxia). S/he may also have trouble speaking. For reasons that are not clear, neuroblastomas that cause this syndrome tend to be less life-threatening than other forms of the disease.

## A11. Molecular Pathogenesis, Genetics and Genomics of Neuroblastoma

Two major causes have been identified in the origin of NB, namely, (i) *familial origin* which is identified in the loss-of-function mutation in the *PHOX2B* gene and (ii) *sporadic origin* which results in chromosomal losses.<sup>131</sup> These will be explained in the following subsections.

### A11.1 Neural Development and Neuroblastoma

In considering neural development, the contribution of the neural crest to sympathetic ganglia and the adrenal gland is important. The majority of NB tumours appear to arise from neural crest-derived cells in the abdomen adjacent to the aorta in the region of the kidney or in the medullary region of the adrenal gland.<sup>6,132,133</sup> Thus, NB is a sympathoadrenal lineage neural crest-derived tumour.<sup>134</sup> The neural crest arises from the dorsal region of the closing neural tube beneath the ectoderm.<sup>135</sup> This transient population of cells produces multipotential progenitor cells that give rise to the peripheral nervous system, the enteric nervous system, pigment cells, Schwann cells, adrenal medullary cells, and cells of the craniofacial skeleton.<sup>135</sup>

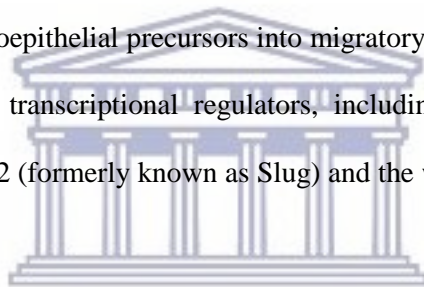
This process is regulated by both extrinsic and intrinsic factors. The Hedgehog and Wnt signaling pathways are especially crucial for proper neural crest development.<sup>5,135,136</sup> Lineage studies in the developing embryo have shown that neural crest cells within the trunk region generate multiple neural crest derivatives such as melanocytes, Schwann cells, glia, and neurons of the dorsal root ganglia. A subset of these trunk crest cells, commonly referred to as the sympathoadrenal lineage, contributes to the sympathetic ganglia and medullary region of the adrenal gland.

This lineage of cells is thought to be the origin of NB.<sup>132,133</sup> However, given the fact that NB can develop anywhere along the sympathetic axis, it is likely that NB can also arise from earlier crest derivatives, before development of the sympathoadrenal lineage but after the initial fate specification. This could contribute to the heterogeneous histology and pathology of NB.<sup>137</sup>

## A11.2 EMT and MET Transitions in the Neural Crest

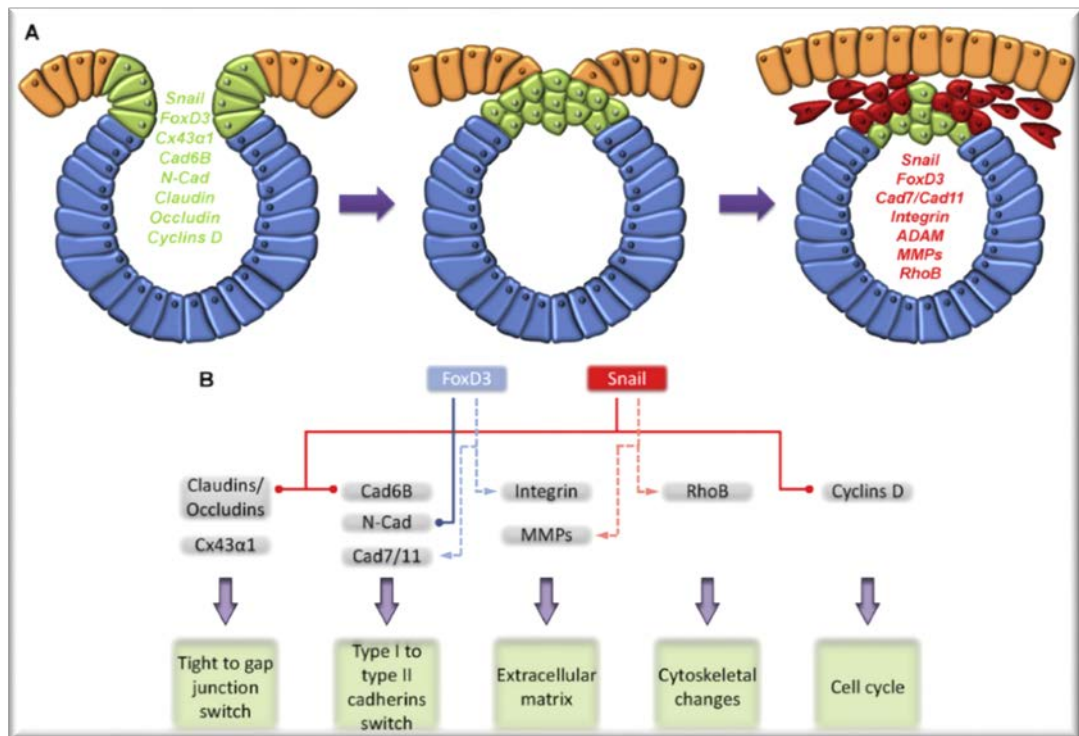
During maturation, the neural crest undergoes programmed epithelial-to-mesenchymal transition (EMT).<sup>138,139</sup> Figure 1.6 is a schematic representation of this process. The progression of NC EMT is synchronized by (i) the coordinated activity of transcription factors and molecular signalling pathways, (ii) changes in cell junctions and polarity, (iii) changes in adhesion properties, and (iv) changes in the extracellular matrix (ECM).

Signalling pathways activated during the course of EMT in the NC are triggered by the integration of ECM signalling molecules and any number of secreted ligands such as members of transforming growth factor beta (TGF $\beta$ ), wingless/integrated proto-oncogene (Wnt) and fibroblast growth factor (FGF) families. These early cellular changes during EMT are essential for the switch from neuroepithelial precursors into migratory NC cells through activation and coordination of several transcriptional regulators, including the zinc finger transcription factors, Snail1 and Snail2 (formerly known as Slug) and the winged-helix transcription factor FoxD3.<sup>139,140</sup>



During embryonic development, mesenchymal transformation involves, among other processes, loss of E-cadherins, loss of cell contacts, activation of matrix metalloproteinases (MMPs). Bone morphogenetic proteins (BMP, multi-functional growth factors that belong to the TGF $\beta$  superfamily), Wnt and FGF signaling within the microenvironment further drive differentiation of these mesenchymal migratory NC cells. The early neural crest is similar to other pluripotent cell populations with regard to their committed self-renewal capacity and selective propensity to generate many different tissue types. Expression of pro-survival and pluripotency factors such as SOX10, FOXD3, C-Myc and MYCN confer on these cells an increased proliferative advantage coupled to an aggressive apoptosis evasive potential.<sup>138,141</sup> Neuroblastoma tumour-initiating cells (TICs) or cancer stem cells (CSCs) derived from diverse environments may direct the clonal evolution of distinct tumour phenotypes according to the developmental stage of their crest precursors.<sup>6,19,142-144</sup>





**Source:**<sup>139</sup> (A) Genes expressed on neural crest cells—prior (green) and after (red)—epithelial-to-mesenchymal transition. (B) Neural crest epithelial-to-mesenchymal transition regulation. NC specifiers, FoxD3 and Snail down-regulate expression of molecules that are associated with epithelial static cell populations, such as N-Cad and E-Cad (or Cad6B in chick and mouse), respectively, to relinquish space to the upregulation of mesenchymal migratory proteins, such as Cad7. Similarly, Snail down-regulates tight junction claudins/occludins to permit the upregulation of gap junction protein connexin-43α1 (Cx43α1), which may also depend on Snail expression. Gene regulation in which the repressors Snail or FoxD3 up-regulate the expression of matrix metalloproteases (MMPs), integrins, Cad7 or RhoB may denote indirect regulatory interactions, possibly mediated by other repressors (denoted by dotted lines). Reproduced from Strobl-Mazzulla PH, Bronner ME. Epithelial to mesenchymal transition: New and old insights from the classical neural crest model. *Seminars in Cancer Biology* 2012;22(5-6):411-416, with permission from Elsevier®. See Appendix 3 for copyright clearance.

**Figure 1.6:** Neural crest programmed epithelial-to-mesenchymal transition

It is likely that the clinicopathologic correlations of neuroblastoma such as genomic instability, tumour heterogeneity and disparate treatment outcomes may be direct consequences of complex molecular signalling pathways that coordinate neural crest differentiation, EMT and maturation/specialization (Figure 1.7).

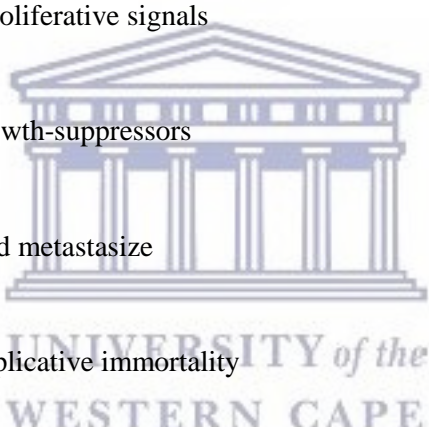
### A11.3 Hallmarks of the Neuroblastoma Tumour Microenvironment

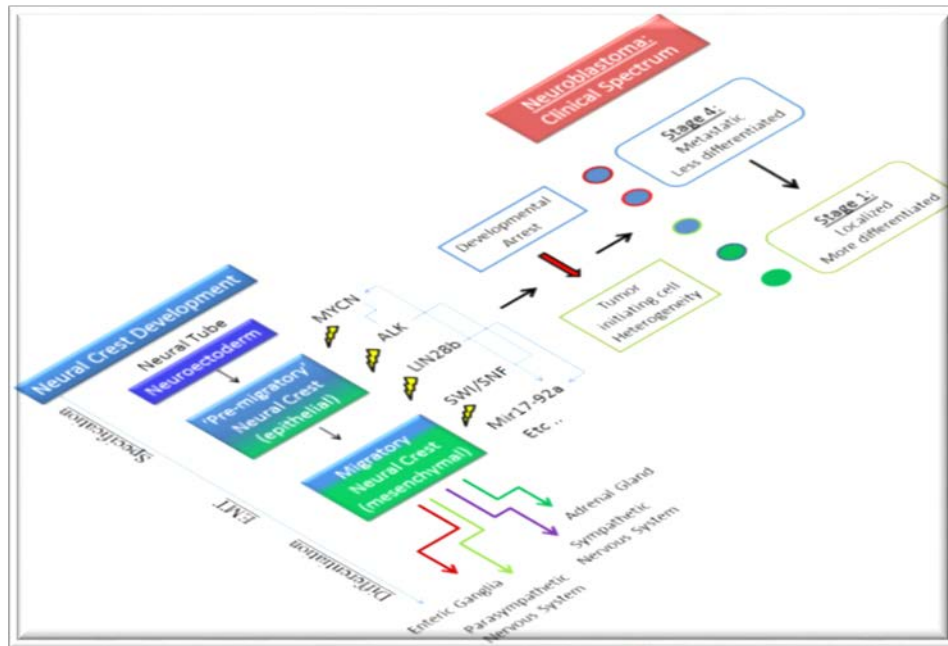
Drawing on the hallmarks of cancer expounded by Douglas Hanahan and Robert A. Weinberg,<sup>145,146</sup> a recent article reviewed how an integrated biological systems repertoire, encompassed by the tumour microenvironment (TME), regulates tumour progression and



metastasis in NB. The authors views converge on the respective contributions of innate [TAMs, neutrophils, natural killer cells (NK), dendritic cells (DC)] and adaptive (T- and B-lymphocytes, and natural killer T cells (NKT)] immune cells, tumour-associated fibroblasts (TAFs), bone marrow-derived mesenchymal stromal cells (MSCs), endothelial cells, Schwann cells, and the extracellular matrix (ECM). Neuroblastoma cells exploit the cell-cell and cell-ECM communication apparatus to “instruct” the TME and TME cells to activate neuroblastoma signalling pathways to express and maintain their neoplastic behaviour (Figures 1.8 and 1.9).<sup>147</sup>

The ten hallmarks of cancer<sup>145-147</sup> are the ability of cancer cells to:

- 
- ❧ Sustain proliferative signals
  - ❧ Evade growth-suppressors
  - ❧ Invade and metastasize
  - ❧ Enable replicative immortality
  - ❧ Induce angiogenesis
  - ❧ Resist cell death or apoptosis
  - ❧ Escape immune destruction
  - ❧ Deregulate cellular metabolism
  - ❧ Confer and express genomic instability
  - ❧ Induce tumour-promoting inflammation



Neuroblastoma is a spectrum of diseases with a wide range of clinical behaviours. Disruption of the normal maturation progression with different genetic drivers at different times leads to heterogeneity of tumour-initiating cells. Interaction between different epigenetic and genetic factors complicates the task of defining a primary oncogenic driver or pathway for this disease. This results in a wide range of pathologies with highly variable responses to treatment.

**Source:**<sup>6</sup> Reproduced and adapted from Louis CU, Shohet JM. Neuroblastoma: Molecular pathogenesis and therapy. *Annual Review of Medicine* 2015;66:49-63, permission not required as stipulated by the Annual Review of Medicine. See Appendix 4 for copyright clearance.

**Figure 1.7:** Clinicopathologic correlations of neuroblastoma

Since these hallmarks of NB have been discussed thoroughly in the cited references in the context of their contribution to the neuroblastoma malignant phenotype and current clinical trials that target the TME in neuroblastoma patients,<sup>147,148</sup> no further consideration will be accorded to them in this section. An essential description of neuroblastoma oncogenic drivers and transcriptional networks is provided in the next section.

## A11.4 Genetic Lesions, Transcriptional Networks and Oncogenic Drivers in Neuroblastoma

Somatic alterations, including mutations, gain of alleles, loss of alleles, or conversions in tumour-cell ploidy, have long been regarded as critical factors in the development and progression of NB.

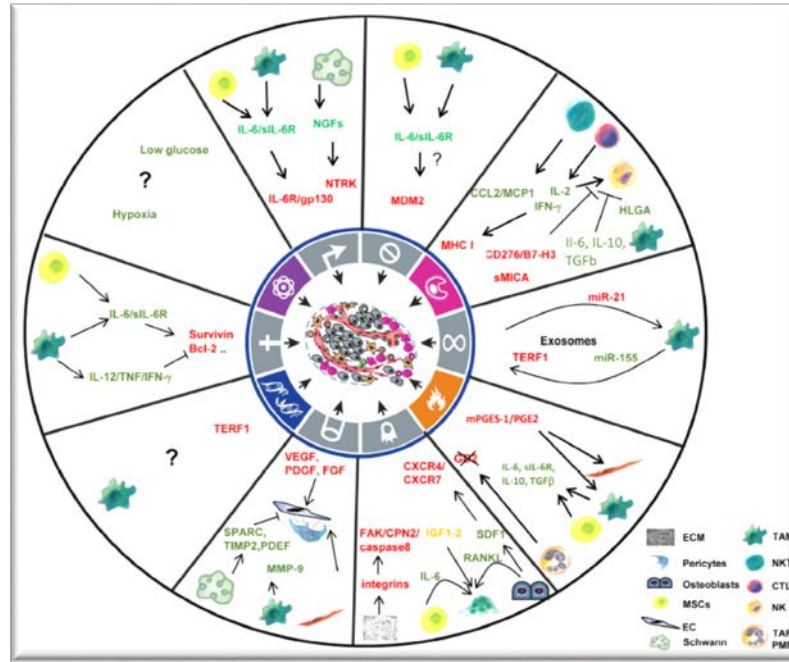
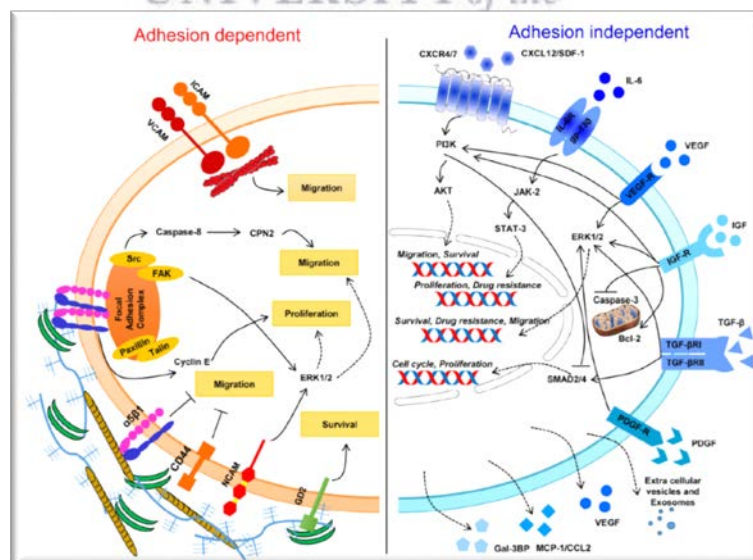


Diagram summarizing the contribution of the cells and ECM in the TME to the ten hallmarks of cancer shown at the centre of the wheel. The central graph was reproduced from Hanahan and Weinberg.<sup>146</sup>

**Source:**<sup>147</sup> Reproduced from Borriello L, Seeger RC, Asgharzadeh S, DeClerck YA. More than the genes, the tumour microenvironment in neuroblastoma. *Cancer Letters* 2015;doi: 10.1016/j.canlet.2015.11.017, with permission from Cancer Letters, Elsevier Ireland Ltd. See Appendix 5 for copyright clearance.

**Figure 1.8:** Contribution of the cells and ECM in the TME to the ten hallmarks of neuroblastoma



**Source:**<sup>147</sup> Reproduced from Borriello L, Seeger RC, Asgharzadeh S, DeClerck YA. More than the genes, the tumour microenvironment in neuroblastoma. *Cancer Letters* 2015;doi: 10.1016/j.canlet.2015.11.017, with permission from Cancer Letters, Elsevier Ireland Ltd. See Appendix 5 for copyright clearance.

**Figure 1.9:** Pathways activated via communication between neuroblastoma and TME cells in the ECM

Many of these chromosomal aberrations are strong prognostic markers that can be used separate from clinical traits in risk stratification and treatment of NB patients.<sup>93,137,149,150</sup> Chromosome regions and genes known to be involved in NB oncogenesis is outlined schematically in Figure 1.10. Some of these gene expression profiles of NB are described in the subsections that follow.

### A11.4.1 Familial Genetic Lesions

Neuroblastoma originates from neuroepithelial cells that migrate from the neural crest to form the sympathetic nervous system.<sup>151</sup> Even though intratumour heterogeneity represents the predictable stage of oncogenesis,<sup>143</sup> it is well-established that neuroblastoma is not exclusively initiated by gene signatures.<sup>147</sup> As such, hereditary NB is both rare and heterogeneous, accounting for less than 5% of all NBs. Hereditary NB predisposition loci have been mapped to chromosomes 16p12–13 and 4p16, indicating that other familial predisposition mutations may exist, but hitherto no specific genes have been unequivocally shown to be inactivated or mutated in these chromosomal regions.<sup>133,137</sup>

Neuroblastoma presents as a locoregional tumour with no detectable amplification of the *MYCN* oncogene in 50% of children, but it correlates with extraordinary prognosis—overall survival (OS) >90%.<sup>147</sup> By contrast, in children older than 18 months of age diagnosed with NB, with or without *MYCN* amplification and metastatic disease, the chance of event-free long term survival despite an intensive combination therapy (myeloablative chemotherapy, radiation therapy, progenitor cell transplantation, surgery, isotretinoin and antibody-based immunotherapy) is less than 50%, and is thus indicative of high-risk disease.<sup>152,153</sup>

The landmark discovery in the early 1980s that a correlation exists between *MYCN* oncogene amplification and advanced stage NB raised hopes that other similar genetic associations may be identified.<sup>154</sup> A family history of NB occurs in 1–2% of patients and 2 mutated genes have been distinguished as promising cancer biomarkers in this regard—anaplastic lymphoma

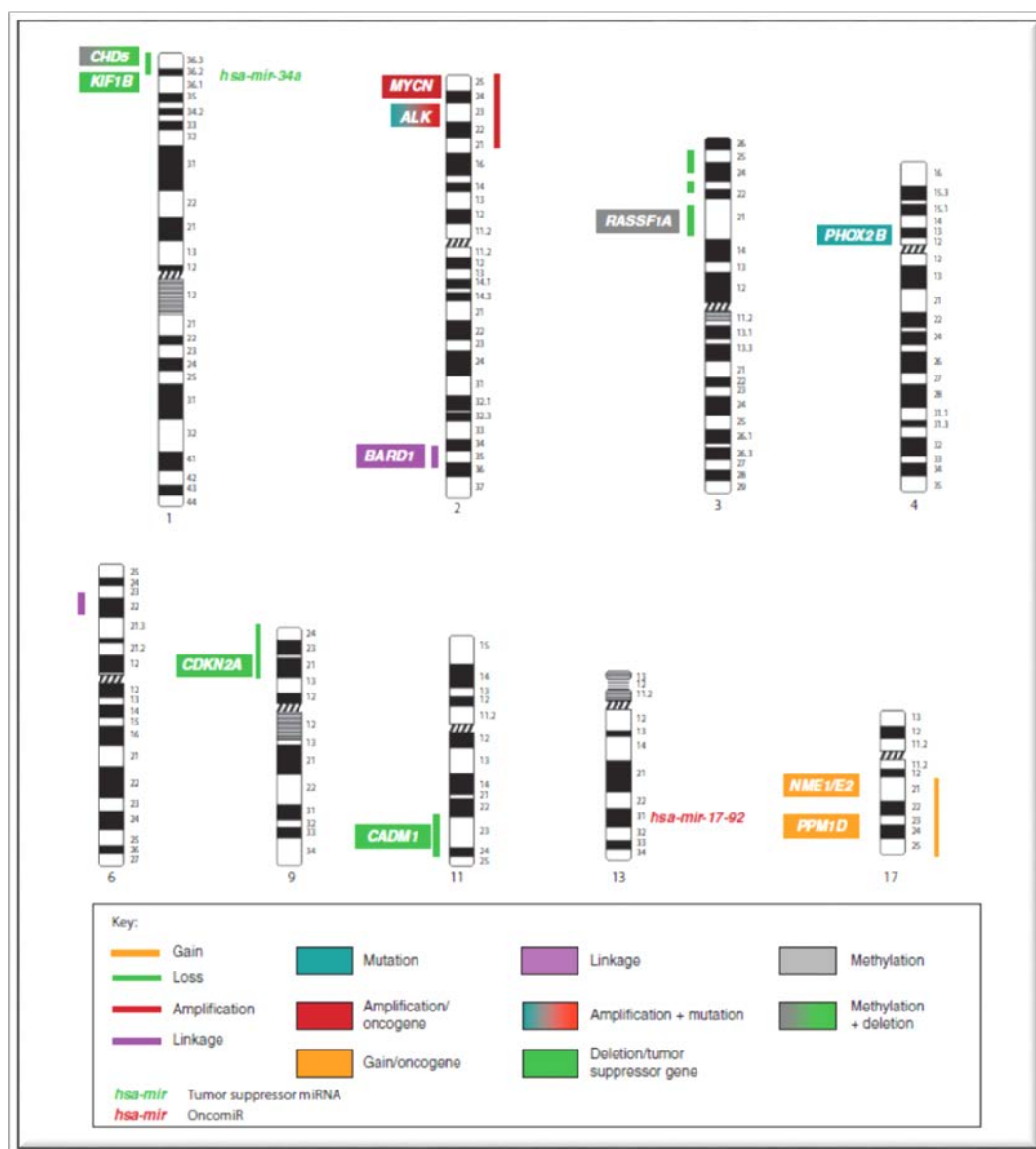
kinase (ALK, exemplifying a gain of function) and the paired-like homeobox 2B (PHOX2B, denoting a loss of function)—in 80% of the familial cases.<sup>155,156</sup> The advent of genome-wide association studies yielded additional gene polymorphisms with a low, but significant risk of NB, including BARD1, LMO1 and LIN28B.<sup>157</sup> Genomic analysis of over 200 NBs showed, unpredictably, low levels of recurrent-driver mutations, most notably activation mutation and amplification of ALK (8% of the cases), activation mutations in PTPN11 (a tyrosine phosphatase), inactivating mutations in chromatin remodeling genes (ATRX and ARID1A) and activating mutations in NRAS, in addition to amplification and activation mutations of MYCN.<sup>147,158-160</sup>

Evaluation of a subset of patients with MYCN non-amplified NB showed that infiltration of tumour-associated macrophages (TAMs) was significantly higher in metastatic NBs than their locoregional equivalents. In addition, metastatic tumours diagnosed in patients at age  $\geq 18$  months had higher expression of inflammation-related genes than those in patients diagnosed at age  $< 18$  months. Expression of genes representing TAMs (CD33/CD16/IL-10/FCGR3) in addition to IL-6 receptor (IL-6R) influenced 25% of the accuracy of a novel 14-gene tumour classification score.<sup>161</sup> Moreover, infiltration with Th2-driven macrophages expressing CD163 and CD206 was also recently observed in a subset of high-risk neuroblastoma tumours with deletion of chromosome 11q and high levels of prostaglandin-synthase and elevated levels of PGE2.<sup>162</sup>

### A11.4.2 PHOX2B Germline Mutations

Germline mutations in the paired-like homeobox 2B (PHOX2B) gene on chromosome 4p13 are the first predisposition mutations identified in NB.<sup>156,163,164</sup> PHOX2B, a *master regulator* of sympathetic neuronal development and mainly expressed in sympathetic neural progenitors,<sup>165</sup> as well as mammalian achaete scute homolog-1 (MASH1), are expressed early in the developing sympathoadrenal progenitors.





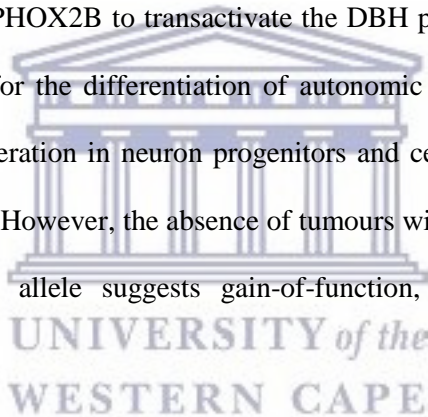
A schematic overview of chromosome regions and genes known to be involved in neuroblastoma oncogenesis. This overview is not comprehensive, and only those regions and genes mentioned in the article are indicated. Gene abbreviations: ALK, anaplastic lymphoma receptor tyrosine kinase; BARD1, BRCA1 associated RING domain 1; CADM1, cell adhesion molecule 1; CDKN2A, cyclin dependent kinase inhibitor 2A; CHD5, chromodomain helicase DNA binding protein 5; KIF1B, kinesin family member 1B; MYCN, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived; NME1/E2, non-metastatic cells 1, protein (NM23A) expressed in/non-metastatic cells 2, protein (NM23A) expressed in; PHOX2B, paired-like homeobox 2b; PPM1D, protein phosphatase 1D magnesium-dependent, delta isoform; RASSF1A, Ras association (RalGDS/AF-6) domain family member 1.

**Source:**<sup>150</sup> Van Roy N, De Preter K, Hoebeek J, Van Maerken T, Pattyn F, Mestdagh P, Vermeulen J, Vandesompele J, Speleman F. The emerging molecular pathogenesis of neuroblastoma: Implications for improved risk assessment and targeted therapy. *Genome Medicine* 2009;1(7):74, with permission from BioMed Central (BMC) Reprints and Permissions (<http://www.biomedcentral.com/about/policies/reprints-and-permissions> [14/06/2016 16:35:15]). See Appendix 6 for copyright clearance.

**Figure 1.10:** Chromosome regions and genes known to be involved in neuroblastoma oncogenesis

Shortly after expression of MASH1 and PHOX2B in the sympathoadrenal lineage, heart- and neural crest derivatives-expressed protein 2 (HAND2), PHOX2A, and GATA2/3 appear. PHOX2B has also been shown to be essential for the expression of the glial family ligand tyrosine kinase coreceptor RET (rearranged during transfection) and for the specification of noradrenergic fates, particularly the biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine beta-hydroxylase promoter (DBH).<sup>137</sup> NB patients with PHOX2B mutations also have familial disorders of the neural crest such as Hirschsprung's disease (HSCR) and congenital hypoventilation syndrome.<sup>156,163</sup>

It is not clear whether the mutations in PHOX2B found in familial NB result in gain or loss of function, although many PHOX2B mutations stabilize the PHOX2B protein and decrease or eliminate the ability of PHOX2B to transactivate the DBH promoter.<sup>166,167</sup> The findings that PHOX2B is necessary for the differentiation of autonomic neurons and overexpression of PHOX2B inhibits proliferation in neuron progenitors and cell lines suggests PHOX2B is a tumour suppressor.<sup>166,167</sup> However, the absence of tumours with loss of heterozygosity (LOH) or mutation in second allele suggests gain-of-function, dominant negative effect, or haploinsufficiency.<sup>164</sup>



### **A11.4.3 Anaplastic Lymphoma Kinase**

Anaplastic lymphoma kinase (ALK) is a member of receptor tyrosine kinases (RTKs) and was first identified as a part of the fusion gene nucleophosmin (NMP)–ALK in anaplastic large cell lymphoma via chromosome translocation of t(2;5)(p23;q25).<sup>168</sup> ALK is thought to play a role in the normal development of the central and peripheral nervous system since ALK mRNA is expressed throughout the nervous system in mouse and rat, but is not present in normal haematopoietic cells.<sup>169,170</sup> Similar patterns of expression are observed in humans although additional ALK transcripts of differing size, most likely due to alternative splicing, have been observed in colon, prostate, testis, small intestine, and brain of adults.<sup>137,171</sup> Full-length ALK protein is comprised of an extracellular region and an intracellular region containing a RTK



domain, linked by a transmembrane (TM)-spanning segment, whereas the NMP–ALK fusion protein generated as a result of the t(2;5)(p23;125) translocation contains the N-terminal of NMP and C-terminal kinase domain of ALK. Translocation of the gene is also evident in other tumours, such as inflammatory myofibroblastic tumour (IMT), and non-small-cell lung carcinoma (NSCLC), but not in NB.<sup>171</sup>

Overexpression of wild-type ALK has also been observed in thyroid carcinoma, breast cancer, NB, melanoma, small cell lung carcinoma, glioblastoma, astrocytoma, retinoblastoma, Ewing sarcoma, and rhabdomyosarcomas NB.<sup>171-173</sup> During 2008, several reports focused attention on ALK point mutations in 8–12% of all NB patients (both hereditary and sporadic) and some NB cell lines.<sup>174-177</sup> Almost all the point mutations identified occurred in the kinase domain and resulted in the constitutive activation of ALK. Two of these activating ALK mutants were able to transform NIH3T3 fibroblasts and induce tumour formation in nude mice.<sup>178</sup> In addition, knockdown of ALK or small molecular ALK inhibitors could reduce cell proliferation and induce apoptosis.<sup>175,176</sup>

Amplification of the ALK gene and/or overexpression of the ALK protein is seen in as many as 77% of all NB tumours, suggesting that overexpression of the ALK protein may also contribute to NB.<sup>179</sup> The downstream effects of ALK in NB need to be elucidated. Current data suggest that ALK may function through the Shc and MAP kinase pathways.<sup>180,181</sup> More recent studies also suggest that activation of ALK enhances RAP1 activity via interaction with C3G, a Crk-binding protein and Crk-like protein (CRKL), and that this complex contributes to NB tumour cell growth and neurite outgrowth.<sup>182</sup>

#### **A11.4.4 Chromosome Gain and Oncogene Activation**

Many genetic abnormalities have been identified in non-familial NB tumours, including amplification of the *MYCN* proto-oncogene (25–33% of patients) and consistent areas of chromosomal deletion and rearrangement that result in loss of 1p36 (25–35%), 11q23 (35–

45%), and 14q23 (16–27%), as well as unbalanced gain of 17q22 (~50%).<sup>132,133</sup> In contrast, known tumour suppressor genes (TSGs) such as p16<sup>INK4a</sup>, pRb, p53 and p14<sup>ARF</sup> are not frequently deleted or mutated in NB, although the nuclear localization of the p16<sup>INK4a</sup> and p53 proteins has been reported to be altered in some tumour cell lines.<sup>132,133,183</sup> Many of these abnormalities are convincing prognostic markers and are highly related to clinical outcome. For example, amplification of *MYCN* in NB patients is correlated with chromosome 1p36 LOH. NB tumours which harbour 1p36 LOH and *MYCN* amplification are usually advanced-stage (stages 3 and 4) aggressive tumours that are frequently metastatic and generally respond poorly to chemotherapy/irradiation.<sup>132,133</sup> In recent years, clinical trials are increasingly based on such tumour genetic markers.

#### **A11.4.5 Amplification of MYCN and the 2p24 Locus**

*MYCN* gene amplification is a hallmark of aggressive neuroblastoma.<sup>184</sup> In 1983, Schwab found that a novel *myc* homologue gene was amplified in several NB cell lines and one NB tumour.<sup>185</sup> Later, several papers termed this gene as *MYCN* based on homology to *c-myc* and expression pattern in the developing nervous system, and identified its location at chromosome 2p24.<sup>185,186</sup> Additional studies have shown that N-myc protein is a nuclear phosphoprotein that is a member of the *myc* family of helix loop-helix transcription factors.<sup>187</sup> Amplification of the *MYCN* gene in patient tumours ranges from 10-fold to more than 500-fold, although the majority of tumours exhibit 50- to 100-fold *MYCN* gene amplification levels. The amplified DNA typically contains a large region of chromosome 2 ranging from 100 kb to 1 Mb which includes the entire *MYCN* gene and varying amounts of adjacent DNA.<sup>188</sup>

Although other genes may be co-amplified with *MYCN*, it is the only consistent amplified gene from this region.<sup>189</sup> *MYCN* amplification is rarely observed on chromosome 2p24 in primary tumours, but is found to be at homogeneously staining regions (HSRs) on different chromosomes or, more frequently, as double minutes (DMs; which are small fragments of extrachromosomal DNA).<sup>185,190</sup> In cell culture, the amplification unit frequently integrates into

chromosomes to become HSRs. The reason for the differences in the location of the amplicon in primary tumours and cultured cells remains unclear. Amplification of *MYCN* is highly associated with aggressive NB tumours and poor outcome. The precise role of *MYCN* in NB is still sketchy, however, amplification of the gene is frequently associated with the overexpression of the N-myc protein. Studies on *MYCN* regulation suggest that the transcription factor and signalling pathways controlling the upregulation of *MYCN* are dependent on cell type.<sup>191</sup> These factors include IL-7 and Pax-5, NF- $\kappa$ B in pre-B cells, and insulin-like growth factors I and II (IGFI and IGFII) in NB cells.<sup>192</sup>

In contrast, *MYCN* transcription is repressed by retinoic acid (RA) in association with E2F binding, nerve growth factor (NGF) binding to TrkA receptor, the iron chelator deferoxamine mesylate and transforming growth factor-beta 1 (TGF- $\beta$ 1).<sup>192</sup> Myc proteins form heterodimers with the Max protein. These heterodimers bind to E-box elements (CACGTG) to activate transcription. However, Myc–Max dimers can also associate with other transcription factors such as Miz-1 and Smad and bind to Inr (initiator) elements to repress transcription. Max can also form homodimers or heterodimers with Mad to compete or suppress Myc–Max binding.<sup>137,187,193,194</sup>

The targets of Myc–Max are involved in various cellular processes, including cell growth, proliferation, loss of differentiation, and apoptosis, and include proteins such as MASH1 and important molecules in the normal development of sympathocoadrenal lineage cells, such as the multidrug resistance protein 1 (MRP1) and MDM2.<sup>187,193,195</sup> *MDM2*, which negatively regulates p53, is a direct transcriptional target of *MYCN* in NB and modulates cell cycle and transcriptional events as demonstrated by targeted inhibition of *MYCN* in a *MYCN*-amplified neuroblastoma cell line which concomitantly decreased *MDM2* expression, stabilized p53 and induced apoptosis.<sup>196,197</sup> Hence, manipulating the paradoxical apoptosis-promoting function of *MYCN* amplification in NB could be a valuable line of attack in the high-risk, *MYCN*-amplified subset of neuroblastoma.<sup>198</sup>

The transgenic mouse model sustains that *MYCN* overexpression is a primordial stage in NB tumorigenesis. In this model, overexpression of the human *MYCN*, followed by NB tumour formation, is driven by the rat TH promoter, which is expressed in migrating cells of the neural crest early in development.<sup>199</sup> However, other factors are also likely to be involved in the early stages of tumour formation since amplification of the *MYCN* oncogene occurs in only about one-third of NBs. Moreover, the tumours in these transgenic mice rarely exhibit significant metastasis despite the presence of high levels of N-myc protein suggesting that the other genetic alterations and/or epigenetic changes are critical for tumour formation and metastasis.

The pioneering of a unique Cre-conditional human *MYCN*-driven mouse model for NB that robustly recapitulates features of the human disease, including tumour localization, histology, marker expression and genomic profile sets a significant benchmark for advance translational approaches to preclinical and molecularly targeted therapies for NBs.<sup>200</sup> Likewise, it has recently been proposed that human neural crest stem cells (hNCSCs) isolated from *in vitro*-differentiating human embryonic stem cells (hESCs) may be an invaluable model system to study human neural crest development and diseases, since suppression of *MYCN* in hNCSCs not only arrests cell growth and cell cycle progression, but its knockdown induces the expression of *Cdkn1a*, *Cdkn2a* and *Cdkn2b*, which encodes the cyclin-dependent kinases p21<sup>CIP1</sup>, p16<sup>INK4a</sup> and p15<sup>INK4b</sup>, highlighting its critical function in stem cell growth and cell cycle progression. Remarkably also, *MYCN* is involved in the regulation of human sympathetic neurogenesis, as knockdown of *MYCN* augments the expression of key transcription factors involved in sympathetic neuron differentiation, including PHOX2A, PHOX2B, MASH1, HAND2 and GATA3, which may have implications for targeted therapy of NB.<sup>165</sup>

Several other genetically engineered mouse models (GEMMs) of NB have been reviewed recently: tyrosine hydroxylase (TH)-*MYCN*, TH-*MYCN*/Trp53(+/-), TH-*MYCN*/TH-Cre/Casp8(flox/flox), TH-*MYCN*/TH-ALK(F1174L) and DBH-iCre/CAG-LSL-Lin28b.<sup>111,201</sup> Correspondingly, studies focusing on *MYCN*-amplified neuroblastoma patient-derived

(human) xenograft models demonstrated sensitivity to the BCL-2 inhibitor ABT-199 which was partly the result of low anti-apoptotic BCL-xL expression, high pro-apoptotic NOXA expression, paradoxical MYCN-driven upregulation of NOXA and widespread induced apoptosis mediated by Aurora kinase A inhibitor MLN8237 combined with ABT-199, which further led to tumour shrinkage and in several instances complete tumour regression.<sup>198</sup>

### A11.4.6 Gain of Chromosome Arm 17q

Gain of genetic material from chromosome arm 17q (gain of segment 17q21–qter) is the most frequent cytogenetic abnormality of neuroblastoma cells. This gain has been linked with progressive disease, infants  $\geq 1$  year old, deletion of chromosome arm 1p, and amplification of the *N-myc* oncogene, all of which predict an adverse outcome.<sup>23</sup> Gain of chromosome arm 17q was originally detected by G-banded cytogenetic analysis in early 1980s. However, this observation was regarded as trivial in comparison to *MYCN* amplification and 1p loss of heterozygosity (LOH).

In the middle 1990s, the significance of 17q abnormalities in NB became increasingly discernible because fluorescence in situ hybridization (FISH) technology revealed that translocation of this chromosome arm was prevalent in 50% of primary NB tumours, resulting in an unbalanced gain of one to three copies of 17q which may confer a selective survival advantage for NB tumour cells.<sup>132,133</sup> It is approximated that multiples of the 17q chromosome fragment (ca. 20 Mb) accounting for more than 200 genes can be translocated in NB tumours, thus making it difficult to spot the genes responsible for the selective persistence advantage.

Several genes in this region have been deemed as good candidate oncogenes or tumour suppressors based on correlations between expression levels and unbalanced gain of 17q. These include survivin, PPM1D and NM23A.<sup>137</sup> Overexpression of *survivin*, an anti-apoptosis gene, mapped to 17q25, is significantly associated with poor prognosis and promotes cell survival in human neuroblastoma.<sup>202</sup> Wip1 (wild-type p53-inducible phosphatase 1) or PPM1D

(protein phosphatase magnesium-dependent 1delta) is a p53-inducible Ser/Thr protein phosphatase which negatively regulates the DNA damage response through the dephosphorylation and inactivation of p53, ATM, p38 and Chk1/2, and hence drives oncogenesis.<sup>204-207</sup> Low expression of NM23A (Nm23/NDP kinase), a metastasis suppressor, has been correlated with poor patient prognosis and survival, lymph node infiltration, and histopathological indicators of high metastatic potential in a number of cancer types, including NB.<sup>149,208-210</sup> Unbalanced gain of 17q correlates with other chromosomal deletions. The most frequent deletion site is the short arm of chromosome 1, followed by 11q. At least 30 translocation sites on 20 different chromosomes have been detected in patient samples and cell lines<sup>211-213</sup> Nevertheless, NB tumours harbouring unbalanced gain of 17q exhibit a more aggressive phenotype and a poorer prognosis than those without this abnormality.<sup>137</sup>

#### **A11.4.7 Amplification and Chromosome Gain of Other Loci**

Besides the amplification of the *MYCN* gene, numerous other regions of gene amplifications have been distinguished in small groups of NB cases. These include amplification of the *MDM2* gene at 12q13, the *DDX* gene at 2p24, the *MYCL* gene at 1p32, and unexplained DNA from chromosome 2p22 and 2p13.<sup>214,215</sup> The mouse double minute 2 homologue (*MDM2*) gene (*MDM2*) is amplified in various NB cell lines and primary tumours. Like the *MYCN* gene amplification, *MDM2* amplification unit first developed within DMs and then integrates into a different chromosome to form HSRs.<sup>215</sup>

The *DDX1* gene, which encodes a RNA helicase, is co-amplified with *MYCN* in 4/6 NB cell lines and 6/16 tumours with *MYCN* amplification, however, *DDX1* amplification was not detected in the absence of *MYCN* amplification.<sup>216</sup> Moreover, the *MYCL* gene is co-amplified with *MYCN* in NB cell lines. *MYCL*, another member of myc gene family, is commonly overexpressed in small cell lung carcinoma.<sup>217</sup> In addition to gain of 17q, other chromosome gains have been seen on 1q, 4q, 5q, 6p, 7q, 18q using comparative genomic hybridization (CGH) methodology, although their clinical significance has yet to be elucidated.<sup>137</sup>



## A12.4 Chromosome Loss and Tumour Suppressor Genes

In addition to mutation, gene amplification and increased chromosome copy number, NB tumours also experience loss of genetic material and deletion of putative tumour suppressor genes (TSGs).<sup>137</sup>

### A12.4.1 Loss of Heterozygosity of Chromosome 1p and CHD5, miR-34, KIF1B $\beta$

Loss of the short arm of chromosome 1 occurs in about 25–35% NB tumours. 1p LOH is correlated with amplification of *MYCN* in NB patients. Loss of 1p correlates with and may stem from unbalanced gain of 17q, but the precise process that underscore these dualistic outcomes is not well-defined. The significance of 1p LOH is borne out by research in which transferring chromosome 1p material into human NB cells caused differentiation and suppression of tumorigenicity.<sup>218</sup> Whereas patients with 1p36 abnormalities without *MYCN* amplification have been identified, the reverse situation virtually never occurs suggesting either that 1p36 LOH provides a permissive environment for *MYCN* amplification or that tumours with these two associated genetic defects have a high degree of genomic instability.<sup>132</sup>

Remarkably, NB tumours with 1p36 LOH and *MYCN* amplification are frequently aggressive with high metastatic potential and generally resistant to chemotherapy/irradiation. Even though the chromosomal regions defined above are crucial in NB, the TSGs that reside within these regions have not been sufficiently characterized. Nevertheless, contemporary studies have classified three new putative tumour suppressors on chromosome 1p36: the chromodomain helicase DNA-binding domain 5 (CHD5), microRNA-34a (mir-34a), and the kinesin superfamily protein 1B beta (KIF1B $\beta$ ).<sup>219-221</sup> These tumour suppressors proteins mediate their effects through cell growth dynamics, e.g., the effects of CHD5 on cell growth were shown to be dependent on p53 and CDH5 positively regulates p53 via p19ARF expression.<sup>219</sup> Thus, overexpression of CHD5 results in enhanced apoptosis and cellular senescence, increased p53 and p19ARF levels, and sequestration of MDM2, the negative regulator of p53, by p19ARF.

On the other hand, cells lacking CHD5 exhibit decreased p16 and p19ARF expression, the latter paralleled a decrease in p53 levels and enhanced cellular proliferation. Therefore, CHD5 acts as a tumour suppressor that controls proliferation, apoptosis, and senescence via effects on the p19ARF/p53 pathway. These effects are largely attributable to changes in the accessibility of the p16/p19ARF gene locus resulting from the chromatin remodelling function of CHD5.<sup>219</sup> By analogy, mir-34a was found to be expressed at very low levels in unfavourable primary tumours and NB cell lines.<sup>222</sup>

Introduction of this microRNA (miRNA) into cell lines diminished cell proliferation and enhanced caspase-dependent apoptosis, by targeting E2F3 mRNA and suppressing its expression.<sup>221</sup> E2F3 a transcription factor that upregulates the expression of countless genes associated with cellular proliferation. On the other hand, overexpression of KIF1B $\beta$  induced cell death while decreased KIF1B $\beta$  levels correlated with cell proliferation and enhanced tumour development in nude mice, implying that KIF1B $\beta$  is also a prospective TSG candidate.<sup>220</sup> Moreover, KIF1B $\beta$  is a downstream target of prolyl hydroxylase EglN3 and an inducer of apoptosis in neuronal progenitor cells or NB cells when NGF is deficient. Missense mutations of KIF1B $\beta$  in inherited NBs and pheochromocytomas strongly support the hypothesis that KIF1B $\beta$  is a conceivable TSG candidate.<sup>223</sup>

#### **A12.4.2 Loss of Heterozygosity of 11q and TSLC1**

Loss of the long arm of chromosome 11 occurs in 35–45% NB primary tumours with a single copy *MYCN* gene. Two large patient studies that analyzed 295 NB primary tumours observed loss of 11q in 44% cases, and common regions of LOH located at 11q23, signifying that putative TSGs reside in this region.<sup>224,225</sup> Loss of 11q correlated with adverse clinical features such as late stage disease, older age of disease onset and unfavourable histology, although it is strongly inversely correlated with *MYNC* amplification and 1p loss. Hence, 11q loss is a valuable and principal marker for verifying the clinical prognosis for those advanced stage tumours without *MYCN* amplification. Transfer of chromosome 11 induced differentiation in

NB cell lines supporting the importance of loss of 11q in tumourigenesis.<sup>218</sup> Another putative tumour suppressor, the *IGSF4* (immunoglobulin superfamily 4) gene, was originally localized to the common 11q23 LOH region in 1999.<sup>226</sup> *IGSF4*, also known as TSLC1/CADM1 (tumour suppressor in lung cancer 1/cell adhesion molecule 1), is a plausible TSG for lung cancers. A recent CGH study which examined 236 primary tumour samples found the TSLC1 LOH locus in 35% tumours. Notably, the level of TSLC1 expression correlated with tumour stage, histological classification, MYCN and TrkA expression levels. Reduced expression of TSLC1 was found in unfavourable tumours. Furthermore, introduction of TSLC1 decreased cell proliferation in NB cell lines and thus a representative NB tumour suppressor candidate.<sup>227</sup> Interestingly, a recent study indicates that expression of both KIF1B $\beta$  and TSLC1 is controlled by the polycomb protein Bmi1, whose expression is regulated by N-myc.<sup>137,228</sup>

### **A12.4.3 Loss of Heterozygosity of 14q**

Loss of the long arm of chromosome 14 is also commonly found in NB primary tumours (~16–27% of the patients).<sup>137</sup> LOH on chromosome 14q was first identified in 1989 using a polymorphic DNA marker which detected allelic deletion at specific 14q23 loci.<sup>229</sup> LOH analysis of 14q in a large number of primary tumours using 11 polymorphic DNA markers found 14q LOH in 83 of 372 tumours (22%).<sup>230,231</sup> 14q LOH was highly correlated with 11q loss and had an inverse relationship with 1p loss and *MYCN* amplification.<sup>230</sup> However, LOH for 14q was present in tumours from all clinical stages, suggesting this abnormality may be a universal early event during tumour development.<sup>137</sup>

## **A13. Treatment and Management of Neuroblastoma**

### **A13.1 Overall Therapeutic Landscape of Neuroblastoma**

Neuroblastoma is the most frequent extracranial solid cancer in paediatric patients and has long puzzled scientists and oncologists alike since its biological and clinical behaviour vary between resistance to multimodal cancer therapies and complete spontaneous regression.<sup>64</sup> Most

children diagnosed with NB are classified as high-risk cases with disseminated metastases and a mortality rate of more than 50%.<sup>33</sup> The cornerstone of treatment of NB consists of chemotherapy, surgical resection and/or radiotherapy. Novel personalized and molecular-guided therapy for the treatment of patients with relapsed or refractory NB are therefore a dire need to stem the tide of NB-related deaths in infants.<sup>19,232,233</sup>

Currently, various efforts are being pursued through innovations in basic medical sciences and translation of promising novel and molecular NB drug targets into successful clinical therapeutic practice.<sup>8,9,37,151,234</sup> Treating NB is complex and frequently entails a multidisciplinary team of health professionals, including a paediatric cancer surgeon, a paediatric oncologist, a paediatric radiation oncologist. However, many other specialists may be involved in the care of children with NB such as physician assistants, nurse practitioners, nurses, psychologists, social workers and rehabilitation specialists.

Treatment of NB depends on the stage of the cancer and the child's age. Current treatment modalities can include any one or several combinations of surgery, chemotherapy, radiation therapy, high-dose chemotherapy/radiation therapy and stem cell transplantation, retinoid therapy and immunotherapy. Complementary and alternative methods to treat cancer or relieve symptoms are optional. These methods can include vitamins, herbs, and special diets, or other methods such as acupuncture or massage. Even though some of these methods might be helpful in relieving symptoms of NB, many have no proven efficacy and might even cause unwanted side effects (*Neuroblastoma*, American Cancer Society, <http://www.cancer.org/>).

### **A13.1.1 Spontaneous Regression and Stage 4S Disease**

Spontaneous regression of cancer is not a new concept and has been defined in the 1950s as “the partial or complete disappearance of a malignant tumour in the absence of all treatment, or in the presence of therapy which is considered inadequate to exert a significant influence on neoplastic disease.”<sup>235,236</sup> However, one of the first allusions to regression of malignant NB

was published in 1927.<sup>237</sup> A significant feature of NB is that occasionally it undergoes spontaneous regression.<sup>238-240</sup> This propensity is consistent with the notion that NB is largely caused by aberrations in the embryonic progressions of the neural crest and thus the sympathetic nervous system.<sup>111</sup> Overexpression of the *MYCN/c-MYC* target gene is a hallmark of malignant NB progression—a process primarily driven by *c-MYC* in stage 4-non-amplified tumours.<sup>241</sup> It has been proposed that moderate gain of *MYCN* function in stage 4S-non-amplified tumours induces a number of target genes that retain their ability to trigger spontaneous regression.<sup>241,242</sup> More importantly, the fifth stage of NB tumours (stage 4S) is said to undergo spontaneous regression with minimum treatment or even without medical intervention.<sup>238,243</sup>

Recent genomic studies of NB corroborated the striking heterogeneity in the clinical behaviour of this disease, which encompasses spontaneous regression or differentiation in some patients, to relentless disease progression in others, in the face of intensive multimodality therapy.<sup>12</sup> Thus, some NBs regress spontaneously without therapy while others progress with a fatal outcome despite therapy. In one such study of infants younger than 12 months, spontaneous regression was noted in almost 50% of the study population within three years of follow-up.<sup>244</sup>

Several conceivable mechanisms may account for the spontaneous regression observed in NBs, including *HOX* gene expression (*HOXC9* expression is downregulated in advanced-stage NB and is involved in cell cycle control and the processes of NB cell differentiation),<sup>245,246</sup> neurotrophin signalling (especially those through nerve growth factor and its receptor, *TrkA*),<sup>247-250</sup> activation of developmentally programmed apoptosis, humoral or cellular immunity, loss of human telomerase (*h-Tert*) activity, epigenetic changes in gene expression controlled by DNA methylation, histone modification, or alterations in chromatin remodelling.<sup>12</sup> A better understanding of the mechanisms of spontaneous regression might help to identify optimal therapeutic approaches for patients with these tumours.<sup>111</sup>

### A13.1.2 Surgery

The goal of surgical resection of NB is to attain macroscopic tumour resection with minimal residual disease (MRD). Surgery in ‘low’ and ‘intermediate risk’ groups is aimed at complete resection—wherever possible—with minimal injury to adjacent structures which are frequently adherent to, if not encased by the tumour mass.<sup>251,252</sup> NB is a highly infiltrative neoplasm and poses several challenges for the paediatric cancer surgeon, e.g., difficulty in obtaining microscopically negative resection margins wherein gross total resection (GTR) or subtotal tumour resection (STR) is desirable.<sup>253</sup> Much controversy and debate subsists on the defining role of surgery in advanced stage 4 disease. Moreover, significant overall survival advantage for radical surgical clearance in stage 4 disease has never been clearly demonstrated.<sup>252-258</sup>

The first recorded successful excision of a NB occurred in 1916, but for many years there was no other form of treatment, and the outlook remained dismal. The use of radiotherapy (1928) and subsequently combination chemotherapy (1965) had a modest impact. Recent advances in accurate disease imaging and staging has allowed a more coherent approach to diagnosis and treatment of NB.<sup>47,251,257</sup> Generally, surgery in NB can be performed safely, but moderate to serious intra- and postoperative surgical complications have been experienced, including massive haemorrhage, major vascular injury, respiratory failure requiring mechanical ventilation after major surgery, cardiac arrest, tumoural rupture, nephrectomies, Bernard-Horner syndrome and pleural effusions.<sup>251</sup> It has been suggested that presurgical chemotherapy may lead to a more extensive and safer removal of locally advanced tumours.<sup>256</sup>

A recent analysis of the SEER database (The Surveillance, Epidemiology, and End Results Programme (SEER, <http://seer.cancer.gov>), after accounting for selection bias, indicated improved survival following surgery and radiation therapy for olfactory neuroblastoma. However, the efficacy, timing, and optimum approach for combining chemotherapy with surgery and radiotherapy could not be established.<sup>259</sup> Also, in intensively treated patients with



stage 4 neuroblastoma age 18 months or older at diagnosis, surgery of the primary tumour site had no impact on local control rate and outcome.<sup>255</sup> Similarly, no substantial survival benefit had been noted in stage IV neuroblastoma patients undergoing complete tumour resection, organ preservation and minimalization of morbidity.<sup>254</sup> Despite these challenges, surgical resection remains a cornerstone of therapy in paediatric patients suffering from this clinically and biologically heterogeneous and complex disease.<sup>253</sup>

### **A13.1.3 Chemotherapy**

About 80% of patients with high-risk NB often survive their primary tumour, i.e., attain remission through high-dose chemotherapy, surgery, radiation and stem cell transplantation. However, those with relapsed metastatic disease after treatment have a discouraging long-term survival outcome.<sup>260</sup> As a paediatric extracranial solid tumour paradigm, NB correlates with a disproportionately high mortality rate, i.e., 15% of all cancer-related deaths in children.<sup>16</sup> In spite of 5-year event-free survival (EFS) and overall survival (OS) rates >90% for low- and intermediate-risk NB groups, the survival rate in children with high-risk NB remains 40%–50%.<sup>3,251,259,261</sup> Also, the manifestation of minimal residual disease (MRD) persists to be a substantial obstacle to bettering the prognosis of patients with high-risk NB.<sup>262-264</sup>

Chemotherapy for high-risk NB entails three empirical stages, viz., (i) induction of remission, (ii) consolidation of remission and ultimately (iii) maintenance phase focused on the eradication of MRD.<sup>64,265</sup> Generally, induction regimens utilize various combinations of anthracyclines, platinum-based compounds, etoposide, microtubule disruptors and alkylating agents. NB combination chemotherapy encompasses dose-intensive cycles of cisplatin and etoposide alternating with vincristine, doxorubicin, and cyclophosphamide.<sup>266</sup> Very low and low-risk patients may require only observation or surgical resection, except in cases of life- or organ-threatening symptoms at diagnosis. Intermediate risk group patients whose tumours are not compatible for primary resection are put on chemotherapy designed to selectively destroy rapidly dividing tumour cells, eliminate life-threatening symptoms or make tumour

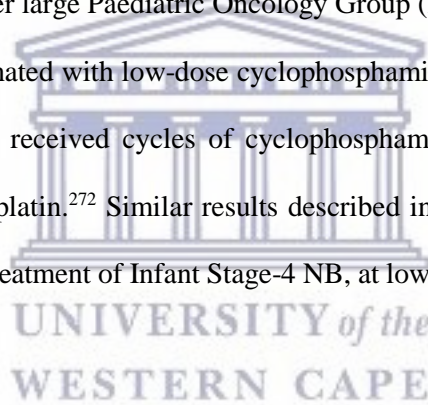
resectability easier. High-risk patients undergo chemotherapy protocols combining carboplatin, etoposide, cyclophosphamide, doxorubicin, and vincristine. Furthermore, patients in the high-risk group also receive myeloablative chemotherapy to impede tumour infiltration (even though normal bone marrow is also suppressed), followed by bone marrow transplantation and granulocyte macrophage colony-stimulating factor (GM-CSF) induction. This sequential therapy regimen has proved beneficial in enhancing EFS.<sup>265</sup> Mild chemotherapy regimens, especially those presently indicated for intermediate-risk NB are deemed ineffective for preventing evolution into advanced-stage (high-risk) disease. There is a disinclination to expose a clinically disease-free infant or child to aggressive, highly toxic multimodal therapy that is only partially effective against advanced-stage disease.

Hence, close clinical monitoring of such patients is warranted.<sup>251</sup> However, chemotherapy is invaluable in the early treatment of patients with stage-2 tumours who present with spinal cord susceptibility from a paraspinal mass or airway weakness from a tumour in the superior mediastinum. Biologic findings strengthen contentions surrounding the use of cytotoxic therapy in localized disease. The salient disparities in chromosomal features of lethal vs low-risk forms of NB lend biologic support for the radical dichotomy in prognosis. In this regard, progression of non-stage-4 NB with low-risk biologic features (triploidy, unamplified *MYCN*) to lethal stage-4 disease is a rare event. Several lines of evidence reinforced the hypothesis that non-stage-4 NB without *MYCN* amplification rarely, if ever, evolves into lethal disease.<sup>251</sup>

Biologically favourable (low-risk) stage-4S disease resolves spontaneously in the majority of cases and surgical resection of primary tumours at diagnosis is no longer recommended since these tumours are likely to regress.<sup>267</sup> Some stage-4S tumours with low-risk prognostic markers, e.g., non-amplified *MYCN*, hyperdiploidy and favourable histopathology, can cause dire life-threatening cardiopulmonary complications and coagulopathies in the neonatal period. Such medical emergencies may abate after treatment with one to two cycles of low-dose chemotherapy and/or modest doses of radiotherapy in order to spare the kidneys and spine.

In the face of persistence of liver lesions, once clinical adjustment has been achieved, supplementary cytotoxic therapy may not be needed as it might pose some risk and the residual disease, even if extensive, is likely to regress.<sup>251</sup> Infants with stage-3 NB lacking *MYCN* amplification have survival rates close to 100%. In multi-institution studies in North America and Europe, these patients have received various modest dose-combinations of platinum compounds, etoposide, cyclophosphamide, doxorubicin and/or vincristine.<sup>268-270</sup>

The French Society of Paediatric Oncology achieved similar success using alternating cycles of carboplatin/etoposide and cyclophosphamide/doxorubicin/vincristine, in moderate doses.<sup>251,271</sup> In a large Children's Cancer Group (CCG) study, a regimen involved 9 months of combination chemotherapy incorporating cisplatin, etoposide, cyclophosphamide, and doxorubicin.<sup>270</sup> In another large Paediatric Oncology Group (POG) study, cycles of high-dose cisplatin/etoposide alternated with low-dose cyclophosphamide/doxorubicin, and in a follow-up POG study, patients received cycles of cyclophosphamide, etoposide, vincristine, plus either cisplatin or carboplatin.<sup>272</sup> Similar results described in clinical trials referred to above have been reported for treatment of Infant Stage-4 NB, at lower but improving cure rates from 10 to 50 to >70%.<sup>268</sup>



Neonatal NB constitutes less than 5% of all cases of the neoplasm and frequently correlates with a good prognosis provided that patients are stratified into low- or intermediate-risk groups for disease recurrence. In neonates less than or older than 30 days, NB has the unusual potential to undergo spontaneous regression and this characteristic is used as a paradigm by several paediatric oncology groups (CCG, COG, POG and INRG) to moderate therapy given to neonates with low-risk NB.<sup>8,44</sup> These groups also strive for the agency of reduced cytotoxic chemotherapy therapy and surgical tumour ablation for certain low- and intermediate-risk patients, but advocate observation approaches for such favourable subsets. By analogy, high-risk patients should receive aggressive chemotherapy, radiation, surgery and myeloablative and immunotherapies.<sup>18,45,273</sup>

### A13.1.4 Radiotherapy

While NB responds favourably to radiotherapy, the efficacy of total body irradiation in paediatric patients remains debateable in the face of long-lasting adverse events. Currently, COG promotes the concept that high-risk patients receive radiation to the primary tumour site irrespective of the coverage of surgical resection and to metastatic sites that display persistent MIBG avidity on pre-transplantation scans.<sup>54,55,260</sup> Radiotherapy is not indicated for low-risk NB, even with local residual disease as risks outweighing potential benefits. In low- and intermediate-risk groups, radiation therapy is reserved for patients with progressive clinical relapse despite chemotherapy and surgery. Infants with stage 4S disease are usually excluded from radiotherapy, except those with severe respiratory distress or abdominal compartment syndrome with precipitous hepatomegaly. Synergistic, additive and antagonistic acute and long-term tumour responses as well side effects may be produced by parallel use of radiosensitizing agents.<sup>260,274</sup> Radiosensitizers include cisplatin, topotecan and irinotecan<sup>260</sup> which are usually safe to give with radiation therapy. Currently, spinal cord compression is managed by chemotherapy, radiotherapy or surgical resection with or without laminectomy, but is contingent on a case by case basis. Radiation therapy is contraindicated in intraspinal tumours because it can trigger gross vertebral impairment and growth arrest resulting in severe scoliosis, but it may be used selectively as an emergency therapy for patients with symptomatic spinal cord compression.<sup>64</sup>

### A13.1.5 Haematopoietic / Peripheral Blood Stem Cell Transplantation

Almost 56% of NB patients present with disseminated disease at the time of diagnosis. The bone, bone marrow, liver, non-contiguous lymph nodes and central nervous system (including the choroid plexus) are the most frequent metastatic foci.<sup>118,133,275-279</sup> The low 5-year survival rate (40–45%) of NB patients with secondary tumours (metastases) underscores the therapeutic hurdles faced by paediatric oncologists in the face of advanced treatment options.<sup>280</sup> Children with bone metastasis have a dismal outcome with survival rates below 7%.<sup>281</sup> Moreover, 40-

50% of patients relapse (presenting with occult NB cells in peripheral blood), often after total remission following multi-modal treatment (surgery, chemotherapy and radiation).<sup>282</sup> The bone marrow is a chief metastatic site in stage IV NB and therefore assessment of MRD in the bone marrow is implicit in disease prognosis. Since high-risk NB correlates with a worse prognosis, autologous bone marrow transplantation (ABMT)<sup>283</sup> and autologous peripheral blood stem cell transplantation (PBSCT) have become a therapeutic mainstay to enhance the prognosis of such patients, in particular to support haematopoietic rescue following high-dose chemotherapy.<sup>264,279,284-287</sup> However, re-infusion of PBSC contaminated with tumour at the time of autologous transplantation may play a significant role in the high proportion of relapse in children with NB who eventually succumb to the disease.<sup>264,285,286,288,289</sup>

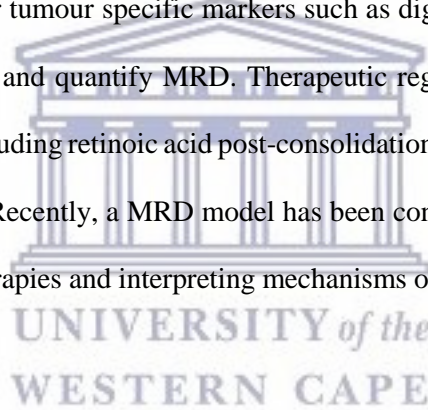
Recently, pulmonary arterial hypertension (PAH) has been acknowledged as a rare condition with high mortality rate after paediatric haematopoietic stem cell transplantation (HSCT). Generally, there is a propensity to overlook PAH in the differential diagnosis of cardiorespiratory failure after HSCT as the clinical presentation is non-specific and may mimic other aetiologies.<sup>290</sup> Accordingly, paediatricians overseeing HSCT recipients should be cognizant of this serious post-transplant complication as appropriate diagnosis and treatment may improve clinical outcomes.<sup>291</sup>

### **A13.1.6 Management of Minimal Residual Disease and Relapse**

Minimal residual disease (MRD) is a major barrier to the obliteration of malignant neoplasms. Even with the high sensitivity of various cancers to therapy, fractions of residual tumour cells persist and give to tumour recurrence and treatment failure.<sup>292,293</sup> The detection of minimal amounts of tumour cells in bone marrow, peripheral blood, putative metastatic sites, lymph nodes or in other tissues, compartments or body fluids has become a major goal in cancer diagnostics. For NB patients, the clinical significance of minimal residual disease (MRD) in bone marrow after induction of chemotherapy or within stem cell harvests prior to autologous transplantation has become a critical clinical context.<sup>262,263</sup> In paediatric patients, relapse is a

frequent occurrence after autologous BMT, indicating the presence of malignant stem cells that are resistant to dose-intensive myeloblastic chemotherapy.<sup>7,118,131,294</sup> MRD status in PBSCs might be crucial in high-risk NB because PBSCs contaminated with tumour cells are thought to contribute to relapse and increased mortality rate. Detection of tyrosine hydroxylase (TH) transcripts by RT-PCR is one way to assess whether PBSCs are contaminated with tumour cells.<sup>264</sup> Re-infusion of PBSC contaminated with tumour at the time of autologous BMT may play a significant role in this relapse.<sup>288</sup>

Early observations that retinoids and other agents are useful to induce differentiation of NB, and, hence improve survival outcomes, have raised interest in these compounds as biological response modifiers against MRD in the bone and bone marrow.<sup>152,262,295</sup> Immunocytology and quantitative RT-PCR for tumour specific markers such as diganglioside (GD2)<sup>296</sup> and TH are currently used to detect and quantify MRD. Therapeutic regimens presently integrate novel biological therapies, including retinoic acid post-consolidation therapy for high-risk NB, aimed at eradicating MRD.<sup>295</sup> Recently, a MRD model has been conceptualized as a novel approach to testing preclinical therapies and interpreting mechanisms of MRD and metastatic disease in experimental NB.<sup>297</sup>



Currently, patients receive six courses of 13-cis-retinoic acid (CRA) to eradicate residual disease that may still be present despite meeting imaging criteria for complete remission. This treatment is guided by the observation that high-dose CRA administered after chemoradiation significantly improved EFS in high-risk NB.<sup>152</sup> Side effects such as skin dryness and cheilitis are the dose-limiting factors, and consequently, CRA therapy involves of 2-week courses alternating with 2 weeks for mucocutaneous recovery.<sup>1,298</sup> CRA appears to be most suited in the setting of MRD.<sup>62</sup> High dose CRA and pulse schedules of other retinoids show therapeutic and chemopreventive efficacy in NB, but low-dose, chronic retinoid administration may not be as effective to treat MRD.<sup>299</sup>



Clinical trials involving myeloablative chemotherapy and  $^{131}\text{I}$ -MIBG have been undertaken in an effort to minimize adverse side effects and rationalize more targeted therapies.<sup>300-302</sup>  $^{131}\text{I}$ -MIBG exhibits activity against refractory NB with response rates ranging from 10-50%. In a phase I trial of  $^{131}\text{I}$ -MIBG therapy for relapsed NB, myelosuppression was the most significant toxicity at doses  $>15$  mCi/kg, as nearly half of the patients enrolled required haematopoietic cell transfusion. Despite this, the response rate (36%), EFS (18% at 1 year), and OS (49% at 1 year; 29% at 2 years) were found to be significantly higher in patients older than 12 years and who had fewer than three prior treatment regimens.<sup>303</sup> Subsequently, a phase I dose escalation study of  $^{131}\text{I}$ -MIBG with myeloablative chemotherapy and stem cell rescue showed a significant response rate of 25% in patients with primary refractory disease.

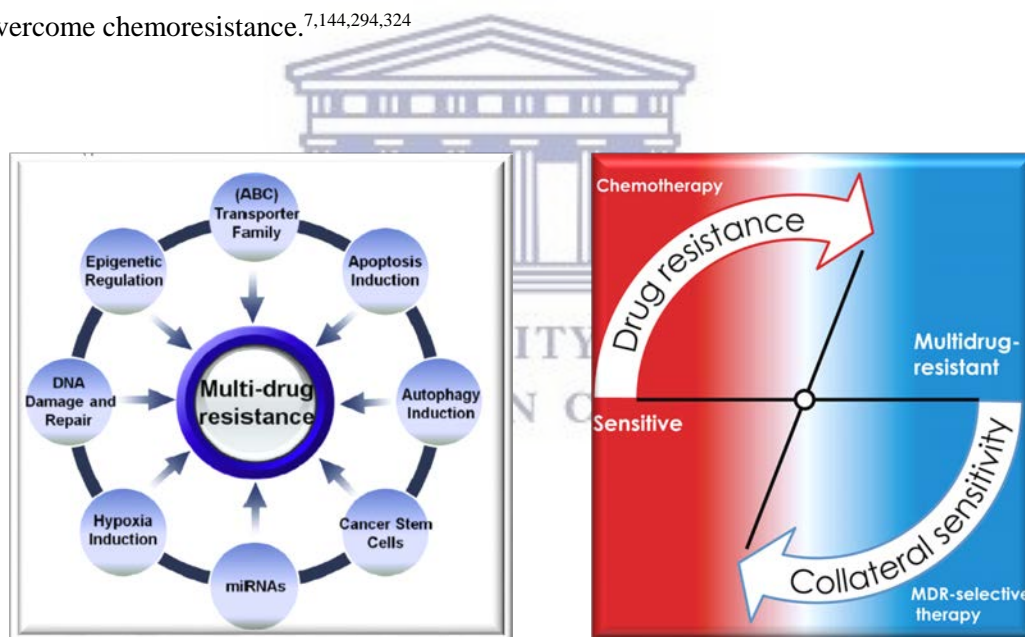
These observations, together with recent research, indicate that  $^{131}\text{I}$ -MIBG may prove useful in conjunction with other treatment modalities,<sup>1,49,80,300,302,304,305</sup> as corroborated also by a recent that autologous stem-cell transplantation (ASCT) may not be indispensable for better outcomes when anti-GD2 immunotherapy is used for consolidation after dose-intensive conventional chemotherapy.<sup>306</sup> However, in certain cases, even after 12 months of high-dose chemotherapy, treatment failures due to MRD are still widespread because of the acquisition of drug resistance and most patients who relapse eventually die from disease progression. Also, those patients who achieve a cure with initial therapy persist to be at risk for developing long-term complications related to treatment, including blindness, hearing loss, infertility, and secondary malignancies.<sup>307,308</sup>

### **A13.1.7 Multidrug Resistance and Monitoring Response to Treatment**

The term multidrug resistance (MDR), as applied to cancer, is a phenomenon in which tumour cells have developed decreased sensitivity to a wide variety of unrelated drugs (cross-resistance) with different modes of pharmacological activity.<sup>309-314</sup> Many of the mechanisms that decrease cell sensitivity to chemotherapeutic agents are caused by well-defined genotypic and phenotypic alterations, including overexpression of the ATP-binding cassette (ABC)

transporter family, apoptosis induction, autophagy induction, cancer stem cell (CSC) regulation, miRNA regulation, hypoxia induction, DNA damage and repair, and epigenetic regulation (Figure 1.11).<sup>312,315,316</sup>

The molecular basis of MDR generally involves specific members of the ABC drug transporters or efflux pumps such as ABCB1 (P-glycoprotein/P-gp/MDR1), ABCG2 (breast cancer resistance protein/BCRP) and ABCC1 (multidrug resistance protein 1/MRP1) and ABCC4/MRP4.<sup>311,313,317,318</sup> In paediatric malignancies, different CSC phenotypes have been detected—including those that overexpress ABC transporters<sup>293,319-323</sup> — not only accounting for the tumour heterogeneity associated with NB, but also raising hopes that further insight into the mechanisms that control the traits of CSCs may aid in the design of novel strategies to overcome chemoresistance.<sup>7,144,294,324</sup>



**A: Source:**<sup>315</sup> Wu Q, Yang Z, Nie Y, Shi Y, Fan D. Multi-drug resistance in cancer chemotherapeutics: Mechanisms and lab approaches. *Cancer Letters* 2014;347(2):159-166, with permission from Cancer Letters, Elsevier Ireland Ltd.) See Appendix 7 for copyright clearance.

**B: Source:**<sup>325</sup> Szakacs G, Hall MD, Gottesman MM, Boumendjel A, Kachadourian R, Day BJ, Baubichon-Cortay H, Di Pietro A. Targeting the Achilles heel of multidrug-resistant cancer by exploiting the fitness cost of resistance. *Chemical Reviews* 2014;114(11):5753-5774, with permission from the American Chemical Society (ACS; <http://pubs.acs.org/copyright/permissions.html>).

**Figure 1.11:** Mechanisms of MDR and the concept of MDR targeting based on collateral sensitivity

Overexpression of ABCG2/BCRP and ABCC4/MRP4 in subpopulations of stem cells was demonstrated in NBs.<sup>311,326,327</sup> ABC transporters such as ABCC1, ABCC3 and ABCC4 are subject to direct transcriptional control of MYCN,<sup>328</sup> and their expression compellingly correlates with poor prognosis.<sup>327,329,330</sup> Many aggressive NBs exhibit MDR,<sup>311</sup> attributable to p53 mutations and/or a loss of p53 function acquired during chemotherapy,<sup>331,332</sup> which escalates the likelihood of relapse and thus presents a major obstacle to effective tumour eradication.<sup>333,334</sup> Most metastatic drug-resistant NBs derive from the selection of clones (side population cells) that express the *MDR1* (*ABCB1*), *MRP1/ABCC1* and *MRP4/ABCC4* gene family, which may or may not correlate with *MYCN* amplification and poor outcome.<sup>7,294,323,327,328,330,335</sup> Moreover, MRD, the major cause of tumour recurrence (relapse) and metastasis, is enriched in CSCs with an increased drug efflux capacity mediated through overexpression of ABC transporters.<sup>336,337</sup>

Hence, in the case of refractory NBs, the treatment algorithm is determined by the patient's disease stage and risk stratification.<sup>1</sup> The objective of induction chemotherapy is to promote remission by alleviating the tumour burden, which then simplifies complete resection when indicated—such as stage 1-2B tumours, but in the case of more advanced-stage NB (stages 3 and 4), surgical intervention is limited to an open biopsy, and for infants who are stage 4S, surgical resection is not recommended since these tumours tend to spontaneously differentiate and regress.<sup>338,339</sup> Induction chemotherapy consists of various combinations of cyclophosphamide, doxorubicin, cisplatin, melphalan, carboplatin, etoposide, topotecan, ifosfamide and vincristine, an example of the chemotherapeutic platform of collateral sensitivity (the hypersensitivity of resistant cancer cells to other drugs) that aims to kill MDR cells selectively over the parental cells from which they were derived.<sup>325,340-343</sup> After induction, treatment is consolidated with one or more courses of high-dose chemotherapy to induce bone marrow ablation, which necessitates autologous haematopoietic stem cell support. Rescue is not without complication as it can lead to growth failure, endocrinopathy, and the occurrence of secondary metastases.<sup>6,8,18,44,47,64,80,251,265,273,295,344-346</sup>

### **A13.1.8 Alleviating the Burden of Late Effects**

A standard component of NB management is follow-up of survivors to monitor and treat adverse events that may debilitate their quality of life and increase the rate of early mortality.<sup>8,62,73,347</sup> NB patients invariably are subjected to intensive therapy which are increasingly associated with a number of complications, including: hearing loss linked to platinum compounds and ototoxic antibiotics indicated for neutropaenic infections (may result in learning and speech impediments); compression of renal vessels resulting in hypertension (renal toxicity caused by platinum agents, myeloablative therapy (MAT) with ASCT or HSCT, radiotherapy, nephrectomy, nephrotoxic antibiotics); secondary metastases initiated by alkylating agents or radiotherapy; leukaemias and myelodysplastic syndromes reportedly related to high doses of etoposide and cyclophosphamide (a reduction of these was achieved by limiting the number of cycles; thyroid cancers and other solid tumours; hypothyroidism; growth impairment; musculoskeletal abnormalities (scoliosis, osteoporosis and bony and soft tissue hypoplasia may result from surgery and/or radiotherapy); cardiopulmonary sequelae induced by anthracyclines or thoracic radiotherapy (endocrine complications and reduced fertility.<sup>62,347-353</sup> It is therefore imperative to develop new therapeutic regimens that will improve the survival and quality of life of NB patients.<sup>64</sup>

### **A13.2 Current Research Milestones and Proposed Novel Therapies**

#### **A13.2.1 Differentiation and Retinoids**

Retinoids, including 13-cis-retinoic acid (CRA, isotretinoin), all-trans retinoic acid (ATRA) and N-(4-hydroxyphenyl) retinamide (HPR, fenretinide) are endogenous, lipophilic vitamin A derivatives that arrest cell growth and induce differentiation of cell lines derived from tumours that are resistant to anticancer drugs.<sup>152,298,354-361</sup> In the late 1980s, ATRA has been hailed as a therapeutic advancement for acute promyelocytic leukaemia and high-risk neuroblastoma (HR-NB).<sup>354</sup> Retinoic Acid (RA) is included in multimodal therapies because it stimulates differentiation of NB cells *in vitro* and decreases the risk of tumour recurrence.<sup>362,363</sup>

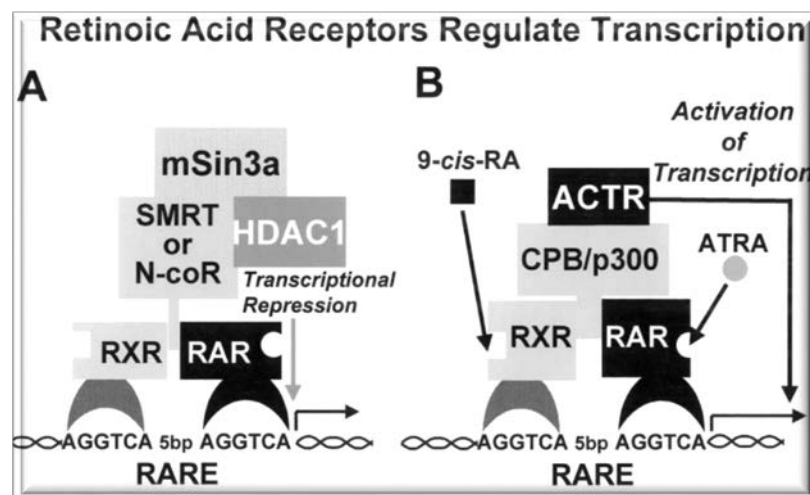
CRA is given at completion of cytotoxic therapy to control MRD in NB.<sup>262,264,295,364-368</sup> ATRA or CRA have been shown to inhibit cell proliferation and induce morphological differentiation of human NB cell lines,<sup>355,356</sup> whereas HPR (an apoptosis inducer) is highly active against retinoic-acid-resistant NB cell lines by deregulating *MYC* expression and removing its transcriptional repression of NB cell differentiation.<sup>369,370</sup>

HPR inhibits NB-induced angiogenesis and may be applied usefully in aggressive HR-NB.<sup>62,371</sup> HPR is also well-tolerated in clinical trials, but has a poor solubility and may decrease night vision.<sup>372</sup> Interestingly, downregulation of the acylated and glycosylated 67-kDa laminin receptor (67LR) by RA has been shown to correlate with reduced metastatic aggressiveness of human NB cells, rendering 67LR as a molecular target in NB.<sup>373</sup>

The retinoids bind to members of the nuclear receptor family of proteins (transcription factors), i.e., the retinoic acid receptors (RARs—multiple isoforms of RAR $\alpha$ ,  $\beta$ , and  $\gamma$ ) and the retinoid X (rexinoid) receptors RXRs—multiple isoforms of RXR $\alpha$ ,  $\beta$ , and  $\gamma$ )—which alter interactions of transcription complexes with numerous cancer stem cell (CSC) genes, thus triggering an exit from the self-renewing, pluripotent CSC reserve into a differentiated mature cell niche.<sup>354,374</sup> The retinoids are activated by RARs and the rexinoids by RXRs—RAR/RXR heterodimers constitutively associate with RA response elements (RAREs) in promoters of target genes (Figure 1.11).<sup>298,354,357,374,375</sup>

The zinc-finger cluster of ZNF423 (also known as Ebfaz, OAZ, or Zfp423) has been shown to be indispensable for retinoid-induced differentiation. ZNF423 combines with the RAR $\alpha$ /RXR $\alpha$  nuclear receptor complex and is critical for transactivation in response to retinoids. Blockade of ZNF423 expression by RNA interference in NB cells confers a growth advantage and acquired resistance to RA-induced differentiation whereas its overexpression triggers growth arrest and amplified differentiation.





The mechanism of action of retinoids is mediated via zinc-finger transcriptional regulators which function as heterodimers to regulate promoter activity of certain target genes. The RAR and RXR proteins bind to specific direct repeat DNA sequences (AGGTCA are separated by either 2 or 5 nucleotides) in gene promoters, known as RA response elements. (A) In the absence of ligand, the RAR/RXR heterodimers interact with nuclear co-repressors including N-CoR and SMRT, which in turn bind to a common adapter protein mSin3 that complexes to proteins with histone deacetylase activity to repress transcription. (B) RA binds to the RAR portion of the complex causing a conformational change in the RAR and RXR proteins which releases the co-repressor complex and facilitates binding of 9-cis-RA to the RXR protein (the latter enhances the activation response). The transcriptional co-regulator CBP/p300 then binds to the receptor complex and recruits the coactivator protein ACTR, which contains histone acetyltransferase activity, that promotes transcription; RARE= retinoic acid response elements.

**Source:**<sup>298</sup> Reynolds CP, Matthay KK, Villablanca JG, Maurer BJ. Retinoid therapy of high-risk neuroblastoma. *Cancer Letters* 2003;197(1-2):185-192, with permission from Cancer Letters, Elsevier Ireland Ltd.) See Appendix 8 for copyright clearance.

**Figure 1.12:** The mechanism of action of retinoids and rexinoids

Correspondingly, deregulation of ZNF423 expression correlates with poor disease outcome in HR-NB patients.<sup>359</sup> Several clinical trials have been conducted to establish the maximal-tolerated doses (MTDs), pharmacokinetics, efficacies and dose-limiting toxicities (DLT) of the retinoids/rexinoids.<sup>357</sup> Current selected clinical trials on various interventions in NB have recently been published.<sup>376</sup> In a phase I trial in children 2 to 12 years of age with NB, treatment with CRA (isotretinoin) doses escalated from 100 to 200 mg/m<sup>2</sup>/day following BMT, DLTs in 6 of 9 patients were observed, including hypercalcaemia (n=3), rash (n=2), and anaemia/thrombocytopenia/emesis/rash (n=1). All toxicities resolved after CRA withdrawal. Three complete responses were observed in bone marrow metastases.<sup>377</sup> Another study was performed on eligible patients which included children and adolescents (1 to 18 years of age) with newly diagnosed HR-NB to assess whether MAT in conjunction with ABMT improved EFS as compared with chemotherapy alone, and whether subsequent treatment with CRA



further improves EFS. In this study, 434 patients had Evans stage IV NB; 72 had stage III disease with one or more of the following: amplification of the *MYCN* oncogene, a serum ferritin level of at least 143 ng/ml and unfavourable histopathological findings; 1 had stage II disease with amplification of *MYCN* (age>1 year); 13 had stage I or II disease with bone metastases before therapy other than surgery; and 19 had had stage IV disease with *MYCN* amplification for less than one year. The conclusion was that treatment with MAT and ABMT improved EFS among children with HR-NB and, significantly, that CRA administered successively to chemotherapy or transplantation had a favourable outcome for patients without progressive disease.<sup>152</sup>

However, a recent phase 2 trial of ATRA, administered orally at a dose of 90 mg/m<sup>2</sup>/day in three divided doses for 3 consecutive days per week—and IFN- $\alpha$ 2a administered subcutaneously daily at a dose of  $3 \times 10^6$  U/m<sup>2</sup>/day for 5 consecutive days per week, in 4 week cycles—was inactive in children with relapsed or refractory NB and Wilms tumour.<sup>378</sup> By comparison, assessment of the long-term outcome of HR-NB patients enrolled on the CCG-3891 study in which patients were randomly assigned to undergo ABMT or to receive chemotherapy and subsequent treatment with CRA indicated that MAT and ABMT, significantly improved 5-year EFS than non-MAT chemotherapy and neither MAT with ABMT nor CRA given after consolidation therapy significantly improved OS.<sup>379</sup> The aforementioned differences may be ascribed to pharmacogenetic variation on CRA disposition in patients with HR-NB and emphasize the need for personalized therapies.<sup>357,380</sup>

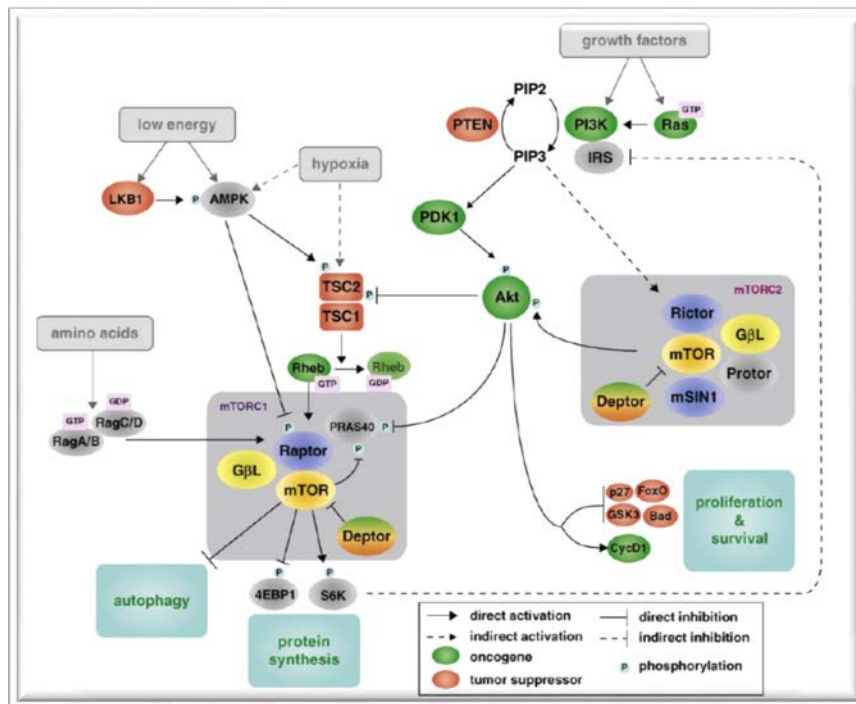
In a retrospective cohort design to verify if intensive chemoradiotherapy with purged ABMT and/or CRA improved outcome for HR-NB patients with no metastatic distant sites, it was deduced that patients with high-risk INSS Stage 3 NB have an overall poor prognosis despite aggressive chemoradiotherapy.<sup>358</sup> Clearly, further research into the efficacies and DLTs of the retinoids is needed to address current controversies surrounding their beneficial status in patients with HR-NB.<sup>295</sup>

### A13.2.2 mTOR Inhibitors

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and downstream effector of the PI3K/AKT pathway and master regulator that orchestrates, via a network of regulatory loops, various signals from nutrient and energy sensors with cell growth and proliferation, survival and motility to ensure that they are activated exclusively during favourable conditions within different organs (Figure 1.12).<sup>381</sup> The mTOR pathway can be activated by various exogenous stimuli such as growth factors, nutrients, energy and stress signals, and essential signalling pathways (e.g., PI3K, MAPK and AMPK) in order to mediate temporal control of physiological processes.<sup>382</sup> Deregulation of the mTOR pathway, including PI3K amplification or mutation, PTEN loss of function, AKT gain of function, and S6K1, 4EBP1 and eIF4E overexpression, has been correlated with oncogenesis, tumour progression and metastases of many cancers, including NB.<sup>381,383-387</sup>

In a benchmark study, AKT and mTOR were found to be overexpressed in primary NB tissue samples, but in non-malignant adrenal medullas this pattern could not be demonstrated. mTOR inhibitors (rapamycin and CCI-779) arrested the growth of NB cells in culture, particularly cell lines with a high *MYCN* gene expression signature. *In vivo*, mTOR inhibitors increased apoptosis, decreased cell proliferation and blocked angiogenesis in established NB tumours. Significantly also, mTOR inhibitors induced downregulation of VEGF-A secretion, cyclin D1 and *MYCN* protein expression *in vitro* and *in vivo*.

Even though mTOR inhibitors may inhibit proliferation of human NB cells without suppression of the *MYCN* oncoprotein,<sup>388</sup> the above findings underscore the therapeutic efficacy of mTOR inhibitors in aggressive *MYCN* amplified NBs and corroborate similar observations in NB tumours with 1p36 aberrations, advanced stage disease at diagnosis and unfavorable histology in which AKT and *MYCN* are co-amplified.<sup>389,390</sup> The mTOR pathway and VEGF signalling are implicated in the regulation of clonal proliferation, angiogenesis and metastasis.



**mTOR signaling pathway.** One branch of mTORC1 activation is mediated by the class I phosphoinositide-3-kinase (PI3K), Akt (also known as Pkb) and the tuberous sclerosis complex (TSC). TSC is formed by TSC1 and TSC2, and inhibits a direct activator of mTORC1, the GTPase Ras-homolog enriched in brain (Rheb) by hydrolyzing its GTP into GDP. TSC2 is activated by phosphorylation by AMP-activated protein kinase (AMPK), which is directly activated by a high AMP versus ATP ratio. AMPK also directly phosphorylates Raptor, so it inhibits mTORC1 by TSC-dependent and TSC-independent manners. The activity of AMPK is regulated by phosphorylation by the tumour suppressor LKB1. This protein, like TSC1/2, was found mutated in the germline of patients with different hamartomatous syndromes. Akt is a serine/threonine kinase and an important player in regulating mTORC1 activity. Akt positively regulates mTORC1 by acting at different levels. First, Akt inactivates TSC1/2 by phosphorylating TSC2. Second, Akt inhibits PRAS40, negative regulator of mTORC1 that counteracts Rheb function. Akt is activated by PI3K, which responds to a variety of growth factors. When activated by insulin or insulin-like growth factors (IGFs), as well as other growth factors, class I PI3K catalyzes the formation of the lipidic second messenger phosphoinositide-3,4,5-tri-phosphate (PIP3) from the bi-phosphate form PIP2. PIP3 triggers the relocation of Akt to the inner surface of the plasma membrane, where it is activated by phosphoinositide-dependent kinase 1 (PDK1) and transduces the signal as described above. Opposing Akt function is the tumour suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN), a lipid phosphatase that converts PIP3 to PIP2, thus shutting off signaling from PI3K. PTEN deficiency causes a series of hamartomatous syndromes collectively classified as PTEN hamartoma tumour syndrome (reviewed in. Amino acids activate mTORC1 by an independent route mediated by the Rag family of proteins. The activation of mTORC2 is not well understood, but this complex directly activates Akt (and Akt-related kinases) by phosphorylation. Akt, in addition, regulates many proteins involved in cell survival and cell-cycle progression.

**Source:**<sup>381</sup> Efeyan A, Sabatini DM. mTOR and cancer: Many loops in one pathway. *Current Opinion in Cell Biology* 2010;22(2):169-176, with permission from Current Opinion in Cell Biology, Copyright Clearance Center's RightsLink service, Elsevier) See Appendix 9 for copyright clearance.

**Figure 1.13:** Overview of the mTOR signalling pathway in cancer

Collateral inhibition, either in a concurrent or successive design, of mTOR and VEGF signalling exemplifies an interesting therapeutic rationale to overcome MDR and optimize efficacious tumour ablation and also to identify prognostic biomarkers for neuroendocrine neoplasms (NENs).<sup>391</sup>

Novel drugs targeting the PI3K/AKT/mTOR cascade in various malignancies, including NB, are currently being refined.<sup>384-387,392-399</sup> The mTOR inhibitor rapamycin (also termed sirolimus) and its analogues (rapalogs), including temsirolimus, everolimus, and ridaforolimus) form a complex with the cytosolic protein FK-binding protein 12 (FKBP12) which attaches directly to mTOR, impeding its function and activating downstream effectors, such as cyclin D1, p21, and HIF1a/b.<sup>197,397</sup> Of concern, however, is the ability of rapamycin to induce the anti-apoptotic protein, survivin which, in NB, may favour clonal proliferation of resistant cells.<sup>203</sup>

Rapamycin and some rapalogs have been approved for clinical trials because of their propensity to inhibit NB cell proliferation.<sup>388,389</sup> Evaluation of the efficacy of temsirolimus in a phase II trial of children with relapsed or refractory high-grade NB did not produce encouraging results despite the observation that disease stabilization occurred which makes it a candidate for combination therapy.<sup>197,400</sup> Similar clinical outcomes have been reported for everolimus in refractory solid NB in paediatric patients.<sup>400-402</sup>

Preclinical evaluation of mTOR inhibitors that mimic ATP-competitive inhibitors (e.g., INK128/MLN0128, AZD2014, and OSI027) have shown limited potential as inhibitors of NB growth,<sup>403</sup> but compounds that target the feedback loops in mTOR/AKT signalling (e.g., MK-2206, an AKT inhibitor) show promise in suppressing tumour growth and increasing survival in mice bearing xenograft NB tumours.<sup>404</sup> Recent efforts focusing on the development and validation of pharmacodynamic biomarkers to evaluate both the mechanism of action of and proof of concept for drugs that block MYCN and PI3K/AKT/mTOR pathways in children with NB may prove useful in future clinical trials.<sup>395,399</sup>

### A13.2.3 Aurora A Kinase and MDM2 as MYCN Targets

Aurora A kinase (AURKA, a serine/threonine kinase), along with p53 and MDM2, are downstream effectors of *MYCN* that regulate cell cycle progression (particularly during the G2 to M phase transition), the DNA damage response, differentiation and apoptosis in NB<sup>405,406</sup> AURKA has been implicated in centrosome maturation, spindle assembly and orientation, meiotic maturation and cytokinesis. Targeted inhibition of AURKA leads to deregulation of autophosphorylation and p53 phosphorylation, monopolar spindles and G2-M arrest. Overexpression of AURKA is widespread in solid tumours and associated with resistance to apoptosis, making it a significant focus for the development of anticancer agents, some of which are currently in early-phase NB clinical trials.<sup>197,407,408</sup>

For example, MLN8237 (alisertib), a reversible AURKA inhibitor, is being investigated in phase I clinical trials by the COG for patients who have experienced relapse. *In vitro* and vivo effects of MLN8237 include apoptosis induction, upregulation of p53 and the tumour suppressor genes p21 and p27.<sup>37,198,409,410</sup> MLN8054 inhibits N-Myc-dependent transcription, correlating with tumour regression and prolonged survival in a mouse model of MYCN-driven NB.<sup>411</sup> AURKA also increases VEGF secretion and NB angiogenesis.<sup>412</sup> Thus, AURKA is a negative prognostic factor in human NB.<sup>413</sup> Dose-escalation and combination therapy studies are currently in progress.<sup>1,11</sup> In a phase 1 trial, alisertib, an oral AURKA inhibitor, in combination with irinotecan and temozolomide, showed promising response and progression-free survival (PFS) rates in patients with advanced NB.<sup>414</sup> Alisertib and a novel pan-AURKA inhibitor, BPR1K653, show potential for the management of patients with MDR1 (ABCB1)-related drug resistance after prolonged chemotherapeutic treatments.<sup>415-417</sup>

### A13.2.4 Tyrosine Receptor Kinase Neurotrophin Receptor Inhibitors

The tyrosine receptor kinase (Trk) neurotrophin receptors (also referred to as neurotrophic tyrosine receptor kinases)—TrkA/NTRK1, TrkB/ NTRK2 and TrkC/NTRK3 (3 isoforms) are crucial modulators of normal central and peripheral nervous system developmental outcomes



(e.g., neuronal differentiation and survival) and NB pathogenesis. Their respective ligands are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) growth factor.<sup>12,247,418-421</sup> Overexpression of TrkA correlates with favourable prognosis and suppressed MYCN amplification,<sup>422,423</sup> whereas upregulation of TrkB and its ligand, BDNF, is associated with aggressive NB—invasion, metastasis, angiogenesis and drug resistance—and unfavourable clinical outcomes.<sup>424</sup>

Tumours isolated from patients with low-stage and 4S disease frequently express elevated levels of TrkA.<sup>12</sup> It is therefore not surprising that the induction of apoptosis and tumour regression in NBs by targeting neurotrophin receptor pathways such as TrkA and p75<sup>NTR</sup>—a member of the tumour necrosis factor (TNF) receptor superfamily—is considered a promising therapeutic paradigm. This is supported by observations that TrkA-expressing tumour cells in primary culture will survive and even differentiate in the presence of NGF, but undergo apoptosis in its absence.<sup>425-427</sup>

Noteworthy also, lestaurtinib (CEP-701), has shown proof-of-principle as a small molecule inhibitor of Trk neurotrophin receptors (TrkA, TrkB and TrkC) against TrkB-expressing NB xenografts,<sup>428-431</sup> and in a phase I trial in children with recurrent and/or refractory neuroblastoma.<sup>432</sup> Several second-generation Trk inhibitors are currently in phase I clinical trials or in preclinical development.<sup>433</sup> Interestingly, oncogenic TRK gene fusions are found across multiple tumour types, and those involving NTRK1, NTRK2 and NTRK3, and in-frame deletions or splice variants of NTRK1 signify newer rational drug targets in cancer and are likely to be actionable oncogenes based on preclinical data.<sup>434</sup>

### **A13.2.5 Targeted Immunotherapy and Disialoganglioside**

Immunotherapy of NB is gaining momentum as a treatment elective to enhance the survival of patients suffering from this challenging paediatric cancer.<sup>435-438</sup> Targeted immunotherapy of MDR microscopic NB offers an approach which exploits tumour selectivity and minimizes



cross-resistance or overlapping side effects (toxicities) with chemotherapy.<sup>62,436</sup>

Disialoganglioside, GD2, is expressed on the surface of tumours of neuroectodermal origin, including NB.<sup>296,439</sup> Anti-GD2 monoclonal antibodies (mAbs) ablate tumour cells through both complement- and cell-mediated lysis (antibody mediated cell cytotoxicity or ADCC), and are therefore exceptional candidates for targeted immunotherapy since they have specificity, high affinity and are relatively nontoxic.<sup>296</sup> A number of anti-GD2 mAbs  $\pm$  GM-CSF  $\pm$  CRA have been tested in clinical trials and favourable therapeutic outcomes were reported.<sup>11,151,440,441</sup>

However, a recent trial inferred a lack of survival advantage with ASCT in HR-NB consolidated by anti-G D2 immunotherapy and CRA, adding to the complexity of developing targeted immunotherapies for NB.<sup>306</sup> Targeting NB immune escape pathways, intrinsic NB cell defects such as impaired expression of the human leukocyte antigen (HLA) class I related antigen processing machinery and functional alterations of the tumour microenvironment (TM) induced by NB cell-derived immunosuppressive molecules such as human major histocompatibility complex (MHC) class I chain-related gene A (MICA) and (HLA-G are critical considerations of such therapeutic interventions.<sup>442</sup>

### **A13.2.6 Angiogenesis and VEGF Signalling Inhibitors**

Angiogenesis or neovascularization—one of the hallmarks of cancer—encompasses the sprouting of new blood vessels in tumours that enable them to grow, survive and metastasize before their metabolic demands are restricted due to diffusion limits of oxygen and nutrients in the tumour microenvironment.<sup>145-148,443</sup> Vascular endothelial cell growth factor (VEGF) is the most potent activator of angiogenesis and comprises six members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor) that bind differentially with three cell surface RTKs, the VEGFRs, or a second class of non-signalling co-receptors, the neuropilins.<sup>443</sup> In NB, elevated expression of pro-angiogenic factors correlates with advanced stage disease while low vascular tumour density is associated with non-metastatic localized

disease and favourable prognosis.<sup>62,444,445</sup> Accordingly, inhibition of angiogenesis has long been regarded as a promising line of attack in the management HR-NB.<sup>446-450</sup> Current anti-vascular NB therapies combining multimodal antiangiogenic, anti-vasculogenic mimicry and anti-lymphangiogenic strategies may yield increased efficacy.<sup>278,391,446,448,449,451-462</sup>

### **A13.2.7 The PI-3 Kinase-Akt-MDM2-Survivin Signalling Axis in High-Risk Neuroblastoma**

The phosphatidylinositol-3 (PI-3) kinase-Akt pathway is a central convergent molecular conduit, and PI-3 kinase is a multiplex signalling hub downstream of various growth factor receptors, including TrkB, VEGFR, PDGFR and EGFR.<sup>463</sup> Activation of the PI3K/Akt pathway in NB correlates with poor patient prognosis, and the forkhead transcription factor, FOXO3a, is a key target of the PI3K/AKT pathway in NB. FOXO3a expression was shown to be upregulated in low-stage NB and normal embryonal neuroblasts, but ablated in late-stage NB. Thus, inactivation of FOXO3a by AKT is essential for neuroblastoma cell survival.<sup>464</sup> The mammalian target of rapamycin (mTOR) protein (section A13.2.2 on mTOR inhibitors) is currently being regarded as a potential therapeutic target in NB patients.<sup>203,391,463,465-467</sup>

Well-defined adjustments are associated with mitochondrial proteins, e.g., VDAC1/Porin protein, an integral part of the mitochondrial permeability transition pore complex, during loss of mitochondrial membrane potential with subsequent cytochrome c release and caspase-3 activation. VDAC1 is negatively regulated by the PI3K/Akt pathway via GSK3beta and inhibition of GSK3beta is activated when Akt is blocked.<sup>468</sup> Similarly, a recent study showed that guanosine offers protection against mitochondrial oxidative stress by a signalling pathway that implicates PI3K/Akt/GSK-3beta proteins and induction of the antioxidant enzyme, haem oxygenase-1.<sup>469</sup> Also, the PI3K/Akt pathway is obligatory for RA-induced NB cell differentiation, and may be exploited as a novel therapeutic strategy against poorly differentiated NB.<sup>394,395,467,470</sup>

### A13.2.8 Gastrin-Releasing Peptide Receptors

Gastrin-releasing peptide (GRP) receptors (GRPR), a member of G-protein coupled receptor family, are overexpressed in undifferentiated NB.<sup>471,472</sup> The decreased expression of the tumour suppressor protein PTEN in aggressive undifferentiated NB is associated with an increase in GRP binding capacity, as a result of GRP-R overexpression.<sup>473-475</sup> It has been suggested that inhibition of the PTEN tumour suppressor gene may be an important regulatory mechanism involved in GRP-induced cell proliferation in NB which offers promising scenarios for the use of radiolabelled and cytotoxic GRP analogues and antagonists for cancer diagnosis and therapy.<sup>476-479</sup> GRPR transactivates the epidermal growth factor receptor (EGFR) and may thus modulate therapeutic responses to EGFR inhibitors, e.g., gefitinib.<sup>480</sup> GRP upregulates proangiogenic IL-8 expression in an Ets1-dependent manner, implying a key role during GRP-induced NB angiogenesis and metastasis,<sup>481,482</sup> and its promise as a NB biomarker and gene silencing therapeutic target.<sup>483-486</sup>

### A13.2.9 Anaplastic Lymphoma Kinase

Germline ALK activating mutations have been implicated in the majority of hereditary NB and somatic ALK activating mutations have also frequently been observed in sporadic cases of advanced NB. Accordingly, gain of function mutations in the gene encoding the anaplastic lymphoma kinase (ALK) is currently deemed the most frequent druggable mutations identified in NB. Preclinical studies warrant the notion of an oncogene addiction of NB cells to mutated ALK and corroborate that ALK inhibitory therapy effectively blocks tumour models. Recently, a paediatric phase I trial for the first approved ALK inhibitor, crizotinib, illustrated significant antitumoural efficacy in NB patients. A successive international phase I study with the second generation ALK inhibitor, LDK-378, has been launched that makes ALK inhibitory therapy accessible to paediatric patients.<sup>487</sup> However, crizotinib is not as effective in blocking the activity of ALK when activating mutations are present within its kinase domain, as with the F1174L mutation. A new ALK inhibitor, AZD3463, effectively suppresses the proliferation of

NB cell lines with wild type ALK (WT) as well as ALK activating mutations (F1174L and D1091N) by blocking the ALK-mediated PI3K/AKT/mTOR pathway and induces apoptosis and autophagy. Moreover, AZD3463 synergistically enhances the cytotoxicity of doxorubicin on NB cells and shows significant therapeutic efficacy on the growth of NB tumours with WT and F1174L activating mutation ALK in orthotopic xenograft mouse models. These results indicate that AZD3463 is a promising therapeutic agent in the treatment of NB.<sup>488</sup>

Interestingly, the ALK/ROS1 inhibitor, PF-06463922, ablates primary resistance to crizotinib in ALK-driven NB. PF-06463922 has high potency across ALK variants and inhibits ALK more effectively than crizotinib *in vitro*. Essentially, PF-06463922 causes complete tumour regression in both crizotinib-resistant and crizotinib-sensitive xenograft mouse models of NB, as well as in patient-derived xenografts harbouring the crizotinib-resistant F1174L or F1245C mutations. Hence, PF-06463922 shows potential to reverse crizotinib resistance and exert significant activity as a single targeted agent against F1174L and F1245C ALK-mutated xenograft tumours, while also inducing responses in an R1275Q xenograft model. These results provide the reasoning to advance PF-06463922 into clinical trials for treatment of patients with ALK-mutated NB.<sup>489,490</sup>

### **A13.2.10 Future Therapeutic Perspective**

In spite of the knowledge provided in the preceding sections, NB remains a therapeutic enigma. As we are driven to improve outcomes and survival, the ideal therapy also remains elusive. However, there are many fronts on which to attack, and it seems unquestionable that the cure will require a multimodal approach. Part of the solution is to effectively eradicate minimal residual disease, as this appears to put patients at highest risk for relapse and progression. Selected clinical trials on current interventions in NB have recently been collated, but most are recruiting and results may not be available yet ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

## SECTION B: GLYCOBIOLOGY AND GLYCOMICS OF NEUROBLASTOMA

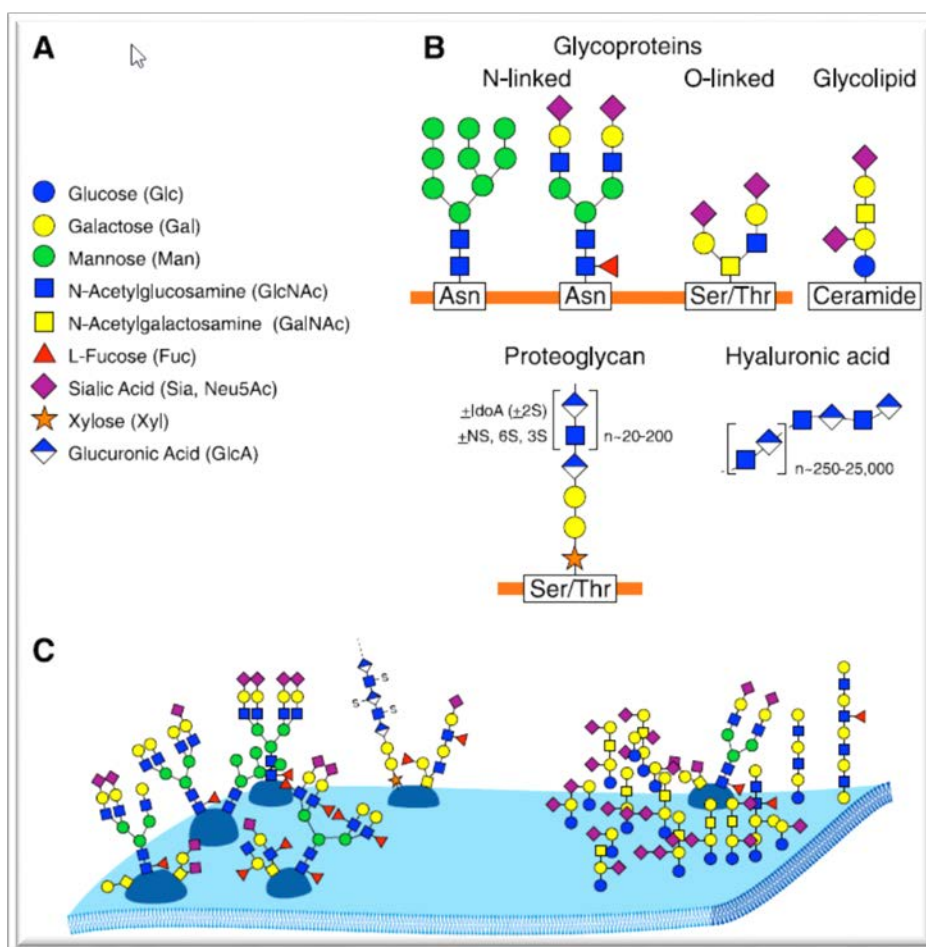
### B1. Orientation to Glycans

Glycans are defined by the International Union of Pure and Applied Chemistry (IUPAC, <https://iupac.org/> and <http://www.chem.qmul.ac.uk/iupac/>) as compounds that consist of monosaccharides or oligosaccharides linked by N- or O glycosidic bonds). Glycans and complementary glycan-binding proteins (GBPs) are indispensable metabolic, structural and modulatory components of various cell functions, including cell-cell communication, cell-matrix interactions, immunity, cancer pathogenesis and progression.<sup>491</sup> The term glycan may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid, or a proteoglycan (Figure 1.14).

The human glycome stems from 9 building blocks that are merged by enzymes (*writers*: glycosyltransferases, glycosidases and glycan modifying enzymes) with precise and regulated biosynthetic functions into a wide diversity of glycan patterns (Figure 1.15) that are functionally read by various human GBPs (*readers*).<sup>492-495</sup> The importance of glycan recognition, for example, in infection and immunity, and advances in our understanding and technologies in the field of glycobiology,<sup>496,497</sup> have already led to the design and use of glycan mimetic anti-infective and anti-inflammatory drugs.<sup>492,498-501</sup>

Glycans are significant regulators of biological homeostasis,<sup>503</sup> playing pivotal roles in protein folding, trafficking and stability,<sup>504,505</sup> and in vertebrate development, morphogenesis and organogenesis,<sup>506-508</sup> and cellular senescence and human aging.<sup>509</sup> Inside cells, protein glycosylation, conceivably in unison with protein phosphorylation, regulates key signal transduction cascades,<sup>503</sup> intercellular communication,<sup>510</sup> pathogen recognition and immunological differentiation of self from non-self.<sup>492,511,512</sup> Glycans are significant regulators of biological homeostasis,<sup>503</sup> playing pivotal roles in protein folding, trafficking and stability,<sup>504,505</sup> and in vertebrate development, morphogenesis and organogenesis,<sup>506-508</sup> and

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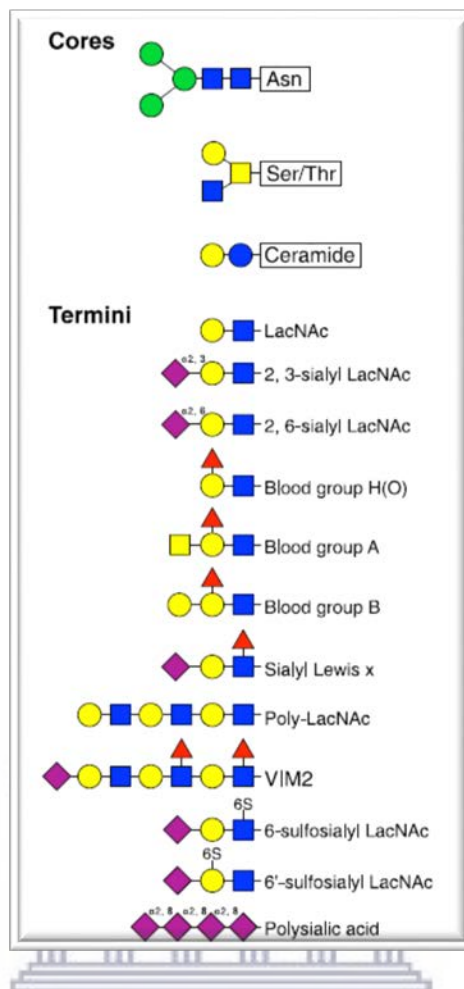


**Major human glycans.** (A) The 9 sugars that comprise most of the human glycome, with their broadly accepted symbol representations.<sup>502</sup> (B) Major classes of human glycans. Linkage details (hydroxyl attachment sites and anomeric configurations at each glycosidic bond) that are keys to structural recognition are omitted here for simplicity. Representative asparagine (N-linked) and serine or threonine (O-linked) glycoprotein structures, a glycosphingolipid (ceramide-linked), a proteoglycan (most frequently O-linked), and hyaluronic acid (HA) (unlinked) are shown. (C) A schematic representation of glycans on a cell surface. Notable features important for understanding glycan recognition include varied glycan branching patterns, variations in terminal glycan structures, and the tendency of glycans to form distinctive, lateral glycan patches.

**Source:**<sup>492</sup> Schnaar RL. Glycobiology simplified: Diverse roles of glycan recognition in inflammation. *Journal of Leukocyte Biology* 2016;99(6):825-838, with permission from *Journal of Leukocyte Biology*. See Appendix 10 for copyright clearance.

**Figure 1.14:** Major human glycans





**Theme and variation in the human glycome.** Glycan recognition often involves variations of terminal glycan structures that are attached to core structures (linkage details are omitted for simplicity). The upper panel provides examples of the invariant, N-linked glycoprotein pentasaccharide core, one of several serine/ threonine-linked glycoprotein cores, and a common glycosphingolipid (ceramide-linked) core. The lower panel provides a sampling of terminal structures. A representation of how these might be grouped on a cell surface is shown in Figure 1.14-C.

**Source:**<sup>492</sup> Schnaar RL. Glycobiology simplified: Diverse roles of glycan recognition in inflammation. *Journal of Leukocyte Biology* 2016;99(6):825-838, with permission from *Journal of Leukocyte Biology*. See Appendix 10 for copyright clearance.

**Figure 1.15:** Theme and variation in the human glycome

Glycans are significant regulators of biological homeostasis,<sup>503</sup> playing pivotal roles in protein folding, trafficking and stability,<sup>504,505</sup> and in vertebrate development, morphogenesis and organogenesis,<sup>506-508</sup> and cellular senescence and human aging.<sup>509</sup> Inside cells, protein glycosylation, conceivably in unison with protein phosphorylation, regulates key signal transduction cascades,<sup>503</sup> intercellular communication,<sup>510</sup> pathogen recognition and immunological differentiation of self from non-self.<sup>492,511,512</sup>

## B2. Protein Glycosylation in Neuroblastoma

### B2.1 General Principles of Glycosylation

Cell-surface and soluble proteins of the secretory pathway are post-translationally glycosylated in the ER.<sup>519</sup> Generally, glycans on membranes, extra cellular matrix (ECM) and secreted proteins are found covalently attached to a protein core at asparagine Asn (N-glycosylation) or at serine/threonine residues (O-glycosylation) (Figure 1.14).<sup>520,521</sup> Glycosaminoglycans (GAGs) are O-linked glycans initiated by a highly conserved tetrasaccharide (GlcA- $\beta$ 1,3-Gal- $\beta$ 1,3-Gal- $\beta$ 1,4 Xyl- $\beta$ ) and classified by the configuration of their disaccharide repeats that consist of either sulfated or non-sulfated monosaccharides.<sup>499,500,522</sup> Typical GAGs are chondroitin sulfate, keratan sulfate, dermatan sulfate and heparan sulfate.

A glycoprotein with one or more GAG chains extending from its protein core is called a proteoglycan which exists as secreted, transmembrane or glycosylphosphatidylinositol (GPI)-anchored units.<sup>523,524</sup> Hyaluronic acid, a GAG-like polysaccharide of the ECM, is the only glycan that is not linked to protein or lipid.<sup>525,526</sup> N-linked glycosylation has been correlated with several physiological and pathological processes such as protein folding and conformation, oligomerization, cell-cell interactions, targeting proteins to sub-cellular or extracellular sites (<http://themedicalbiochemistrypage.org/glycoproteins.php#nglycans> for a quick glance at glycans, glycoproteins and glycosylation).

### B2.2 Gangliosides

Most tumour cells, including those of neuroectodermal cell origin, have upregulated levels of gangliosides.<sup>527,528</sup> Gangliosides also accumulate in activated glia in the developing brain,<sup>529</sup> and may have neuroprotective roles via activation of microglia and astrocytes in response to acute ethanol concentrations in the neonatal brain. However, chronic ethanol exposure can induce an inappropriate proinflammatory glial reaction and neurotoxicity.<sup>530</sup> Gangliosides are glycosphingolipids which comprise of a carbohydrate chain bearing one or several sialic acid

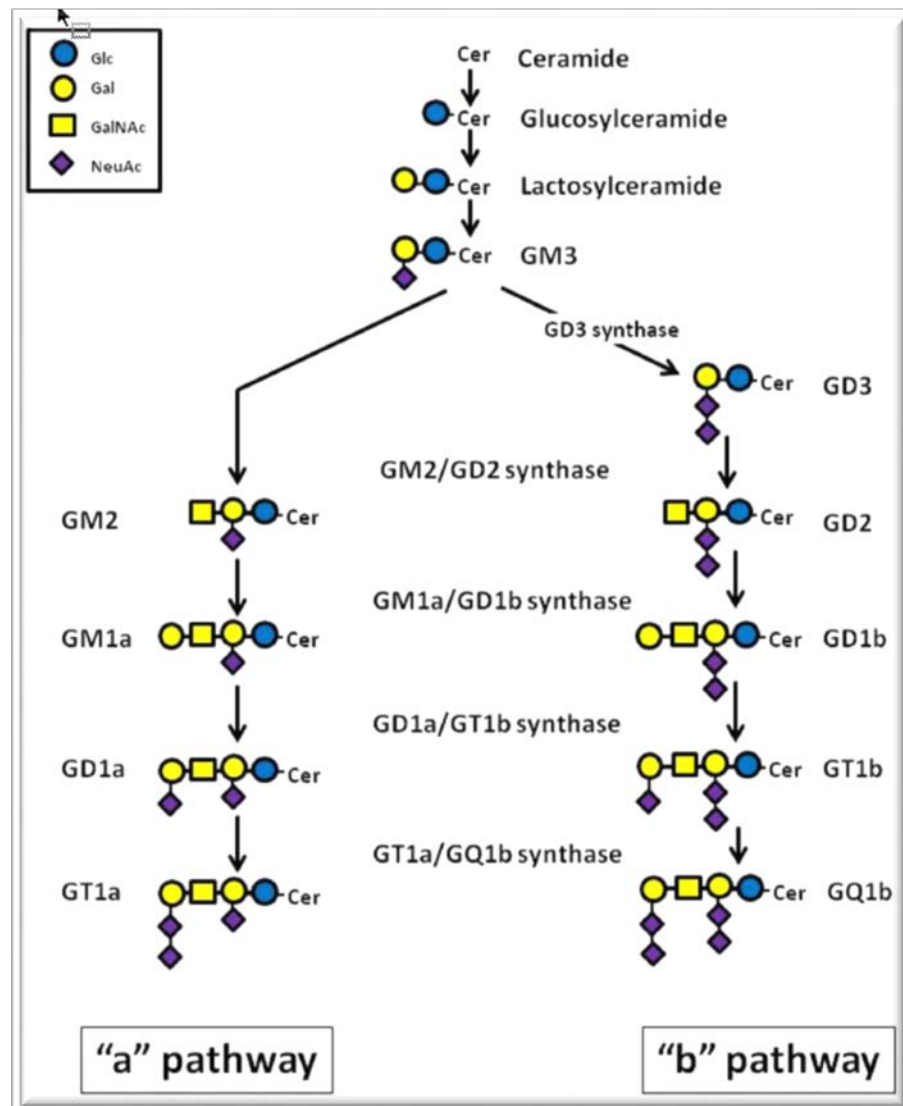
(N-acetyl-neuraminic acid) residues and a lipid portion (ceramide backbone), which attaches (anchors) the ganglioside molecule to the cell membrane.<sup>531,532</sup> Figure 1.16 illustrates the consecutive glycosylation steps in ganglioside biosynthesis which involves two primary pathways indicated as “a” (GM2, GM1a, and GD1a) and “b” (GD3, GD2, GD1b, GT1b and GQ1b), from a common precursor (GM3). GM1a/GD1b synthase (UDP-Gal:betaGlcNAc-beta-1,3-galactosyltransferase) is the key enzyme in ganglioside biosynthesis and has been implicated in human NB.<sup>533</sup>

Even though gangliosides are predominantly expressed on tumour cell surfaces, they may be shed into the tumour microenvironment and ultimately appear in the patient’s plasma to induce the production of anti-glycan antibodies.<sup>527,534-536</sup> In NB, ganglioside signatures or arrays may influence tumour behaviour and clinical outcome.<sup>527,537</sup> For instance, elevated levels of gangliosides of the “b” pathway (GD3, GD2, GD1b, GT1b, GQ1b) are prevalent in infant NB in contrast to NB in older children.<sup>538</sup> These gangliosides correlate with an aggressive NB phenotype and reduced survival in NB patients.<sup>539,540</sup> Overexpression of both complex “a” gangliosides (CaG and CbG), ablates aggressive tumour-cell behaviour *in vitro* (e.g., cellular proliferation and migration) and favours differentiation.<sup>541</sup>

Understandably, complex gangliosides have stimulated interest as diagnostic biomarkers to predict clinical outcome, to stratify NB patients for targeted anticancer therapy and to monitor efficacy of treatment.<sup>542</sup> Thus far, retinoic acid has proved useful for maintenance therapy of disseminated NB as it induces a remarkable shift from synthesis of simple gangliosides toward predominant expression of structurally complex “a” and “b” pathway ganglioside molecules.<sup>527,543</sup>

Targeted immunotherapy of NB with antibodies directed against disialoganglioside (GD2) has been discussed in detail in section A13.2.5. Various other glycans such as polysialic acid (PSA), galectin-1 (Gal-1), and other related processes such as N- and O-protein glycosylation,

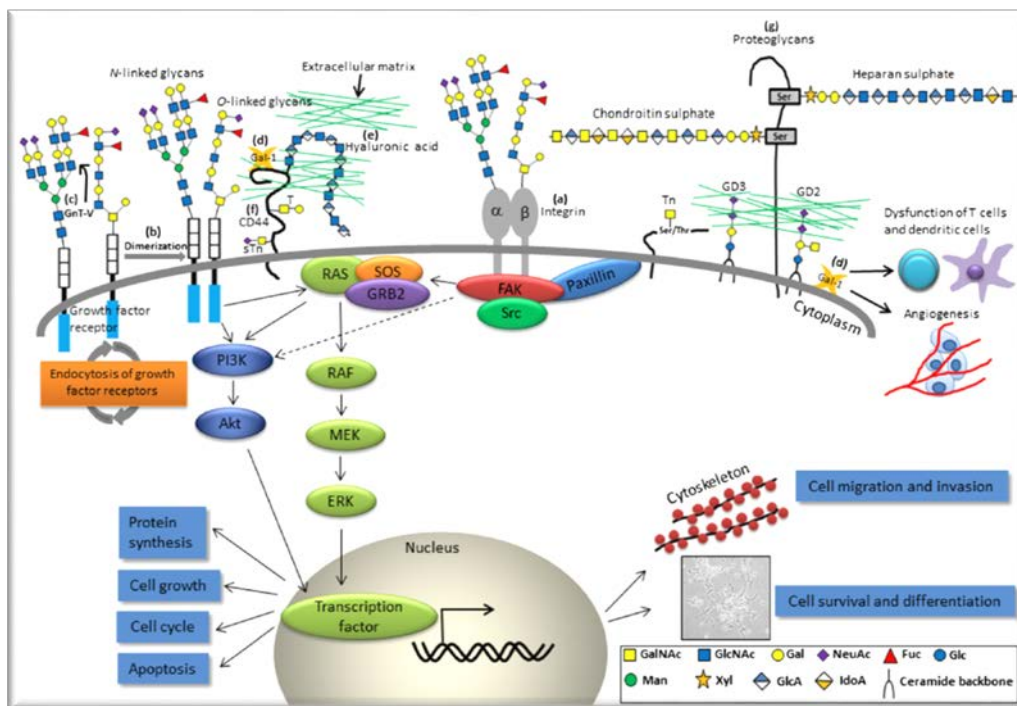
glycosyltransferases and glycosidases, have been amply documented for their respective involvement in NB glycopathobiology and, therefore, share parallel platforms and themes with the gangliosides.<sup>491,492,515,516,527,529,544-547</sup> For the sake of objectivity and some degree of inclusiveness, some of these highlights are encapsulated in Figures 1.17 and 1.18.



Each ganglioside is structurally more complex than its precursor molecule, and the stepwise addition of monosaccharide or sialic acid (N-acetyl-neuraminic acid) residues in the Golgi apparatus is catalyzed by the same specific membrane-bound glycosyltransferases in both pathways.<sup>548</sup> Gangliosides can also be grouped into structurally simple (SG) and complex (CG) molecules. The enzyme GM1a/GD1b synthase (UDP-Gal:betaGlcNAc-beta-1,3-galactosyltransferase) converts its substrates, the simple gangliosides, GM2 and GD2, into the corresponding initial complex ganglioside products, GM1a and GD1b.

**Source:**<sup>527</sup> Berois N, Osinaga E. Glycobiology of neuroblastoma: Impact on tumor behavior, prognosis, and therapeutic strategies. *Frontiers in Oncology* 2014;4:114, with permission from *Frontiers in Oncology*. See Appendix 11 for copyright clearance.

**Figure 1.16:** Schematic representation of the major ganglioside biosynthesis pathways



(a)  $\beta$ 1,4-N-acetylgalactosaminyltransferase 3 (B4GALNT3) and  $\beta$ 1,4-galactosyltransferase 3 (B4GALT3) exhibit differential effects on malignant phenotypes by modification of  $\beta$ 1 integrin in NB cells; (b) N-acetylgalactosaminyltransferase 2 (GALNT2) modifies O-glycans on IGF-1R, thereby suppressing IGF-1-induced IGF-1R dimerization and downstream signaling; (c) N-acetylglucosaminyltransferase V (GnT-V) modulates the sensitivity of NB to apoptosis; (d) Gal-1 promotes attachment of NB cells to the extracellular matrix (ECM) and endothelial cells through binding to CD44. Besides, Gal-1 may dampen the function of T cells and dendritic cells (DC) as well. Glycosaminoglycans present as (e) free polysaccharides (hyaluronic acid), a major counterreceptor for (f) CD44, or (g) as part of proteoglycans (heparan sulfate and chondroitin sulfate). *GalNAc* N-acetylgalactosamine, *GlcNAc* N-acetylglucosamine, *Gal* galactose, *NeuAc*, N-acetylneuraminic acid, *Fuc* fucose, *Glc* glucose, *Man* mannose, *Xyl* xylose, *GlcA* glucuronic acid, *IdoA* iduronic acid.

**Source:**<sup>494</sup> Ho WL, Hsu WM, Huang MC, Kadomatsu K, Nakagawara A. Protein glycosylation in cancers and its potential therapeutic applications in neuroblastoma. *Journal of Hematology & Oncology* 2016;9(1):100.4, with permission from *BioMed Central* in terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

**Figure 1.17:** Altered glycans and related pathophysiological events involved in NB progression

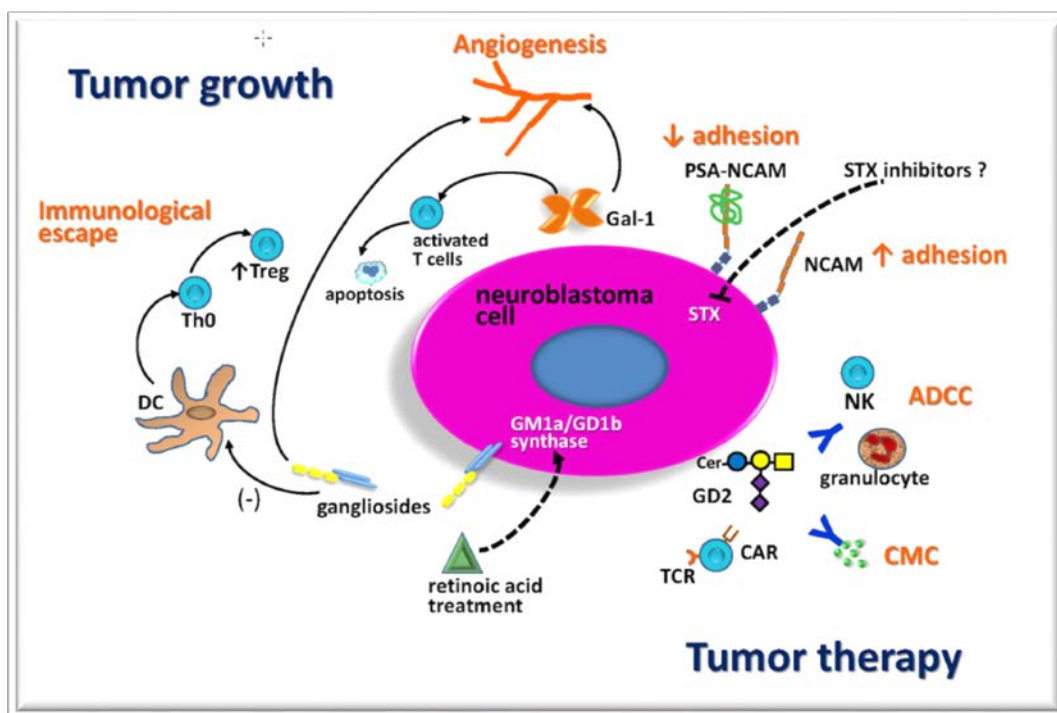
## B2.3 Intercellular Adhesion Molecule-2

Cell adhesion molecules (CAMs, e.g., selectins, integrins, cadherins and immunoglobulin-like CAMs) comprise four ubiquitously occurring families of glycosylated, membrane-bound proteins involved in multiple cellular processes, including cell-cell communication, cell motility, inside-out and outside-in signalling, tumourigenesis, angiogenesis and metastasis.<sup>549-</sup>

<sup>552</sup> In the context of NB, it was found that intercellular adhesion molecule-2 (ICAM-2, a 55-60 kDa transmembrane glycoprotein), also known as CD102 (Cluster of Differentiation 102),



which has six N-linked glycosylation sites at Asn47, Asn82, Asn105, Asn153, Asn178 and Asn87, suppressed tumour cell motility and dissemination, but not tumourigenic potential, *in vivo* in a murine model of metastatic NB.<sup>527,553,554</sup> N-glycosylation of ICAM-2 is critical for these effects,<sup>554</sup> and ICAM-2 confers a non-metastatic phenotype in NB cells by interaction with  $\alpha$ -actinin.<sup>555</sup>



Treatment with retinoic acid markedly enhances the activity of GD1b/GM1a synthase, resulting in increased expression of complex gangliosides, associated with less-aggressive tumours. NB gangliosides promote dendritic cells (DC) to develop with decreased costimulatory signals and IL-12 production. These DC promote differentiation of human T-helper type 0 (Th0) cells toward regulatory T-cells (Treg). Galectin-1 (Gal-1) secreted by NB also contributes to the immunosuppressive tumour microenvironment, limiting T-cell survival and impairing DC function. Both, gangliosides and Gal-1 contribute to tumour angiogenesis. The presence of polysialic acid (PSA) on neural cell-adhesion molecule (NCAM) reduces NCAM-mediated adhesion processes promoting NB cell migration. The fact that sialyltransferase (STX) is the dominant polysialyltransferase for PSA biosynthesis in NB suggests that this enzyme could be a good therapeutic target. Disialoganglioside (GD2) is a relevant antigen for NB immunotherapy. Anti-tumour activity of anti-GD2 antibodies is mediated by antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human natural killer (NK) cells and granulocytes, as well as by complement-mediated cytotoxicity (CMC). Anti-GD2 chimeric antigen receptor T-cells (CAR-T-cells) activity could induce NB tumour regression.

**Source:**<sup>527</sup> Berois N, Osinaga E. Glycobiology of neuroblastoma: Impact on tumor behavior, prognosis, and therapeutic strategies. *Frontiers in Oncology* 2014;4:114, with permission from *Frontiers in Oncology*. See Appendix 11 for copyright clearance.

**Figure 1.18:** Neuroblastoma glyco-biology impact on tumour growth and antitumour therapy



## B2.4 Anaplastic Lymphoma Kinase

The role of ALK as a predisposing gene in NB has been discussed in detail in section A11.4.3. ALK has 16 highly conserved putative sites of N-linked glycosylation in its extracellular domain.<sup>527</sup> Previous studies have observed that perturbation of N-linked glycosylation ablates ALK phosphorylation and blocks downstream pro-survival signalling and cell viability in NB cell lines selected for mutated or amplified ALK,<sup>556</sup> raising hopes that inhibition of this post-translational modification could be applied usefully in NB targeted therapy.

## B2.5 Cell-Surface Mucin-Type O-Glycans

Cell-surface mucins are glycoproteins with large branches of O-linked oligosaccharides. The most abundant mucin-type glycoproteins typically contain an  $\alpha$ -N-acetylgalactosamine residue (GalNAc) covalently linked to the alpha hydroxyl group of Ser/Thr residues.<sup>494,517</sup> Such linkages are catalyzed by UDP-GalNAc:polypeptide-N-acetyl-galactosaminyl-transferases (GalNAc-T). GalNAc-T is a multigene family of 20 or more isoenzymes (<http://www.cazy.org>).<sup>557</sup> Several carcinomas express truncated O-glycosylated tumour-associated glycan antigens (terminal structures arising from sialylation and fucosylation) such as (Tn, sTn, T, and sLe<sup>a/x</sup> and Thomsen–Friedenreich antigen (TF) which correlate with adverse outcome and poor prognosis in cancer patients, thus making them candidate therapeutic targets.<sup>494,514,527,558-560</sup>

In the case of NB, recent evidence suggests that the expression of enzymes encoded by the GALNT [UDP-N-acetyl- $\alpha$ -D-galactosamine:polypeptide N-acetylgalactosaminyltransferase (GALNAC-T)] gene family which catalyze the first step in O-glycosylation, correlates with improved overall survival in low- and high-risk groups and improved clinical outcome (overall and disease-free survival) in low-risk NB patients. Hence, GALNT9 expression may be a valuable prognostic marker for personalized therapy.<sup>561</sup> Likewise, elevated expression of  $\beta$ 1,3-N-acetylglucosaminyltransferase-3 (B3GNT3), the enzyme responsible for adding GlcNAc to core 1 (T antigen), predicted a favourable prognosis in NB patients distinct from other

prognostic markers. B3GNT3 overexpression also interfered with T antigen production and malignant signatures such as migration and invasion of SK-N-SH cells, while B3GNT3 knockdown enhanced these phenotypes of SK-N-SH cells. Additionally, B3GNT3 expression abolished phosphorylation of focal adhesion kinase (FAK), Src, paxillin, Akt and ERK1/2 (Figure 1.17). Thus, B3GNT3 as a modulator of mucin-type O-glycosylation and signalling in NB cells, may be a precise clinical predictor of NB behaviour and therapeutic outcome.<sup>562</sup>

## B2.6 Polysialic Acid

Polysialic acid (PSA) exemplifies a distinctive post-translational modification of the neural cell adhesion molecule (NCAM).<sup>527,563</sup> PSA assembly involves extended linear homopolymerization<sup>564</sup> of 150-200  $\alpha$ 2,8-linked sialic acids on N-glycans of the fifth immunoglobulin-like domain of NCAM. During normal development, PSA mediates cell migration and axonal growth, but in undifferentiated NB, it promotes NB cell proliferation and metastatic potential.<sup>565</sup> PSA expression is upregulated in high-risk NB. In the Golgi apparatus, two homologous polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST), catalyze the synthesis of variable amounts of PSA in tumours.<sup>566</sup>

The ST8SiaII gene is expressed predominantly during embryonic development and thought to be silent in normal tissue, but is highly expressed in metastatic NB.<sup>567,568</sup> ST8SiaIV is the major polysialyltransferase in the adult brain.<sup>527</sup> STX has attracted considerable interest as a molecular marker and therapeutic target for metastatic NB<sup>569</sup> as borne out by recent efforts aimed at reducing STX-mediated polysialylation of NCAM using cytidine monophosphate (CMP)<sup>570</sup> and inhibiting migration of IMR-32 NB cells with the sialic acid precursor, ManNProp.<sup>568</sup>

## B2.7 Lectins (Glycan-Binding Proteins)

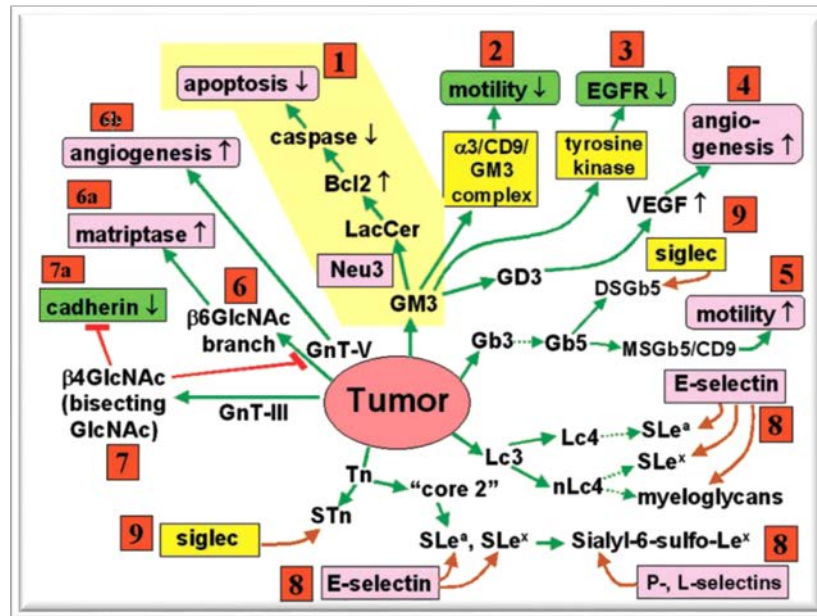
Three main categories of lectins, namely, siglecs (sialic acid binding Ig-like lectins), galectins and selectins are GBPs that have a high specificity for sugar moieties. Endogenous lectins are

involved in processes such as cell-cell recognition, cell adhesion and motility, pathogen-host recognition, and tumour progression and metastasis. Many lectins are expressed on the surface of immune and endothelial cells or exist as ECM components and cytoplasmic adhesion molecules.<sup>571</sup> Normal glycans of colonic epithelial cells, for example, suppress cyclooxygenase-2 expression by resident macrophages, thus maintaining immunological homeostasis in mucosal membranes, whereas loss of immunosuppressive glycans by impaired glycosylation during colonic carcinogenesis triggers inflammatory destruction of colonic mucosa.

Siglec-7 and -9, expressed on resident macrophages in the colonic lamina propriae bind to ligands di-sLe<sup>a</sup> and 6-sulfo sLe<sup>x</sup>, but loss of this function occurs during malignant transformation coupled with a gain of expression of sLe<sup>a</sup> and sLe<sup>x</sup> which have no siglec ligand activity.<sup>572</sup> Siglec-7 is expressed mainly on natural killer (NK) cells and suppresses NK cell-mediated cytotoxicity towards target cells overexpressing  $\alpha$ 2,8-disialic acid-bearing ganglioside, GD3 (see Figure 1.16).<sup>573,574</sup> Malignant melanoma and NB overexpress GD3, a cancer signature that may confer on these tumours the ability to evade immunosurveillance and elimination by NK cells. This concept may indeed be exploited in the targeted inhibition of siglec-7 and NB metastasis.<sup>574</sup>

In recent years, galectin-1 (Gal-1) has gained prominence as a burgeoning target in NB translational therapeutics.<sup>494,527</sup> Gal-1 is an adaptable regulator of multiplex signalling pathways such as tumour–host interaction,<sup>575</sup> angiogenesis,<sup>576</sup> promotion of immunosuppression by T cell apoptosis and impairment of dendritic cell (DC) function in numerous cancers, including NB.<sup>577-579</sup> Aggressive NB tumours express high levels of neurotrophin receptor TrkB (section A13.2.4) and Gal-1 which are not only coupled with invasive behaviour and high metastatic potential, but also associated with therapy resistance and thus poor prognosis<sup>580</sup>. These Gal-1 phenotypic features of NB are meticulously being probed for their glycan-based therapeutic potential.<sup>494,527,547,579,581</sup>

Figure 1.19 summarizes how glycosylation/glycans, in particular, the selectins cast the hallmarks of the cancer phenotype.



Tumour cell malignancy is defined by several key phenotypes: apoptosis (route 1), motility (routes 2 and 5), EGF receptor tyrosine kinase (route 3), angiogenesis (routes 4 and 6b), matrilysin (matrix-destroying enzyme) activity (route 6a), self-adhesion (through cadherin) (route 7a), adhesion to ECM (through integrin), adhesion to ECs and platelets (through E- or P-selectin) (route 8), adhesion to blood cells and other parenchymatous cells (through siglecs) (route 9). Each phenotype is up- or down-regulated (1, 2) by different status of glycosylation. Phenotypes with 1 or 2 and green color inhibit tumour invasiveness. Those with 1 or 2 and pink color promote invasiveness. Glycosyl epitopes capable of binding to specific ligands (pink color without arrow) promote invasiveness. Ligands with yellow color have variable or unclear effect on invasiveness. Note that a given phenotype is produced by different glycosylations, and a given glycosylation produces different phenotypes. Phenotypic changes have cooperative effects on malignancy. For example, GM3 inhibits motility through  $\alpha 3/CD9$  complex and also inhibits EGF receptor tyrosine kinase (routes 2 and 3). Reduction of GM3 inhibits apoptosis (route 1), but promotes motility and proliferation (negative route 2 and 3 effect). Essentially all glycosylation pathways catalyzed by multiple glycosyltransferases and their genes are well established (for review see 2). However, the mechanism by which each type of glycosylation affects the various phenotypes remains to be studied. Structures of GSLs are abbreviated according to International Union of Pure and Applied Chemistry–International Union of Biochemistry nomenclature recommendations. S, sialyl; MS, monosialyl; DS, disialyl.

**Source:**<sup>582</sup> Hakomori S. Glycosylation defining cancer malignancy: New wine in an old bottle. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99(16):10231-10233, with permission from PNAS, <http://www.pnas.org/site/aboutpnas/rightperm.xhtml>, accessed 4 December 2016.

**Figure 1.19:** Glycosylation defining malignancy—invasive and metastatic phenotype of tumours

## B2.8 Glycosyltransferases

Tumour cells exhibit striking changes in cell-surface glycosylation as a consequence of dysregulated glycosyltransferases and glycosidases.<sup>583</sup> Aberrant glycosylation is a cancer hallmark which correlates with differential expression of cell-surface and cytosolic glycans

and tumour-associated antigens.<sup>527,561</sup> Glycosyltransferases have clinical relevance as cancer biomarkers for different tumours, markers for minimal residual disease (MRD) detection, risk group assignment and as prognostic predictors (<https://pob.abcc.ncifcrf.gov/cgi-bin/JK>).<sup>494,527,567,584</sup>

Elevated  $\beta$ 1,6-N-acetylglucosaminyltransferase V (GnT-V) expression, for example, predicts a favourable prognosis and treatment outcome in NB.<sup>585</sup> The expression, clinical relevance and functional significance of several glycosyltransferases, including  $\beta$ 1,4-N-acetylgalactosaminyltransferase (GD2synthase), sialyltransferase (STX or ST8SiaII),  $\beta$ 1,3-N-acetylglucosaminyltransferase 3 (B3GNT3), UDP-polypeptide GalNAc-transferase 13 (GalNAc-T13, GALNT13),  $\beta$ 1,4-galactosyl-transferase 3 (B4GALT3), and N-acetylgalactosaminyltransferase 2 (GALNT2), in NB and other cancers, have been widely documented.<sup>494,527,562,586-588</sup>

## B2.9 ATP-Binding Cassette Multidrug Transporters

Direct and coordinate transcriptional targets of MYCN (section A11.4.5) include several of the ATP-binding cassette (ABC) transporters—ABCB1 (P-glycoprotein/P-gp/MDR1), ABCG2 (breast cancer resistance protein/BCRP) and ABCC1 (multidrug resistance protein 1/MRP1), ABCC3 (MRP3) and ABCC4/MRP4.<sup>311,328,589</sup> The expression of these multidrug resistance (MDR) transporters are strongly prognostic of NB outcome (section A13.1.7) since they extrude a wide array of structurally- and functionally-related or -unrelated chemotherapeutic drugs.<sup>311,329,330</sup> Moreover, endogenous substrates of MDR transporters such as bioactive lipid mediators (e.g., prostaglandins and leukotrienes) may modify normal neural development by switching on processes (angiogenesis, cell signalling, inflammation, proliferation, and migration and invasion) that promote NB initiation and progression. The ABC transporters are thus promising candidates for therapeutic suppression in HR-NB, the rationale behind increasing drug bioavailability (therapeutic efficacy) in refractory tumours which overexpress these glycans.<sup>326</sup> The glycosylation of ABCB1 (P-glycoprotein/P-gp) has been studied widely



and will thus be considered as the representative drug transporter. Previous studies have shown that the most strongly upregulated genes associated with acquired drug resistance and an important cause of NB treatment failure was *GALNT13*, followed by *ABCB1 (MDR1)*.<sup>527,590,591</sup> *GALNT13* encodes the UDP-GalNAc:polypeptide GalNAc-transferase-13 (GalNAc-T13), constitutively expressed in neural tissue.<sup>584,592</sup> It has been demonstrated unequivocally that inhibition of protein glycosylation reverses the MDR phenotype of cancer cell lines.<sup>593</sup> Likewise, inhibition of N-linked glycosylation impairs ALK phosphorylation and perturbs pro-survival signalling in NB cell lines.<sup>556</sup> Glycosylation of P-gp corresponds to the en bloc transfer of the oligosaccharide portion of a lipid-linked oligosaccharide onto the acceptor asparagine of nascent proteins, typical for all N-glycans.<sup>513</sup>

P-gp is synthesized as a 140–150 kDa precursor protein which is escorted by chaperones (calnexin and Hsp70) in the ER lumen to the Golgi. P-gp is modified post-translationally by N-glycosylation encompassing various sugar moieties—a process essential for its destination docking (dynamic integration into the membrane) and, ultimately, mature functioning (drug efflux pump activity), as inferred from experiments with cDNA encoding N-glycosylation-deficient P-gp showing that the immature or non-glycosylated protein is trapped in subcellular compartments.<sup>594,595</sup>

Tunicamycin (one of the prototype inhibitors of glycosylation) suppresses P-gp activity thereby triggering the accumulation of cytostatic drugs in the cells, and thus providing evidence that inhibitors of glycosylation ablate the P-gp-mediated MDR phenotype.<sup>596,597</sup> By contrast, some researchers assert that while N-glycosylation may stabilize correct folding of P-gp, guiding its proper subcellular localization and protecting it from luminal protease degradation, its precise role in P-gp function remains open-ended as tunicamycin treatment either altered P-gp cellular localization to the plasma membrane nor the P-gp drug efflux activity.<sup>598-600</sup>



## B2.10 Inhibitors of N-Linked Glycosylation

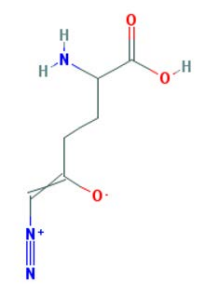
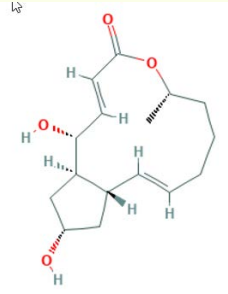
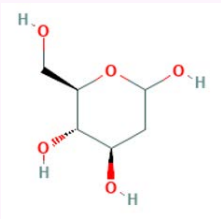
The myriad types of N-linked oligosaccharides are formed by two sequential reactions: 1) the formation of the lipid-linked saccharide precursor, Glc<sub>3</sub>Man<sub>9</sub>(GlcNAc)-2-pyrophosphoryl-dolichol, by the stepwise addition of GlcNAc, mannose and glucose to dolichyl-P, and 2) the removal of glucose and mannose by membrane-bound glycosidases and the addition of GlcNAc, galactose, sialic acid, and fucose by Golgi-localized glycosyltransferases to generate diverse complex oligosaccharide structures. Many glycoproteins contain more than one N-linked oligosaccharide structure—one oligosaccharide may be of the high-mannose type whereas another may be a complex chain.

Various methodologies are used to establish the role of specific structures in glycoprotein function, including inhibitors that hinder the different modification steps, resulting in the production of aberrant glycoproteins with altered carbohydrate structures. Several alkaloid-mimetic/specific inhibitors of the glucosidases and mannosidases involved in glycoprotein processing have been characterized. These inhibitors trigger the assembly of glycoproteins with glucose-containing high mannose structures, or various high-mannose or hybrid chains, depending on the site of inhibition. These inhibitors have also been useful for studying the glycan processing pathways and for comparing processing enzymes from different organisms.<sup>545,594,601-604</sup> N-linked glycosylation inhibitors are categorized according to their mechanism of action and target.<sup>594,605-607</sup> They may prevent N-glycosylation through:

1. Interference with the turnover of the process precursors;
2. Inhibition of glycosyltransferases and glycosylases;
3. Inhibition of transport of modified proteins between cellular compartments engaged in N-glycosylation, endoplasmic reticulum, Golgi apparatus; and
4. Functioning as substrate analogues.

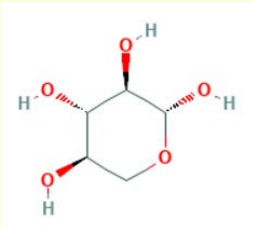

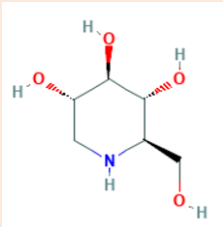
Table 1.13 summarizes some of the known inhibitor classes of N-glycosylation.

**Table 1.13:** Specific classes and examples of N-glycosylation inhibitors

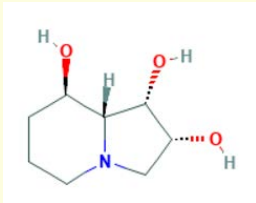
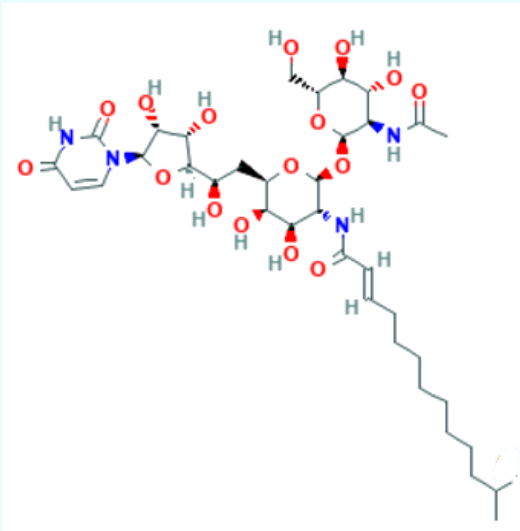
Inhibitor Class/Example(s)	Structure	Mode of Action	References
<p><i>Metabolic Inhibitors</i></p> <p><b>6-Diazo-5-Oxo-L-Norleucine (DON)</b></p>		<p>Affects turnover of glycosylation precursors, primarily at the stage of their formation. DON blocks glutamine:fructose-6-phosphate aminotransferase which catalyzes the synthesis of glucosamine from glutamine and fructose. Effects include disruption of mitochondrial internal membrane, permeabilization and dilation of the endoplasmic reticulum and induction of apoptosis. DON exhibits antitumour effects.</p>	594,607-609
<p><b>Brefeldin A (BFA)</b></p>		<p>A macrocyclic lactone synthesized from palmitate by various fungi. Inhibits the early transport of proteins from the endoplasmic reticulum to the Golgi apparatus. BFA also disrupts organization of the microtubule and actin cytoskeletons.</p>	610,611
<p><i>Sugar Analogues</i></p> <p><b>2-Deoxyglucose (2dGlc/2DG)</b></p>		<p>Inhibits glycosyltransferases, impacts signalling pathways and it blocks the cell cycle progression, DNA repair which culminates in apoptosis.</p>	594,612-614

*Continued/...*

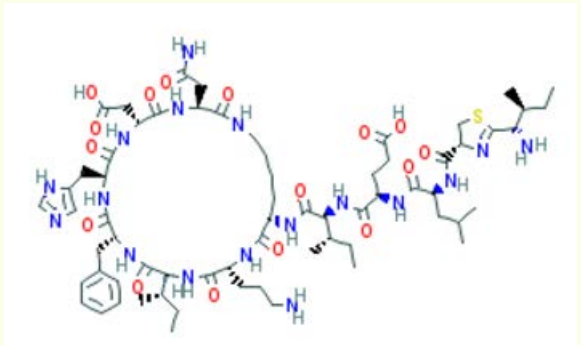
**Table 1.13:** Specific classes and examples of N-glycosylation inhibitors (continued)

Inhibitor Class/Example(s)	Structure	Mode of Action	References
<b>Glycoside Primers</b> <b><math>\beta</math>-D-Xyloside (<math>\beta</math>-D-Xyl)</b>		$\beta$ -D-Xyl blocks synthesis of glycosaminoglycans on growing proteoglycans. Disrupts glycoprotein assembly by elongating the oligosaccharide chains with exogenous primers instead of the endogenous glycoprotein core with specific sugar moieties which causes premature inhibition of glycan synthesis. $\beta$ -D-Xyl also reversibly inhibits cell proliferation by arresting cells in the G <sub>1</sub> phase of the cell cycle.	607,615-617
<b>Plant Alkaloids</b> <b>Castanospermine (CST)</b>		CST specifically inhibits $\alpha$ -glycosidase I and II, thus impeding elongation of saccharide chains. Inhibition occurs particularly at the stage of glycosylation, after formation of a 14 monomer-long chain (Glc <sub>3</sub> Man- <sub>9</sub> GlcNAc <sub>2</sub> ). Hence, plant alkaloids block formation of mature glycoproteins.	594,602,604,607,618-620
<b>Deoxynojirimycin (DNJ)/ Deoxymannojirimycin (DMJ)</b>		Castanospermine and deoxynojirimycin both obstruct angiogenesis <i>in vitro</i> . 1-Deoxymannojirimycin predominantly inhibits Golgi mannosidase I. 1-Deoxynojirimycin (1-DNJ) has been shown to possess antimetastatic potential.	602,607,621-623

**Table 1.13:** Specific classes and examples of N-glycosylation inhibitors (continued)

Inhibitor Class/Example(s)	Structure	Mechanism of Action	References
<p><i>Plant Alkaloids</i></p> <p><b>Swainsonine (SWSN)</b></p>		<p>Blocks Golgi <math>\alpha</math>-mannosidase II and lysosomal <math>\alpha</math>-mannosidase.</p> <p>It has a neurotoxic effect (“syndrome loco”) and intoxication with it occasions in accumulation of glycoproteins in lymph nodes. It also abrogates metastasis of melanoma cells by triggering natural killer cell proliferation or their anti-tumour activity. The drug is the first glycosidase inhibitor to have undergone anticancer clinical testing.</p>	602,604,607,618,624
<p><i>Antibiotics</i></p> <p><b>Tunicamycin (TM)</b></p>		<p>It is a nucleoside antibiotic which blocks the enzyme responsible for the transfer of 1-phospho-N-acetylglucosamine from UDP-N-acetylglucosamine to dolichol phosphate, i.e., the first glycosylation step. Tunicamycin induces E-cadherin-mediated cell–cell interactions and apoptosis in neoplastic cells. The antibiotics also exerts synergistic effects in combination with doxorubicin, cisplatin and vincristine.</p>	594,597,615,621,625,626

**Table 1.13:** Specific classes and examples of N-glycosylation inhibitors (continued)

Inhibitor Class/Example(s)	Structure	Mechanism of Action	References
<p><i>Antibiotics</i></p> <p><b>Bacitracin (BAC)</b></p>		<p>Prevents dolichol pyrophosphate (Dol-PP) hydrolysis to dolichol phosphate which, the after-effect being ablation of glycoprotein synthesis at its first stage. Bacitracin interferes with P-gp expression and localization. Bacitracin is a competitive inhibitor of protein disulfide isomerase and reduces phosphorylated focal adhesion kinase (p-FAK) and secreted matrix metalloproteinase-2 (MMP-2), which are the downstream of integrin and play a major role in cell migration and invasion, considered a therapeutic target for glioblastoma. Bacitracin has significant effects on both non-catalyzed protein folding and on other molecular chaperones. Bacitracin further disrupts ER function and causes higher ERS-mediated apoptosis in melanoma cells as demonstrated by the upregulation of ER chaperones.</p>	<p>627-631</p>
<p>2-D structures of the N-glycosylation inhibitors were downloaded from <a href="https://pubchem.ncbi.nlm.nih.gov/">https://pubchem.ncbi.nlm.nih.gov/</a>.</p>			

## SECTION C: PROTEIN GLYCOSYLATION, ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE

### C1. Introduction

In eukaryotic cells, many proteins are covalently modified during or immediately after translation. These modifications (e.g., phosphorylation, acetylation, glycosylation, methylation, sumoylation, sulfation, nitrosylation and ubiquitylation) collectively referred to as post-translational modifications (PTMs), regulate protein maturation, stability, dynamics, assembly, translocation, molecular interactions and cellular functions.<sup>632-636</sup> Modifications of a protein at asparagine Asn (N-glycosylation) or at serine/threonine residues (O-glycosylation) (Figure 1.14) are arguably the most prevalent PTMs which impact protein folding, maturation and activity.<sup>491,637</sup> Protein glycosylation occurs in the eukaryotic secretory pathway and encompasses discrete biosynthetic transitions between the endoplasmic reticulum (ER) and Golgi apparatus.<sup>491,606</sup> Unlike nucleic acids and proteins, glycan structures are not directly determined by genes or synthesized from a template, and may be linear or branched, and even undergo additional modification by acetylation, sulfation or phosphorylation.<sup>632</sup>

Glycosylation is an important component of the ER protein quality control system (ERQC) which precisely sorts and corrects misfolded proteins for reprocessing.<sup>638</sup> The ERQC, starting in the ER and ending at the trans-Golgi, directs the dispatch, transit, secretion and fate (final localization) of properly folded and glycosylated proteins to the cell surface or external environment (terminal compartments). This progression is imperative in the development and homeostasis, as well as cell-to-cell communication in complex multicellular organisms.<sup>632,637,639,640</sup> The glycan moieties of glycoproteins play crucial roles in intricate processes ranging from protein solubility, stability, conformation and function—and thus affect their half-life in the blood (circulation)—to their altered expression in most chronic or acquired infectious diseases, ERS and cancer.<sup>491,497,606,641-646</sup>

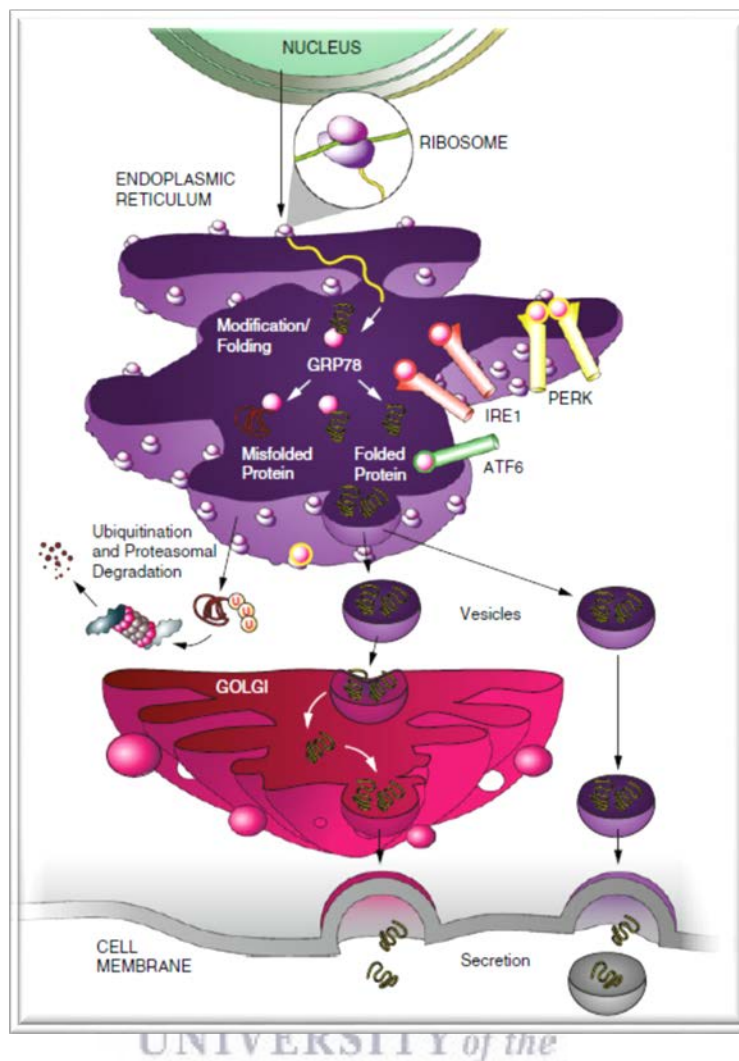


## C2. Endoplasmic Reticulum Stress and the Unfolded Protein Response

The ER is a major cellular compartment for protein synthesis, assembly and trafficking. Within the lumen and membranous network of the ER, a robust protein quality control system (ERQC) verifies whether secretory and membrane proteins are properly folded and modified before they are dispatched to their final destinations (cytosol, membrane and extracellular milieu).<sup>640,647-649</sup> Incorrectly or misfolded proteins cannot assume their final conformation and functionally active structures, and are, therefore, retained in the lumen of ER until they are reconfigured into their proper conformations.<sup>650-652</sup>

If the mature tertiary structure cannot be synthesized, misfolded proteins are then redirected to the cytoplasm to undergo ubiquitination and proteasome-mediated degradation (Figure 1.20), a process referred to as ER-associated degradation (ERAD).<sup>651-654</sup> ERAD is indispensable in cells that cannot constitutively induce the unfolded protein response (UPR). Equally, loss of ERAD function leads to constitutive UPR induction. Ultimately, concurrent loss of ERAD and the UPR significantly decreases cell viability, suggesting that the UPR and ERAD are dynamic responses vital for the synchronized clearance of misfolded proteins, even in the absence of acute stress.<sup>655,656</sup>

Under normal physiological conditions, the ERQC can cope with cellular demands, but extreme conditions of intracellular and extracellular stress (e.g., increased protein synthesis, genetic mutations that cause defects in folding, alteration in calcium homeostasis, and nutrient starvation such as glucose deprivation),<sup>650,651</sup> neurodegenerative disorders, heart disease, smoking, diabetes and malignancy may overwhelm the ERQC capacity, leading to ERS.<sup>638,657-660</sup> Thus, perturbations of ER homeostasis, in particular, protein homeostasis (proteostasis), results in the accumulation of unfolded proteins which then activates the ERAD and the UPR—integrated transcriptional and translational systems for transmitting information about the status of protein folding to the cytosol and nucleus (Figure 1.21).<sup>661</sup>



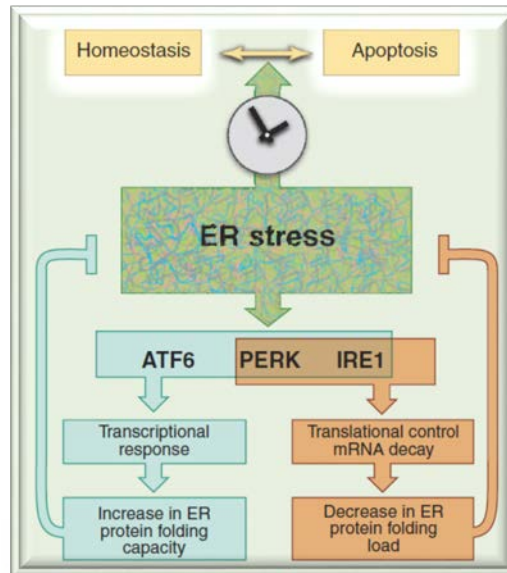
**Normal state.** Proteins that enter the ER are folded and transported to the Golgi apparatus or other destinations. GRP78 is bound to the luminal domains of PERK, IRE1, and domains of PERK, IRE1 and ATF6.

**Source:**<sup>662</sup> Park SW, Ozcan U. Potential for therapeutic manipulation of the UPR in disease. *Seminars in Immunopathology* 2013;35(3):351-373, permission granted by *Springer*, under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited; <http://link.springer.com/article/10.1007%2Fs00281-013-0370-z#copyrightInformation>; accessed 8 January 2017.

**Figure 1.20:** Endoplasmic reticulum protein folding function under normal physiological conditions

In eukaryotic cells, three ER transmembrane components (Figure 1.22)—inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6), initiate distinct UPR signalling arms.<sup>663-665</sup> The UPR triggers upregulation of genes encoding ER chaperones [e.g., heat shock protein 90 (HSP90), HSP70, CAAT/enhancer binding protein- $\alpha$  homologous protein (CHOP)/ growth arrest and DNA damage-inducible protein (GADD153), XBP1 (X-box binding protein 1),<sup>666</sup> calreticulin (CRT),<sup>667</sup> ER HSP40

ERdj3/DNAJB11,<sup>668,669</sup> and ERdj5,<sup>670</sup> and calnexin],<sup>671</sup> attenuation of translation, and initiation of the ERQC to reinstate ER homeostasis.<sup>672,673</sup>

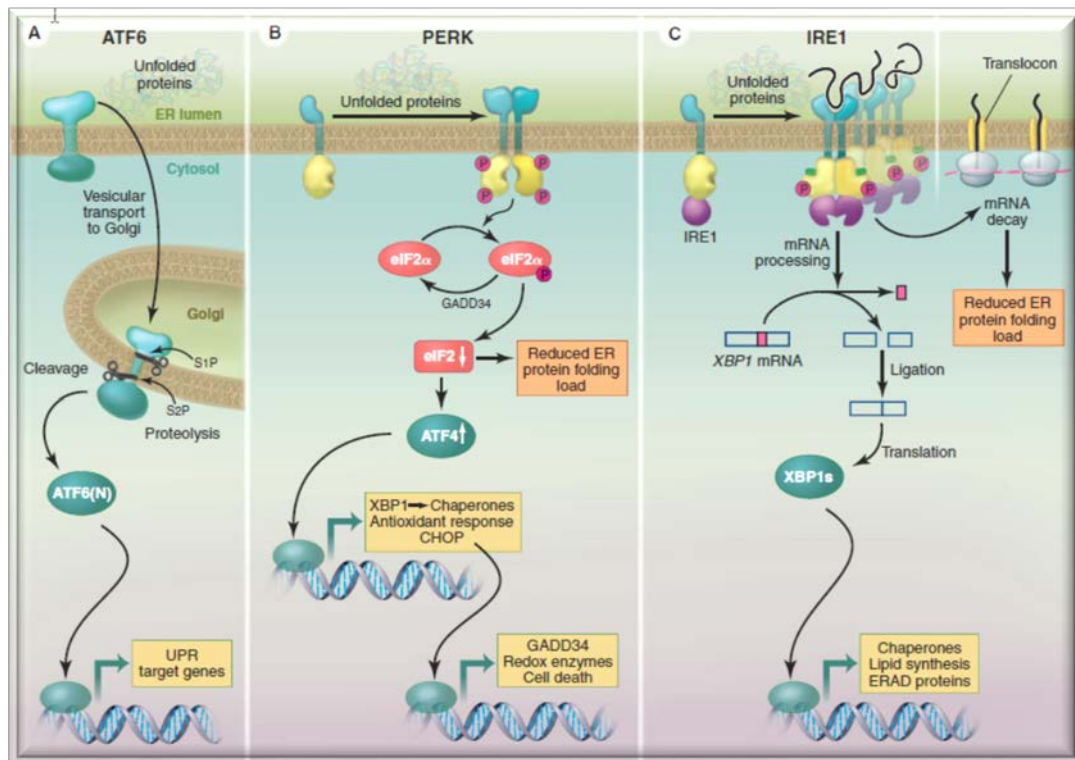


ERS activates the stress sensors ATF6, IRE1, and PERK, representing the three branches of the UPR. Activation of each sensor produces a transcription factor [ATF6(N), XBP1, and ATF4, respectively] that activates genes to increase the protein-folding capacity in the ER. IRE1 (via RIDD) and PERK (via eIF2a phosphorylation) also decrease the load of proteins entering the ER. Both outcomes work as feedback loops that mitigate ERS. If cells cannot reestablish homeostasis, but continue to experience prolonged and unmitigated ERS (depicted by the timer), they apoptose.

**Source:**<sup>651</sup> Walter P, Ron D. The unfolded protein response: From stress pathway to homeostatic regulation. *Science* 2011;334(6059):1081-1086. Permission granted by *Science* (American Association for the Advancement of Science), <http://www.sciencemag.org/help/reprints-and-permissions>; accessed 8 January 2017.

**Figure 1.21:** Core elements of the UPR signalling network

The ER luminal binding protein—immunoglobulin heavy chain-binding protein (BiP)—also called glucose-regulated protein, 78kDa (GRP78), a member of the heat shock protein 70 (HSP70) family, is the most abundant ER-chaperone, and a central regulator of the ERQC machinery. BiP binds to and suppresses the activity of the mammalian ERS sensors, PERK, IRE1, and ATF6.<sup>674,675</sup> Pro-survival (yang) GRP78 and pro-apoptotic (yin) CHOP are quintessential antagonistic mediators of the ERS response.<sup>676</sup> When the UPR is insufficient to restore the steady state in the ER, programmed cell death (PCD) or apoptosis ensues, but chronic ERS can lead to pathological states.<sup>521,647,677-680</sup> ERS is also a strong inducer of autophagy, a self-degradative process that has an adaptive function.<sup>681-683</sup>



Three families of signal transducers (ATF6, PERK, and IRE1) sense the protein-folding conditions in the ER lumen and transmit that information, resulting in production of bZIP transcription regulators that enter the nucleus to drive transcription of UPR target genes. Each pathway uses a different mechanism of signal transduction: ATF6 by regulated proteolysis, PERK by translational control, and IRE1 by non-conventional mRNA splicing. In addition to the transcriptional responses that largely serve to increase the protein-folding capacity in the ER, both PERK and IRE1 reduce the ER folding load by down-tuning translation and degrading ER bound mRNAs, respectively.

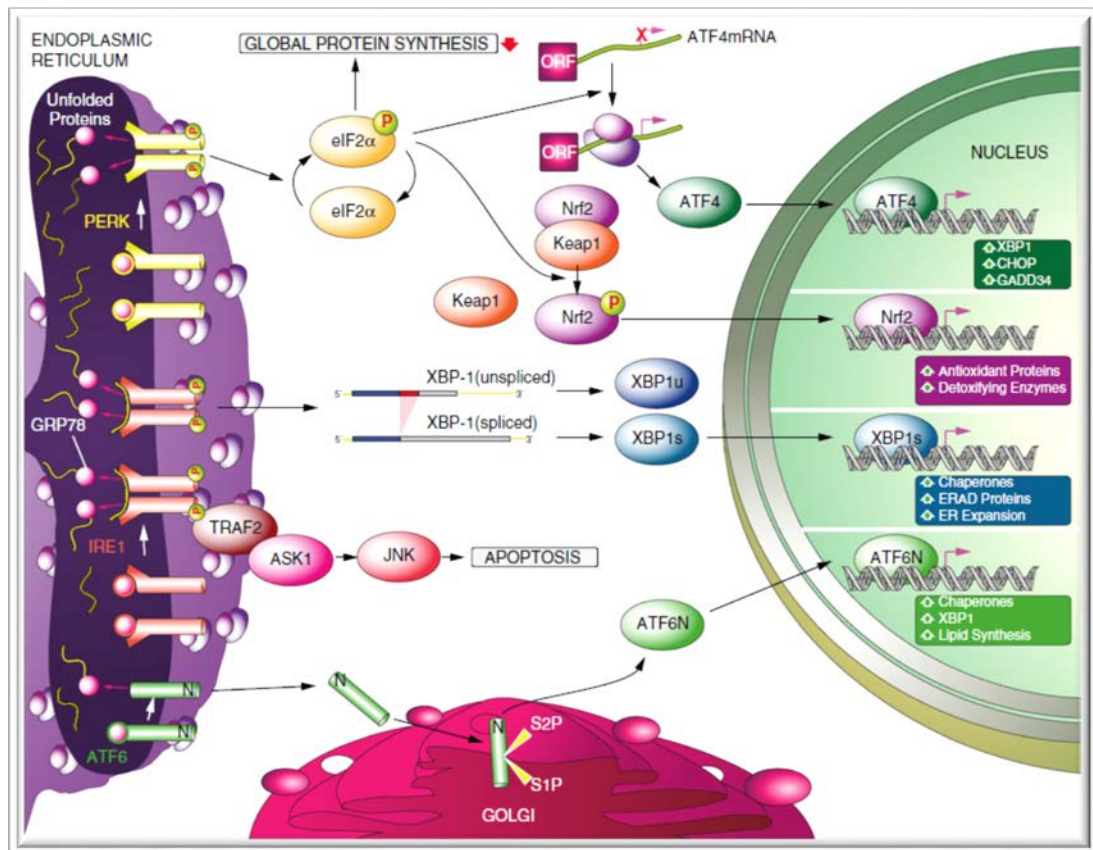
**Source:**<sup>651</sup> Walter P, Ron D. The unfolded protein response: From stress pathway to homeostatic regulation. *Science* 2011;334(6059):1081-1086. Permission granted by *Science (American Association for the Advancement of Science)*, <http://www.sciencemag.org/help/reprints-and-permissions>; accessed 8 January 2017.

**Figure 1.22:** The three branches of the UPR

### C3. ER Stress and the UPR in Cancer

Most of the functions mediated by the UPR in cellular homeostasis are also displayed in the role of ERS response in diseases that include cancer,<sup>684</sup> diabetes, and metabolic, genetic, inflammatory, and neurodegenerative disorders.<sup>672,683,685</sup> Several lines of evidence suggest that all branches of the UPR either promote or impede cancer initiation and progression, implicating various hallmarks of cancer,<sup>145,146,686,687</sup> including sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Figure 1.24).



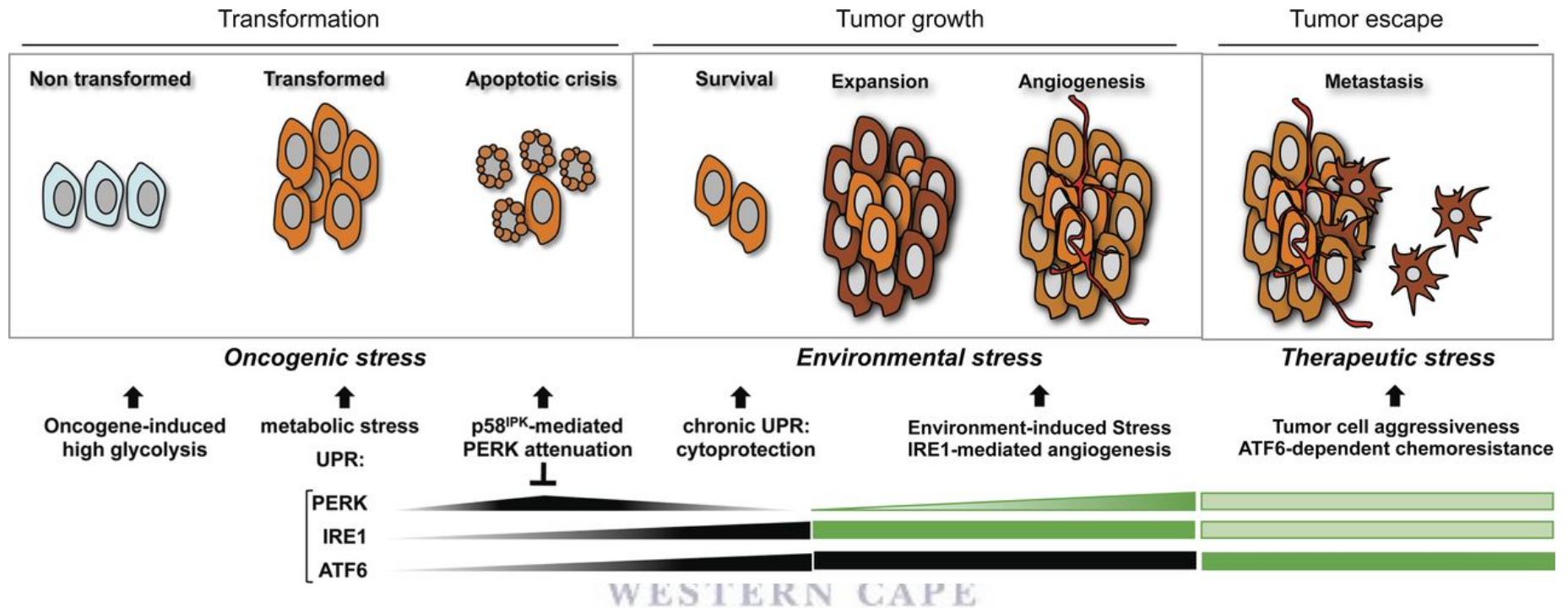


**ERS state.** GRP78 dissociates from PERK, IRE1, and ATF6. PERK and IRE1 oligomerize, forming a dimeric structure with a deep groove where peptide can bind. Upon oligomerization, PERK and IRE1 are auto-phosphorylated. PERK phosphorylates eIF2 $\alpha$ , leading to attenuation in global protein synthesis. Phosphorylated eIF2 $\alpha$  leads to translation and nuclear translocation of ATF4 and Nrf2. Activated IRE1 mediates unconventional mRNA splicing of XBP1 to generate XBP1s. IRE1 also recruits TRAF2 and ASK1 and leads to activation of JNK. ATF6 translocates to the Golgi apparatus and the cytoplasmic tail of ATF6 acts as a transcription factor to regulate UPR target genes.

**Source:**<sup>662</sup> Park SW, Ozcan U. Potential for therapeutic manipulation of the UPR in disease. *Seminars in Immunopathology* 2013;35(3):351-373, permission granted by Springer, under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited; <http://link.springer.com/article/10.1007%2Fs00281-013-0370-z#copyrightInformation>; accessed 8 January 2017.

**Figure 1.23:** Endoplasmic reticulum protein folding function under ERS conditions

Both intrinsic and extrinsic factors can activate the UPR in cancer cells, including hyperactivation of oncogenes and loss-of-function mutations in tumour suppressor genes, which may inappropriately amplify protein synthesis and translocation into the ER in response to excessive metabolic demands. Furthermore, mutations in oncogenes and tumour suppressor genes are known to inhibit ERS-induced apoptosis.<sup>676,684,688</sup> Cancer cells exploit the ERS responses to promote survival and growth.



The activation status of the three UPR arms is shown at the bottom of the scheme. Green indicates a predominant role of the arm concerned in the tumorigenic process indicated at the top of the scheme (the gradient in green indicates the relative contribution of each arm).

**Source:**<sup>685</sup> Manie SN, Lebeau J, Chevet E. Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 3. Orchestrating the unfolded protein response in oncogenesis: An update. *American Journal of Physiology Cell Physiology* 2014;307(10):C901-907, permission granted by *American Journal of Physiology Cell Physiology, The American Physiological Society*, <https://s100.copyright.com/AppDispatchServlet#formTop>; accessed 8 January 2017.

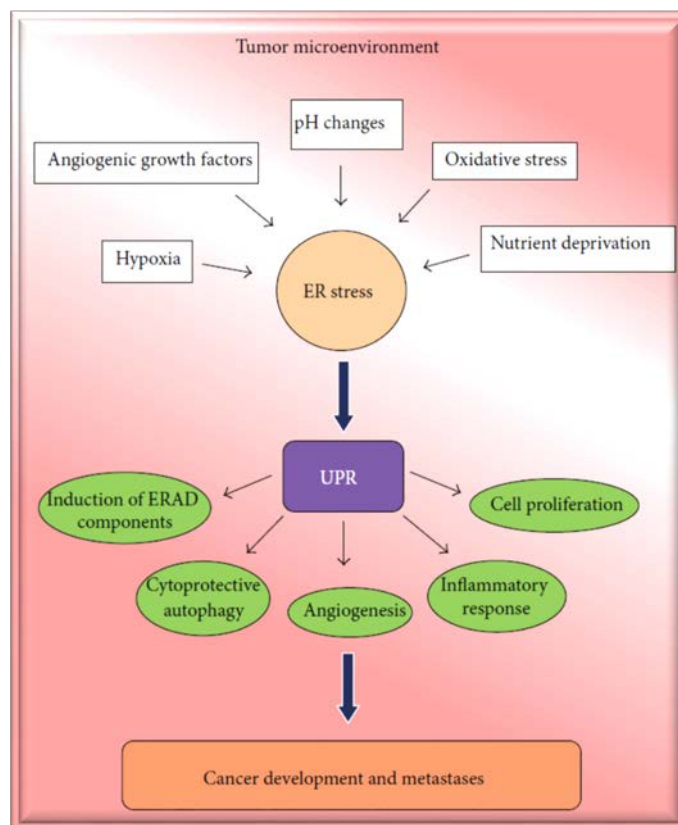
**Figure 1.24:** Involvement of UPR signaling during cell transformation and tumour growth



For example, the ER protein chaperone BiP is commonly overexpressed in breast cancer, lung cancer, prostate cancer, melanoma, and other malignancies to mediate the prosurvival response of cancer cells to major environmental stress.<sup>666,689,690</sup> Surprisingly, unremitting ERS and UPR activation may interchange the cytoprotective functions of UPR into cell death programmes, a principle that can be exploited as a line of attack against cancer cells.<sup>662,676,691,692</sup>

#### C4. Targeting ER Stress and the UPR

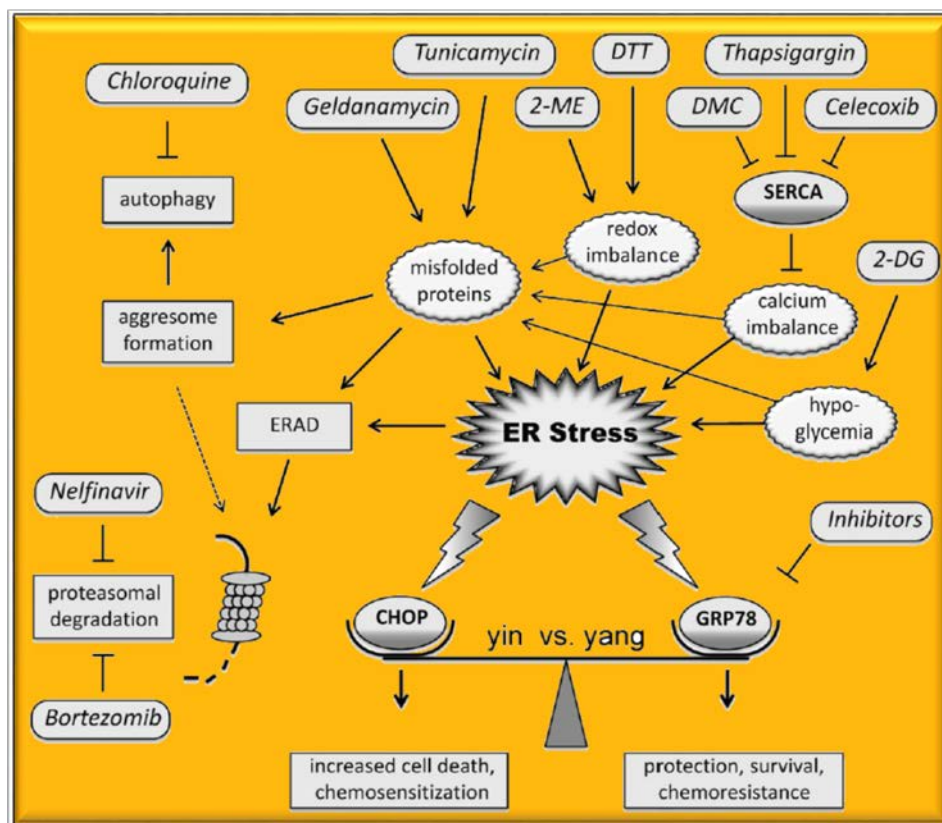
Cancer cells are resistant to extreme environmental stress conditions that induce ERS and UPR responses leading to cancer initiation and metastases (Figure 1.25).<sup>684</sup>



**Source:**<sup>684</sup> Giampietri C, Petrunaro S, Conti S, Facchiano A, Filippini A, Ziparo E. Cancer microenvironment and endoplasmic reticulum stress response. *Mediators of Inflammation* 2015;2015:417281, permission granted (Open Access) by *Hindawi Publishing Corporation*; <https://www.hindawi.com/oa/>; accessed 9 January 2017.

**Figure 1.25:** Tumour microenvironment and activation of ERS and UPR responses in cancer

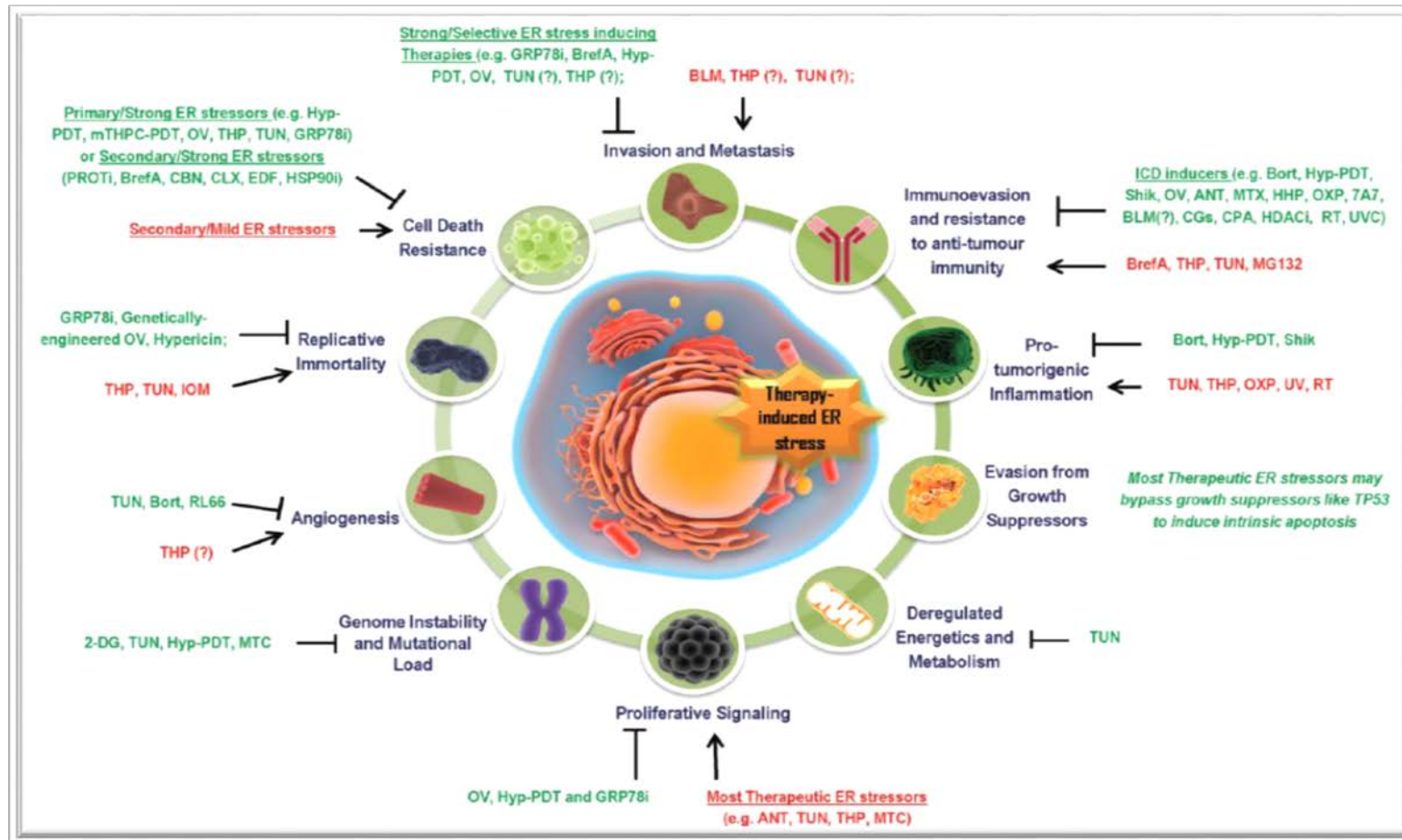
Therefore, the various molecular interconnections defining ERS and UPR in cancer, neurodegenerative and metabolic diseases offer a promising targeted therapeutic raison d'être. Several excellent reviews have recently described the merits of such pharmacological targeting of the UPR and the literature on this topic is expanding at an alarming rate.<sup>661,662,664,676,693-698</sup> Selected examples of ERS aggravators (ERSAs) and UPR responses are indicated in Figures 1.26 and 1.27, and Table 1.14.<sup>676,687</sup>



Pharmacological agents (shown in rounded rectangles) cause physiological imbalances (shown in undulated ovals) or directly block SERCA, autophagy, or the proteasome, thus causing the accumulation of misfolded proteins and resulting in aggravated ERS. ERS can be further exacerbated via inhibition of GRP78 with specific inhibitors (left, bottom; see text for details). Activation of the ERS response system/UPR involves GRP78 as key pro-survival and CHOP as key pro-apoptotic components, and these two proteins are representatives of the antagonistic cellular struggle for survival vs. cell death. Shifting this yin–yang balance towards dominance of CHOP will ensure cell death and abort the cell. However, if the yin module (in particular, GRP78) prevails, cell survival and, in the case of tumour cells, increased chemoresistance, will be favoured. CHOP, C/EBP homology protein; 2-DG, 2-deoxy-d-glucose; DMC, 2,5-dimethyl-celecoxib; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; ERAD, endoplasmic reticulum-associated degradation.

**Source:**<sup>676</sup> Schönthal AH. Pharmacological targeting of endoplasmic reticulum stress signaling in cancer. *Biochemical Pharmacology* 2013;85(5):653-666, permission granted by *Biochemical Pharmacology, Elsevier* (Appendix 12).

**Figure 1.26:** Cellular impact of ERS aggravators that weigh on the yin vs yang balance



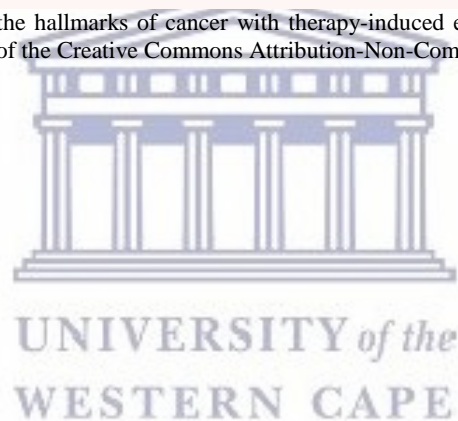
**Figure 1.27:** An overview of therapeutic ERS-based targeting of the main hallmarks of cancer

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**Figure 1.27:** An overview of therapeutic ERS-based targeting of the main hallmarks of cancer (continued)

Respective therapy-based ERS inducers have been segregated into 2 categories (wherever possible) based on their ability to target each of the hallmarks of cancer; such that therapies or drugs labeled with green inhibit the hallmark (thereby inhibiting or suppressing tumourigenesis) whereas those labeled with red support the hallmark (thereby enabling or supporting tumourigenesis). The question mark in parenthesis (?) indicates that data supporting the ability of the given therapy or drug to target or support a hallmark of cancer are not conclusive but are evidenced by either contradictory or incomplete observations. Please see the text for further details. 2-DG, 2-deoxyglucose; 7A7, murine anti-EGFR antibody; ANT, anthracycline; BLM, bleomycin; Bort, bortezomib; BrefA, brefeldin A; CBN, cannabinoids; CG, cardiac glycoside; CLX, celecoxib; CPA, cyclophosphamide; EDF, edelfosine; GRP78i, BiP/GRP78 inhibitor; HDACi, HDAC inhibitor; HHP, high hydrostatic pressure; HSP90i, HSP90 inhibitor; Hyp-PDT, hypericin-based photodynamic therapy; ICD, immunogenic cell death; IOM, ionomycin; MTC, microtubule-targeting chemotherapy; MTX, mitoxantrone; OV, oncolytic viruses; OXP, oxaliplatin; PROTi, proteasome inhibitor; RL66, an analog of curcumin; RT, radiotherapy; Shik, shikonin; THP, thapsigargin; TUN, tunicamycin; UVC, UV irradiation of C-band wavelength.

**Source:**<sup>687</sup> Garg AD, Maes H, van Vliet AR, Agostinis P. Targeting the hallmarks of cancer with therapy-induced endoplasmic reticulum (ER) stress. *Molecular and Cellular Oncology* 2015;2(1):e975089; permission granted (Open Access, under the terms of the Creative Commons Attribution-Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>)).



**Table 1.14:** Pharmacologic modulators commonly used in targeting ERS and UPR signalling

Biologic or Pharmacologic Modulator	Endoplasmic Reticulum Stress Mechanistic Principles and Therapeutic Targeting Strategy	References
<b>Thapsigargin</b>	<p>Thapsigargin, derived from <i>Thapsia garganica</i>, is a potent inducer of GRP78 expression and endoplasmic reticulum stress (ERS) and activator of the UPR through non-competitive inhibition of SERCA (sarcolemmal/endoplasmic reticulum calcium ATPase). SERCA inhibition causes extensive efflux of calcium from ER stores into the cytosol and is thus a strong inducer of ERS—a potent trigger for autophagy, a self-degradative process that has an adaptive function. ERS mediated by thapsigargin promotes CHOP and death receptor 5 (DR5, also referred to as Apo2) synthesis, thus sensitizing TRAIL treatment, which induces oesophageal squamous cell carcinoma cell (ESCC) apoptosis. Thapsigargin synergistically enhances the anticancer activity of drugs against human ESCCs, including inhibition of tumour cell proliferation, invasion and metastasis, and induction of apoptosis. However, thapsigargin causes systemic toxicity, including potent tumour promoter, causes histamine release, and stimulates arachidonic acid metabolism. Combination of bortezomib with SERCA inhibitors, such as thapsigargin, celecoxib, or 2,5-dimethyl-celecoxib (DMC), aggravates ERS and greatly increases glioblastoma cell death <i>in vitro</i> and <i>in vivo</i>, pointing to the potential of thapsigargin as a combination agent in therapeutic regimens.</p>	676,681,687,699-703
<b>Tunicamycin</b>	<p>Tunicamycin is an asparagine-linked (N-linked) glycosylation inhibitor which causes impairment of protein folding and thus ERS. Tunicamycin blocks cell surface receptor tyrosine kinases (RTKs), thereby interrupting mitogenic and pro-survival signalling pathways and sensitizing tumour cells to cytotoxic therapies.</p>	597,704-710

Continued/...

**Table 1.14:** Pharmacologic modulators commonly used in targeting ERS and UPR signalling (continued)

Biologic or Pharmacologic Modulator	Endoplasmic Reticulum Stress Mechanistic Principles and Therapeutic Targeting Strategy	References
<b>Tunicamycin</b>	Tunicamycin inhibits angiogenesis <i>in vitro</i> and <i>in vivo</i> by arresting cells in the G <sub>1</sub> phase of the cell cycle. It prevents the progression of a double- and a triple-negative breast tumour in athymic nude mice by inducing ERS, followed by apoptosis. Tunicamycin is efficacious alone or in combination with radiation/radiotherapy.	687
<b>Brefeldin A</b>	Brefeldin A (BFA) is an inhibitor of protein transport from ER to Golgi, and thus the secretory pathway. It is also an ADP-ribosylation factor (ARF) inhibitor. BFA is also a known perturbant of P-glycoprotein (P-gp), an ATP-dependent efflux pump encoded by the <i>MDR1</i> gene which mediates multidrug resistance of tumour cells to cancer therapy. BFA induces caspase activation and apoptosis and triggers GRP78 upregulation and ER dilation, markers of ERS.	610,676,686,687,711,712
<b>GRP78/BiP Inhibitors</b>	The ER chaperone, GRP78, is one of the most dynamic components of cancer cells. Overexpression of GRP78 correlates with apoptosis, angiogenesis, proliferation, tumourigenesis, invasion/metastasis, inflammation, immunity and drug resistance. GRP78 inhibitors are not generally available for clinical testing, but are developed under screening platform licences ( <a href="https://nuevolution.com/wp-content/uploads/2016/08/EFMC-ISMC-2016_Poster_Final-PDF.pdf">https://nuevolution.com/wp-content/uploads/2016/08/EFMC-ISMC-2016_Poster_Final-PDF.pdf</a> ; <a href="https://nuevolution.com/pipeline/1097/">https://nuevolution.com/pipeline/1097/</a> ;). GRP78 levels can be reduced using a GRP78-specific small interfering RNA (siRNA). In NB cells, Akt increases the accumulation of GRP78 in response to 2-DG. Inhibition of GRP78 is of therapeutic utility for cancer and for bacterial and viral infections.	686,687,713-718

Continued/...



**Table 1.14:** Pharmacologic modulators commonly used in targeting ERS and UPR signalling (continued)

Biologic or Pharmacologic Modulator	Endoplasmic Reticulum Stress Mechanistic Principles and Therapeutic Targeting Strategy	References
<p><b>Celecoxib (CEL)</b> <b>2,5-Dimethyl-Celecoxib (DMC)</b></p>	<p>Cyclooxygenase 2 inhibitor. Postulated to impede SERCA and perturb intracellular calcium homeostasis. Relentless Ca<sup>2+</sup> dysregulation can induce ERS-mediated apoptosis. Celecoxib induces apoptosis independently from its COX-2 inhibitory action via a mitochondrial apoptosis pathway. It also prevents neuroblastoma tumour initiation and progression and potentiates the effect of chemotherapeutic drugs <i>in vitro</i> and <i>in vivo</i>. ERS inducer DMC augments TRAIL-induced apoptosis in glioblastoma and inhibits cell cycle progression and induces apoptosis in human leukaemia cells.</p>	676,709,719-723
<p><b>NSAIDs (Aspirin, Salicylates and Diclofenac)</b></p>	<p>Some nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin (acetyl salicylic acid) and its metabolite, sodium salicylate, have profound effects on cellular functions and survival. Aspirin activates PERK and upregulates expression of the pro-apoptotic transcription factor CHOP (GADD153), a downstream event to eIF2<math>\alpha</math> phosphorylation which, together with cleavage of caspase-12, are hallmarks of ERS-mediated responses. Salicylates inhibit prostaglandin H synthase (cyclooxygenase/COX) activity. By contrast, diclofenac (another NSAID) has been reported to exert protective effects against ER-stress-induced apoptosis in human neuroblastoma SH-SY5Y cells, by suppressing the activation of caspases in the intrinsic apoptotic pathway. NSAIDs perturb ER homeostasis by upregulating the expression of GRP78 and CHOP, and the activation of PERK and ATF6, but rarely the other UPR arm, viz. IRE-1. Inhibition of PGE<sub>2</sub> production with diclofenac, resulted in reduced tumour growth in an <i>in vivo</i> model of 11q-deleted neuroblastoma.</p>	162,724-728

Continued/...

**Table 1.14:** Pharmacologic modulators commonly used in targeting ERS and UPR signalling (continued)

Biologic or Pharmacologic Modulator	Endoplasmic Reticulum Stress Mechanistic Principles and Therapeutic Targeting Strategy	References
<b>Geldanamycin</b>	HSP90 and GRP94 inhibitor	233,666,728,729
<b>Irestatin</b>	Inhibits IRE-1 $\alpha$ activity	666,695,730
<b>Bortezomib</b>	Reversible inhibitor of the 26S proteasome. Bortezomib exerts synergistic cytotoxic effects in cancer cells by turning off the prosurvival ER chaperone BIP/Grp78 and turning on the pro-apoptotic NF-kappaB. Both nelfinavir and bortezomib lead to autophagy-dependent growth arrest and the radiosensitization of cancer cells.	689,731-733
<b>Ritonavir and Nelfinavir</b>	HIV protease inhibitors that activate certain UPR components such as CHOP and GRP78 and hence induce accumulation of misfolded proteins. Nelfinavir, in combination with various NSAIDs, causes reduction in cell survival and an increase in apoptosis. Also, HIV protease inhibitors significantly impede ABC transporters, including P-gp.	310,644,687,726,734-737
<b>Resveratrol</b>	Induces GRP78 and CHOP, p-eIF2 $\alpha$ and XBP1 splicing. Downregulate P-gp expression via inhibiting PI3K/Akt/mTOR pathway. Augments ER stress and the cytotoxic effects of glycolytic inhibition in neuroblastoma by downregulating Akt. Enhances mitochondrial biogenesis and riggers UPR.	658,714,738,739
<b>Epidermal Growth Factor (EGF)-SubA</b>	Targets GRP78, impeding its function and affecting proteostasis in ER.	687
<b>Oncolytic Viruses</b>	Stress the ER through viral protein overload.	687
<b>Protein Disulphide Isomerase (PDI) Inhibitors</b>	Protein disulfide isomerase (PDI) is an essential enzyme of disulphide bond formation in the ER. PDI inhibitors cause rapid accumulation of misfolded or unfolded proteins in the ER. There is an interrelation of ER stress and ROS with redox signalling mediators such as PDI-ER oxidoreductin (ERO)-1, glutathione (GSH)/glutathione disulphide (GSSG), NADPH oxidase 4 (Nox4), NADPH-P450 reductase (NPR) and calcium.	687,740-742

*Continued/...*

**Table 1.14:** Pharmacologic modulators commonly used in targeting ERS and UPR signalling (continued)

Biologic or Pharmacologic Modulator	Endoplasmic Reticulum Stress Mechanistic Principles and Therapeutic Targeting Strategy	References
<b>Versipelostatin/Epigallocatechin Gallate</b>	Derived from green tea extract and causes inhibition of GRP78.	676,686,687,743
<b>Anthracyclines/Mitoxantrone/Carboplatin</b>	Cause ROS production leading to ROS-based ERS.	687,695,744
<b>Chloroquine</b>	Lysosomotropic agent and inhibitor of autophagy. Combination of nelfinavir and chloroquine significantly increased ER stress and caused selective cell death in multiple cell line models with hyperactive mTORC1.	745-747
<b>BRAF Inhibitor</b>	Interferes with cytosolic Ca <sup>2+</sup> homeostasis causing ERS. Predominantly causes activation of the PERK–eIF2 $\alpha$ –ATF4/ATF3 pathway, which in turn promotes cytoprotective autophagy. Combined BRAF and autophagy inhibition promotes tumour regression in BRAFi-resistant xenografts.	312,687,746,748-750
<b>Cannabinoids</b>	Cause ERS through ceramide accumulation and eIF2 $\alpha$ phosphorylation. Plant-derived cannabinoids are moderately effective in reversing MDR in CEM/VLB100 cells by decreasing P-gp expression.	687,751
<b>Curcumin (Turmeric)</b>	Plant polyphenols that have been identified to possess proteasome-inhibitory activity include (-)-epigallocatechins-3-gallate (EGCG), genistein, luteolin, apigenin, chrysin, quercetin, curcumin and tannic acid. SERCA inhibition causes ER Ca <sup>2+</sup> imbalance and ERS. Down regulates calreticulin. Liposome-encapsulated curcumin suppresses neuroblastoma growth through nuclear factor-kappa B inhibition. Curcumin down-regulates transcription factors important for cell growth and survival, through modulation of the NF-kB and PI3K/AKT pathways.	662,687,743,752
<b>HDAC inhibitors (HDACi) e.g. Vorinostat</b>	Cause GRP78 acetylation, inhibiting GRP78 function and compromises ER protein folding, causing ERS.	
<b>Ceapins</b>	Selectively targets the ATF6 $\alpha$ branch.	753
<b>Chemotherapeutic Agents</b>	Induce ERS through various mechanisms.	676,686,687,692,699,730-732

## C5. The ERS and UPR in Perspective

Pro-survival (yang) GRP78 and pro-apoptotic (yin) CHOP are quintessential opposing regulators of the ERS response. Whereas suprabasal levels of GRP78 are commonly found in many tumour cell lines and primary tumour tissues (and levitate upon ERS), CHOP is predominantly infrabasal, but intensifies in response to short-term, acute ERS. Factoring in the differential in baseline ERS levels in tumour vs. normal cells, manipulated pharmacological aggravation of pre-existing ERS in tumour cells can be exploited to ‘‘overload’’ this already burdened system to eclipse the tumour cells’ capacity for adaptation. During this process, the ERS system’s pro-apoptotic module would surpass the pro-survival module, leading to increased chemosensitivity of the tumour. By analogy, normal cells should be reasonably safeguarded from the toxic outcomes of pharmacologically increased ERS. Their ERS response system, which had not been subjected to chronic activation, would be triggered from significantly lower baseline levels and therefore would have the competence to stem increased ERS levels. Generally, the protective module of normal cells would presumably dominate and shield the cells from stress-induced toxicity significantly longer than is the case in tumour cells. This is the sine qua non of probing ERS and UPR dynamics and hence targeting the hallmarks of cancer with therapy-induced ERS.

### SECTION D: RESEARCH CONTEXT

#### D1. Problem Statement and Research Questions

Despite recent advances in cancer cell glycomics and the arsenal of investigational and approved drugs against NB therapeutic targets, successful treatment of high-risk neuroblastoma (HR-NB) remains a challenging task since 40 % of patients still relapse during or after glycan-based immunotherapy following standard therapy.<sup>153,494</sup> Therefore, a dire need exists to develop novel treatment modalities that target the NB glycome, proteome and transcriptome. Neuroblastoma (NB) is the most common paediatric cancer and accounts for

15% of all oncology deaths in infants. NB continues to perplex scientists and oncologists alike because its biological and clinical behaviour fluctuate between complete spontaneous regression and clinical multidrug resistance, strongly indicating that besides genetic events, the tumour microenvironment (TME) significantly influences these characteristics of NB.<sup>64,147</sup> Survival rates of HR-NB remain less than 50%, with amplification of the *MYCN* oncogene being the most significant hallmark associated with aggressive NB and poor survival outcome.<sup>184,185</sup>

Transcriptionally, the ABC transporters are directly and coordinately regulated by *MYCN*<sup>328</sup> and, correspondingly, their overexpression correlates with poor prognosis.<sup>327,329</sup> Most aggressive NBs exhibit MDR,<sup>311</sup> attributable to p53 mutations and/or a loss of p53 function induced during chemotherapy,<sup>754</sup> which further exacerbates the probability of relapse.<sup>333,334</sup> While the prognostic merit of the ABC transporters in childhood NB is generally ascribed to their role in cytotoxic drug efflux, several reports claim that they might promote the malignant phenotype independent of this function, thus unlocking their potential as therapeutic targets,<sup>330</sup> and strengthening the less well understood, but evolving theme of the drug efflux-independent contributions of ABC transporters to cancer biology and treatment failure.<sup>311</sup>

Similarly, some metastatic MDR NBs derive from the clonal selection of side population cells that constitutively express the *MDR1* (*P-gp*, *ABCB1*), *MRP1/ABCC1* and *MRP4/ABCC4* gene family, which may or may not correlate with *MYCN* amplification and poor outcome.<sup>7,294,323,327,328,330,335</sup> Moreover, minimal residual disease (MRD), the major cause of tumour recurrence (relapse) and metastasis, is enriched in cancer stem cells (CSCs) with an increased drug efflux capacity mediated through overexpression of ABC transporters.<sup>336,337</sup>

Charging into the fray are elevated levels of different types of gangliosides that profoundly contribute to aggressive NB behaviour and poor patient survival.<sup>527,537,539,540</sup> By contrast, overexpression of both complex “a” gangliosides (CaG and CbG), eradicates aggressive

tumour-cell behaviour *in vitro* (e.g., cellular proliferation and migration) and promotes differentiation.<sup>541</sup> In recent years, interest in gangliosides has been revived, mainly as prognostic biomarkers to stratify NB patients for targeted anticancer immunotherapy and to monitor efficacy of treatment.<sup>542</sup> Diverse molecules involved in NB glycobiology play key roles in tumour growth and are therefore potential targets for anti-tumour therapy.<sup>527</sup>

As mentioned above, the overexpression of MDR transporters strongly correlates with poor NB therapeutic outcome since they efflux a wide array of endogenous compounds and anticancer drugs from cancer cells.<sup>311,329,330</sup> Theoretical and contextual issues emerging from the literature point to a multitude of unclarified roles of endogenous compounds and anticancer drugs at the intersection of ABC transporters and NB behaviour and cancer cell responses to chemotherapy. Perturbation of ABC transporters may provide insightful options for therapeutic repression of HR-NB, and proof of concept for increasing drug bioavailability (therapeutic efficacy) in refractory tumours which overexpress these glycans.<sup>326</sup>

The MDR transporter, P-glycoprotein (ABCB1, P-gp), has been shown to be one of the most strongly upregulated genes associated with acquired drug resistance and NB treatment failure.<sup>527,590,591</sup> Inhibition of protein glycosylation reverses the MDR phenotype of several cancer cell lines.<sup>593</sup> Equally, inhibition of N-linked glycosylation hampers ALK phosphorylation and pro-survival signalling in NB cell lines.<sup>556</sup> Inhibitors of N-glycosylation, e.g., tunicamycin, hinder P-gp-mediated MDR phenotype.<sup>596,597</sup> The precise role of N-glycosylation in P-gp function remains to be fully unravelled.<sup>598-600</sup> Several classes of N-linked glycosylation inhibitors are available that need to be evaluated further for their potential to alter NB behaviour.<sup>594</sup>

The glycan moieties of glycoproteins are critical for various cellular processes such as protein solubility, stability, conformation and function. Thus, altered expression of glycans has been implicated in chronic or acquired infectious diseases, endoplasmic reticulum stress (ERS) and



cancer.<sup>491,497,606,641-646</sup> Perturbation of N-linked glycosylation can also result in the accumulation of unfolded/misfolded proteins which, in turn, may trigger ERS, the unfolded protein response (UPR) and, ultimately, decreased cell viability and apoptosis.<sup>651,662</sup> Severe ERS may as well induce autophagy, a self-degradative process that has a life-saving adaptive function.<sup>681-683</sup> The TME is the arena for ERS and UPR responses that sustain various hallmarks of cancer.<sup>684,685,687</sup>

It is clear from the above considerations that NB glycopathobiology, particularly the diverse cancer landscape exemplified by the N-glycoproteome in eukaryotic cells (N-linked protein glycosylation in the ER coupled with MDR, ERS, UPR activation, apoptosis and autophagy), offers an emerging theme in the therapeutic targeting of cancer, including NB.<sup>326,493,494,519,527,545,547,570,589,594,600,646,755-763</sup> To this end, we have set out to explore the effects of various glycosylation inhibitors and ERS inducers on SK-N-BE(2) NB cell survival and ability to efflux calcein-AM, a P-gp substrate.

## **D2. Purpose of the Study**

The purpose of the study was to investigate the effects of various glycosylation inhibitors and ERS inducers on the behaviour of NB cells in culture. For this study, we have selected the continuous SK-N-BE(2) cell line as representative of human NB cells *in vitro* that overexpress readily detectable levels of P-glycoprotein (P-gp, ABCB1) and other ABC transporters.<sup>764,765</sup> The SK-N-BE(2) cell line was derived from a bone marrow metastases in a patient refractory to chemotherapy.<sup>766-769</sup> Figure 2.1 in Chapter 2 shows the experimental design of the project presented in this thesis.

## **D3. Aims of the Study**

The aims of the study were to determine the effects of N-glycosylation inhibition and ERS induction on SK-N-BE(2) cell proliferation and viability, apoptosis and P-glycoprotein drug efflux function.

#### D4. Objectives of the Study

In this study, the following N-glycosylation inhibitors and ERS inducers—tunicamycin, deoxynojirimycin, celecoxib, swainsonine and  $\beta$ -D-xyloside—were evaluated for their effects on:

1. SK-N-BE(2) NB cell growth and viability, using the CCK-8 assay.
2. P-glycoprotein (P-gp, ABCB1)-mediated cellular drug efflux function in SK-N-BE(2) NB cells, using Cayman's Calcein-AM multidrug resistance assay.
3. SK-N-BE(2) NB cell viability, cytotoxicity and apoptosis induction by caspase-3 activation, using the Apotox-Glo Triplex assay.
4. SK-N-BE(2) NB cell apoptosis induction by morphological staining of cells with Annexin-FITC.

#### D5. Hypothesis

We hypothesize that N-glycosylation inhibitors and ERS inducers will antagonize the manifestation of SK-N-BE(2) cancer cell hallmarks evaluated, namely cell survival (proliferation, viability and apoptosis) and P-glycoprotein-mediated drug efflux function.

### SECTION E: SUMMARY

This chapter provided the introduction and literature review on NB encompassing the epidemiology of the disease, risk factors and staging, prognostic markers, histopathological characteristics, detection, diagnosis and prognosis, clinical presentation, signs and symptoms, molecular pathogenesis, genetics and genomics and therapeutic landscape. In addition, the chapter underscored the significance of glycans and protein glycosylation in NB and the targeting strategies for ER stress and the UPR. Finally, the chapter outlined the research context of the study in terms of problem statement, aims and objectives and hypothesis.

## CHAPTER 2

### RESEARCH METHODOLOGY

#### 2.1 Experimental Design

The focus of this chapter is to outline and describe the research methodology and experimental design that have been chosen for the study. It summarizes the materials and methods used such as chemicals required, drugs tested and the maintenance of the parental SK-N-BE(2) (American Type Culture Collection / ATCC® CRL2271™) neuroblastoma cell line. Analyses of the SK-N-BE(2) neuroblastoma cells exposed to brefeldin A (BFA), thapsigargin (TG), aspirin (AS), castanospermin (CST) and bacitracin a (BAC) included growth curves, cell viability and cytotoxicity assays by means of the Cell Counting Kit-8 (CCK-8), measurement of P-glycoprotein (P-gp, ABCB1) cellular drug efflux pump function using the Calcein-AM (Cayman's multidrug resistance) assay kit, the Apotox-Glo™ triplex cell viability, cytotoxicity and apoptosis assays, caspase-3 activation and morphological staining of apoptotic cells using the Annexin V-FITC kit (Figure 2.1). Further details of the experimental design are covered in the subsections that follow. The statistical methods used for data analysis are also described.

#### 2.2 Drugs and Chemicals

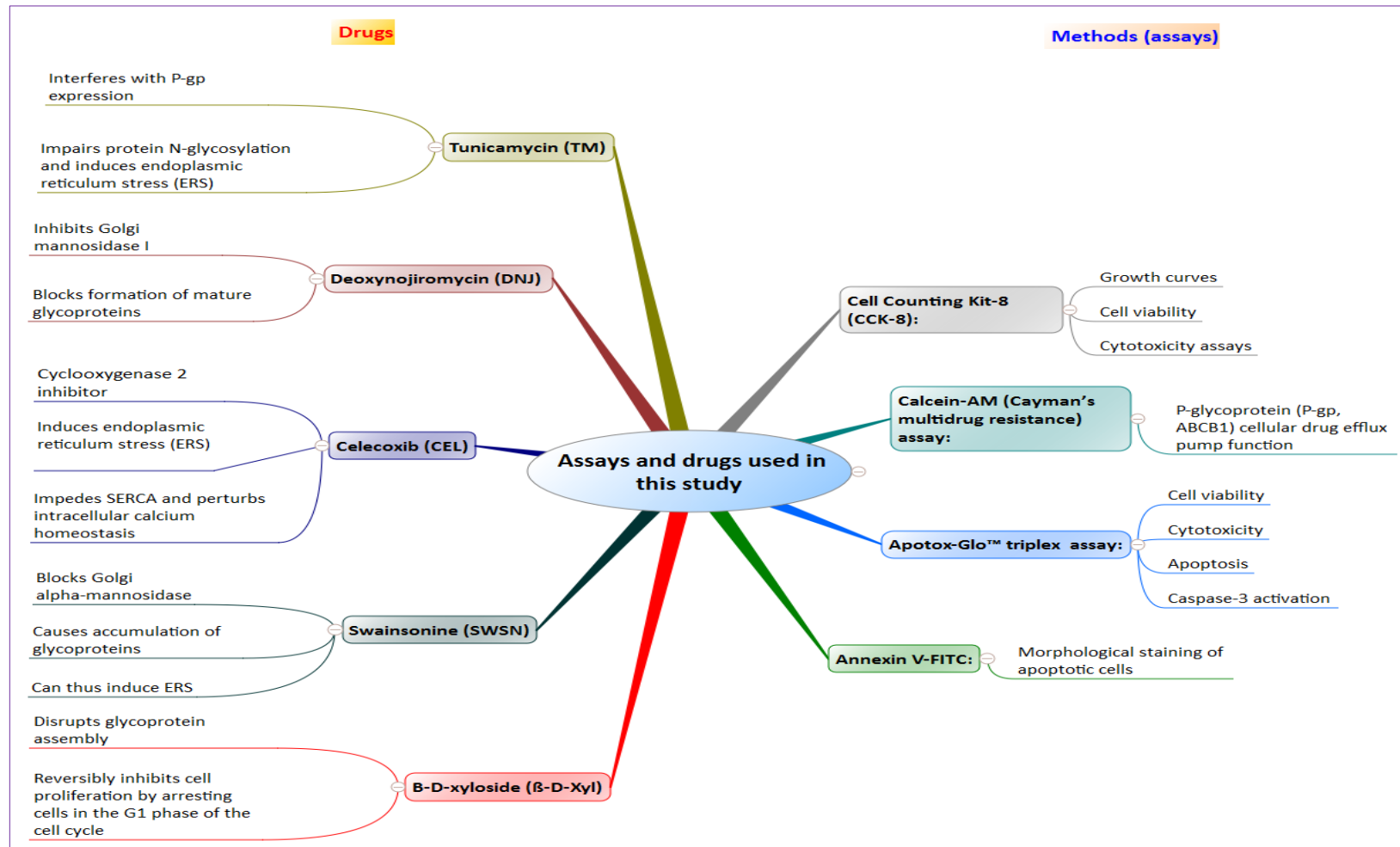
Drugs and chemicals used in this study included thapsigargin from plant *Thapsia garganica* (CAS 67526-95-8, Sigma-Aldrich, St Louis, MO, USA), brefeldin A from *Penicillium brefeldianum* (CAS 20350-15-6, Sigma-Aldrich, St Louis, MO, USA), castanospermin from *Castanospermum australe* seeds (CAS 79831-76-8, Sigma-Aldrich, St Louis, MO, USA), bacitracin (CAS 1405-87-4, Sigma-Aldrich, St Louis, MO, USA), aspirin (CAS 50.78.2, Sigma-Aldrich, St Louis, MO, USA), heat inactivated foetal bovine serum (Biochrome, The Scientific Group), phosphate buffered saline (PBS) (Gibco, Life Technologies), Dulbecco's

Modified Eagles Medium supplemented with F-12 glutamax (DMEM F-12 glutamax) (Gibco Life Technologies), penicillin/streptomycin (Invitrogen or Gibco, Life Technologies), trypsin-EDTA (Gibco, Life Technologies), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA), trypan blue (CAS 72-57-1, Sigma-Aldrich, St Louis, MO, USA), Cell Countin Kit-8 (CCK-8)(Item no 06041406, Enzo Life Sciences) Cayman Chemicals Multi Drug Resistance kit (Calcein-AM) (Item no. 600370), Annexin V-CY3 (Cat: APOAC; Sigma-Aldrich, St Louis, MO, USA), ApoTox-Glo<sup>®</sup> triplex assay kit (Cat: G6320, Promega).

### 2.3 Culture and Maintenance of SK-N-BE(2) Neuroblastoma Cells

The continuous human neuroblastoma (NB) SK-N-BE(2) cell line, originally purchased from the American Type Culture Collection (ATCC, Rockville, MA), was kindly provided by Dr AM Serafin, Radiobiology Laboratory, Department of Medical Imaging and Clinical Oncology, Faculty of Medicine and Health Sciences, University of Stellenbosch, South Africa. The SK-N-BE(2) cell line is known to overexpress readily detectable levels of P-glycoprotein (P-gp, ABCB1) and other ABC transporters.<sup>764,765</sup> The SK-N-BE(2) cell line was established from a bone marrow biopsy of a metastases in November 1972 of a patient refractory to chemotherapy.<sup>766-769</sup>

All tissue culture operations were carried out in a model NU-5510E NuAire DHD autoflow automatic CO<sub>2</sub> air-jacketed incubator and an AireGard NU-201-430E horizontal laminar airflow cabinet with a HEPA-filtered clean work area (NuAire). SK-N-BE(2) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% heat-inactivated foetal bovine serum (HIFBS), 1% penicillin/streptomycin (100 µg/ml penicillin and 10 µg/ml streptomycin) and grown as monolayer cultures at 37 °C in a of 80%) in an atmosphere of 5% CO<sub>2</sub>:95% air. Routinely, cryovials containing frozen SK-N-BE(2) cells in 40% HIFB, 50% DMEM, 10% DMSO were removed from -80 °C freezer and thawed in a 37°C water bath.



**Figure 2.1:** Experimental design: Assays and drugs used in this study

The caps were wiped with 70% ethanol and the contents of the vial transferred aseptically to a 15-ml conical centrifuge tube containing 1 ml of Modified Eagles Medium (MEM)/F-12 supplemented with 1% penicillin-streptomycin and 10% HIFBS, centrifuged for 5 minutes at 2500 rpm. After centrifugation, the supernatant was discarded and the cell pellet resuspended in 2 ml of complete medium. The cells were mixed thoroughly to ensure a homogeneous cell suspension, 1 ml of which was transferred to T-25 culture flask (surface area 2500 mm<sup>2</sup>) containing 5 ml complete medium to maintain stock cultures.

The flask was placed on a PrimoVert phase-contrast microscope to visualize the presence of suspended cells, and then placed in a 37°C incubator at 5% CO<sub>2</sub> and 80% RH, the incubation specifications were kept constant throughout for cells to acclimatize and attach to the substratum of the flask. The cells were allowed to attach for 24 h, after which the flask was removed from the incubator and attachment confirmed by microscopy. The flask was incubated under ideal tissue culture conditions and growth medium periodically changed until approximately 80-90% of the flask substratum had been occupied by SK-N-BE(2) cells.

Once confluency had been reached, cells were gently trypsinized. The medium was aspirated and the cells rinsed with 2 ml PBS. After 1 minute, the PBS was aspirated and replaced with 2 ml of 0.25% Trypsin-EDTA and placed in the incubator for 5-15 minutes in order for detachment of the cell monolayer to be achieved. The flask was then removed from the incubator and placed in a laminar flow cabinet. Thereafter, 4 ml complete medium was added to the flask to deactivate the trypsin. The cells were gently mixed using an electronic pipette aid and detached cells aspirated and transferred to a 15 -ml conical centrifuge tube, centrifuged 5 minutes at 2500 rpm to separate the cells from the medium-trypsin solution. After centrifugation, the supernatant was discarded and the cell pellet resuspended in 5 ml of complete medium. The cells were mixed to ensure a homogeneous cell suspension, 1ml of which was transferred to T-25 culture flask (surface area 2500 mm<sup>2</sup>) containing 5 ml complete medium to maintain stock cultures or for use in experiments.



## 2.4 Growth Curve Analysis of SK-N-BE (2) Neuroblastoma Cells

SK-N-BE(2) neuroblastoma cells were trypsinized and transferred to a 15-ml centrifuge tube and spun at 2500 rpm for 5 min. The supernatant was removed and the pellet resuspended in 5 ml of complete fresh medium. Cells were seeded into 24-well plates at density of  $1 \times 10^5$  cells/ml per well (100  $\mu$ l of suspension was mixed with 1.9 ml of fresh media to obtain a final volume of 2 ml per well. The plates were incubated for 24 hours (24h), 48h, 72h and 96h, respectively. After the incubation period, cells were harvested with 1 ml trypsin-EDTA from wells every 24h for the duration of the experiment. Viable cells were counted using the Bio-Rad TC-20 cell counter at a ratio 1:1 cell suspension: 0.4  $\mu$ M trypan blue. The experiments were conducted in quadruplicate and the results pooled.

## 2.5 Cell Counting Kit-8 (CCK-8) Cell Viability Assay

The Cell Counting Kit-8 (CCK-8, CCK-8; Dojindo Laboratories, Japan) permits precise assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron carrier.<sup>770-772</sup> CCK-8, being non-radioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give a yellow-coloured product (formazan), which is soluble in tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Cell viability was measured using the CCK-8 kit, according to manufacturer's protocol.

All CCK-8 assays were carried out in 96-well flat bottom tissue culture plates. SK-N-BE(2) NB cells were seeded at density of  $5 \times 10^4$  cells/ml. A 100  $\mu$ l of cell suspension was added to each well and cells were allowed to attach for 24h under normal incubation conditions. After 24h, the media was aspirated from all wells, first and second columns were replaced with 100  $\mu$ l of complete media alone while the other columns were replaced with increasing  $\log_{10}$

concentrations of test compounds: tunicamycin (0.0001, 0.001, 0.01, 0.1, 1, 10  $\mu\text{M}$ ), deoxynojirimycin (0.02, 0.2, 2, 20, 200, 2000  $\mu\text{M}$ ), celecoxib (0.001, 0.01, 0.1, 1, 10, 100  $\mu\text{M}$ ), swainsonine (0.002, 0.02, 0.2, 2, 20, 200  $\mu\text{M}$ ) and  $\beta$ -D-xyloside (0.01, 0.1, 1, 10, 100, 1000  $\mu\text{M}$ ) to the appropriate wells and with negative control of untreated cells (cells and vehicle) and of medium only in four replicate wells. Following incubation with the compounds for various time periods, 100  $\mu\text{l}$  of CCK-8 solution was added to each well of the plate, and then plates were placed in a 37°C incubator at 5%  $\text{CO}_2$  and RH 80%. The optical density (OD) was obtained at 450 nm using a Promega GloMax™ Multiscan plate reader. The mean blank-corrected absorbance (MBCA) was derived from the following equation:

$$MBCA = \frac{1}{4} \sum_{i=1}^4 (A_i - A_0)$$

where  $A_i$  represents the absorbance reading of well  $i$  and  $A_0$  is the absorbance reading of the blank well (inoculated cells without test compound=untreated controls with variable molar concentrations of vehicle approximating final concentrations present in the test wells).

## 2.6 Apotox-Glo™ Triplex Cytotoxicity, Cell Viability and Apoptosis Assays

### 2.6.1 Principle of the Apotox-Glo™ Triplex Assay

A number of 96-well assays are available for high throughput screening of cytotoxicity of drugs (<https://www.promega.com/-/media/files/promega-worldwide/north-america/promega-us/webinars-and-events/assessmentcellhealthwebinfo0412.pdf>).<sup>773-777</sup> The Promega ApoTox-Glo™ Triplex Assay combines three assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. The first part of the assay simultaneously measures two protease activities; one is a marker of cell viability and the other is a marker of cytotoxicity. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycylphenylalanyl aminofluorocoumarin, GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell

protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic cell-impermeant peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Because bis-AAF-R110 is not cell-permeant, essentially no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously.

The second part of the assay uses a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD (aspartic acid, glutamic acid, valine, aspartic acid), in a reagent optimized for caspase activity, luciferase activity and cell lysis. Adding the Caspase-Glo® 3/7 reagent in an “add-mix-measure” format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase activity present. The Caspase-Glo® 3/7 reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which is formulated to generate a stable “glow-type” luminescent signal and improve performance across a wide range of assay conditions.

## 2.6.2 Assay Conditions for the ApoTox-Glo™ Triplex Assay

All Apoptox-Glo™ Triplex assays were carried out in white opaque bottom 96-well plates.  $1 \times 10^5$  cells/ml were seeded into each well in a final volume of 100  $\mu$ l per well and allowed to attach for 24h. After the attachment period, culture medium was removed, first and second columns were replaced with 100  $\mu$ l medium containing vehicle and vehicle control (untreated cells and vehicle), while the other columns were replaced with increasing concentrations of test compounds in four replicate wells as described for the CCK-8 assay. Plates were placed in a 37°C incubator at 5% CO<sub>2</sub> and RH 80% appropriate time intervals. After exposure of SK-N-

BE(2) cells for the time intervals indicated, 20  $\mu\text{l}$  of viability/cytotoxicity reagent containing both GF-AFC substrate and bis-AFF-R110 substrate was added to all wells, plates were covered in foil and briefly mixed by orbital shaking at 300 rpm for 30 seconds(s). After the reagents were added and allowed to mix, plates were placed in the CO<sub>2</sub> incubator at 37°C for 1 hour. Following the incubation period, plates were removed from the incubator, the foil removed and the fluorescence measured at excitation (Ex) wavelength of 400 nm ( $\lambda_{\text{ex}}$  400 nm) and emission wavelength of 505 nm ( $\lambda_{\text{em}}$  505 nm) and  $\lambda_{\text{ex}}$  485 nm /  $\lambda_{\text{em}}$  520 nm for viability and cytotoxicity, respectively using the Promega GloMax™ Multiscan plate reader. To determine apoptosis, 25  $\mu\text{l}$  of Caspase-Glo® 3/7 reagent was added to each well, the plate was covered in foil, and briefly mixed by orbital shaking at 300 rpm for 30 seconds. Thereafter, the plate was incubated for 1 hour and luminescence was measured (caspase-3 activation, a hallmark of apoptosis) using the Promega GloMax™ Multiscan plate reader.

## 2.7 Measurement of P-Glycoprotein-Mediated Efflux Function

A number of *in vitro* assays have been used to identify compounds as MDR protein modulators, either as a substrate or as inhibitors of P-glycoprotein (Pgp; ABCB1), a member of the ATP-binding cassette (ABC) superfamily which actively exports structurally diverse hydrophobic compounds from the cell by ATP hydrolysis. Of these, the calcein–acetoxymethylester (Calcein-AM) assay has been shown to identify both substrates and inhibitors of MDR proteins, and therefore offer an advantage over other assays.<sup>778-784</sup> Calcein-AM is cell-permeable non-fluorescent dye. Upon transport into live cells, its acetomethoxy group is removed by intracellular esterases, thereby trapping the compound inside the cell where it exhibits strong green fluorescence.

As an MDR protein substrate, calcein-AM is rapidly excluded from cells expressing MDR protein, thus reducing fluorescent calcein in the cytosol. This property makes calcein-AM an ideal probe for identifying MDR protein overexpressing cells. Cayman's Multi-Drug Resistance Assay Kit provides a convenient tool for studying MDR protein modulators. The

kit employs calcein-AM, a substrate for MDR proteins, including P-gp and MRP, as a probe for the detection of chemical compounds interacting with MDR proteins. Cyclosporin A, a competitive inhibitor, and verapamil, a non-competitive inhibitor of P-gp, are included as positive controls.

All experiments using the calcein-AM kit were carried out in black, clear bottom 96-well tissue culture treated plates. SK-N-BE(2) cells were seeded at a density of  $5 \times 10^5$  cells/well in 100  $\mu$ l of complete DMEM cell culture medium and incubated allowing for cells to attach and grow overnight (24h). On the day of the experiment, the plate was centrifuged for 5 min at 400 x g at room temperature, the medium aspirated from all the wells and replaced with 100  $\mu$ l concentrations of test compounds: tunicamycin (1, 2.5, 5  $\mu$ M), deoxynojirimycin (200, 1200, 2400  $\mu$ M), celecoxib (0.1, 0.4, 0.8  $\mu$ M), swainsonine (2, 20, 200  $\mu$ M) and  $\beta$ -D-xyloside (400, 1200, 2000  $\mu$ M). Included in the kit were cyclosporin A and verapamil which were used as positive controls diluted 1:1000 and 1:2000, respectively, into culture medium.

The plates were incubated for 24h in CO<sub>2</sub> incubator at 37°C. It is recommended for positive controls to be incubated for 30 min. At the end of the specified treatment interval, 100  $\mu$ l of the prepared calcein-AM solution (2X) was added to each of the sample wells and incubated for additional 30 min in in CO<sub>2</sub> incubator at 37°C. Then, the plates were centrifuged for 5 min at 400 x g at room temperature. The supernatants were aspirated and another 100  $\mu$ l of the prepared calcein-AM solution (2X) added to each of sample wells and incubated for an additional 30 min in a CO<sub>2</sub> incubator at 37°C. The plates were again centrifuged for 5 min at 400 x g at room temperature, supernatants aspirated and finally 200  $\mu$ l of ice cold medium added to each well. The plates were analyzed immediately with a fluorescent plate reader (Promega GloMax™ Multiscan). Cells that have taken up calcein-AM display strong fluorescence intensity with excitation and emission wavelengths of 485 nm and 535 nm, respectively.

## 2.8 Annexin-V Cy3™ Apoptosis Assay

### 2.8.1 Principle of Annexin-V Cy3™ Apoptosis Assay

The annexins are a group of homologous proteins that bind phospholipids in the presence of calcium. Apoptosis, or programmed cell death (PCD), is an important mechanism that most cells use to negatively select cells deleterious to the host. Many cells of the immune system such as thymocytes, self-reactive B- and T-cells undergo apoptosis as a result of normal cell selection processes. The cellular changes involved in the process include loss of cell membrane phospholipid asymmetry during early stages of apoptosis.

In living cells, phosphatidylserine (PS) is transported to the inner plasma membrane leaflet by the enzyme Mg-ATP dependent aminophospholipid translocase. However, during the onset of apoptosis, PS is transported to the external leaflet of the plasma membrane. PS is then available for binding to annexin-V and any of its conjugates in the presence of  $Ca^{2+}$  ions. Apoptotic cells can be differentiated from necrotic cells in several ways.

The method employed by this kit involves the use of two labels: Annexin-Cy3 (AnnCy3) binds to PS present in the outer leaflet of the plasma membrane of cells starting the apoptotic process. The binding is observed as red fluorescence. 6-Carboxyfluorescein diacetate (6-CFDA) is used to measure viability. When this non-fluorescent compound enters living cells, esterases present hydrolyze it, producing the fluorescent compound, 6-carboxyfluorescein (6-CF). This appears as green fluorescence.

Cells can be incubated either with AnnCy3 or 6-CFDA separately, or with the two compounds simultaneously. After labelling at room temperature, the cells are immediately observed by fluorescence microscopy. Live cells will be labelled only with 6-CF (*green*), while necrotic cells will label only with AnnCy3 (*red*). Cells in the early stage of apoptosis, however, will be labelled with both AnnCy3 (*red*) and 6-CF (*green*).



## 2.8.2 Assay Conditions for Annexin-V Cy3™ Apoptosis Assay

SK-N-BE(2) neuroblastoma cells were seeded into 24-well plates, at density of  $5 \times 10^5$  cells per well in 1 ml of culture medium. Cells were then incubated and allowed to attach for 24h. After 24h, cells were exposed to 100  $\mu$ l of relative concentrations of test compounds: tunicamycin (1, 2.5, 5  $\mu$ M), deoxynojirimycin (200, 1200, 2400  $\mu$ M), celecoxib (0.1, 0.4, 0.8  $\mu$ M), swainsonine (2, 20, 200  $\mu$ M),  $\beta$ -D-xyloside (400, 1200, 2000  $\mu$ M). Staurosporine (1  $\mu$ g/ml) was used as positive control. After inducing apoptosis using the specified concentrations of ER stress inducers, cells were washed in PBS.

Thereafter, cells were trypsinized (500  $\mu$ l/well) and detached cells were transferred to a 15-ml conical centrifuge tube and centrifuged for 5 min. The cell pellet was resuspended in PBS (1 ml). A 2-mm tip PAP pen (Sigma-Aldrich), a special marking pen that delivers a thin film-like green-tinged hydrophobic barrier when a circle is drawn around a specimen on a slide, was used to draw circles of 1 cm diameter on poly-prep-poly-L-lysine-coated slides to restrict movement of cell suspension to the slide. A droplet (50  $\mu$ l) of cell suspension was deposited inside the circle and cells were allowed to attach to the slide by incubating at room temperature.

The cells were washed twice with 50  $\mu$ l of binding buffer (10 mM HEPES/NaOH, pH 7.5, containing 150 mM NaCl, 5 mM KCl and 2.5 mM  $\text{CaCl}_2$ ) and 50  $\mu$ l of a double label staining solution (Sigma-Aldrich; 1  $\mu$ g/ml Annexin-V Cy3.18 and 500  $\mu$ M 6-CFDA in 1X binding buffer) added onto each circle and covered with foil.

Cells were incubated at room temperature for 10 min. Slides were washed three times with 50  $\mu$ l 1X binding buffer in order to remove excess unbound staining solution. A drop of binding buffer (35  $\mu$ l) was added to the centre of each slide. A cover slip (24 x 50 mm) was placed onto the slide and results were viewed and recorded using a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa, <http://www.imp.co.za/>).

## 2.9 Statistical Analysis

The Apotox-Glo™ Triplex cell viability, cytotoxicity and apoptosis assays were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, using GraphPad Prism version 7.02 for Windows, GraphPad Software, La Jolla California USA ([www.graphpad.com](http://www.graphpad.com)) or SigmaPlot 13 (<https://systatsoftware.com/products/sigmaplot/>), where indicated. Results are expressed as the mean  $\pm$  SEM (n=4), from three independent experiments. A difference of  $p < 0.05$  was considered to be significant as compared to untreated SK-N-BE(2) cells (vehicle-treated controls). ANOVA followed by Tukey's multiple comparisons test and with the significance criterion set a priori at 0.05 was used for transformed raw data of P-gp ATPase activity (Calcein-AM assay) and are presented as mean  $\pm$  95% confidence interval (CI) (n=4).



## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1 Introduction

In this study, specific classes of N-glycosylation inhibitors (Table 1.13, Chapter 1) and pharmacologic modulators commonly used in targeting endoplasmic reticulum stress (ERS) and the unfolded protein response (UPR) signalling (Table 1.14, Chapter 1) were used to evaluate their effects on SK-N-BE(2) neuroblastoma cell proliferation, viability and induction of apoptosis.

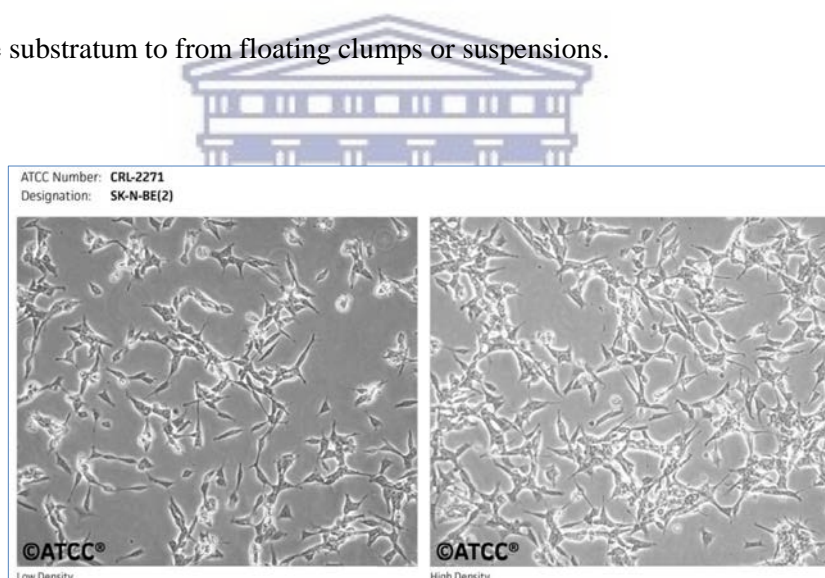
The compounds are: tunicamycin (an antibiotic that blocks the rate-limiting step of protein N-glycosylation and induces ERS and apoptosis in cells), deoxynojirimycin (inhibits Golgi mannosidases and thus maturation of glycoproteins, causing ERS), celecoxib (a potent cyclooxygenase 2 inhibitor, known to impede sarcoplasmic/endoplasmic reticulum calcium ATPase/SERCA) and so triggering relentless  $\text{Ca}^{2+}$  dysregulation that can induce ERS-mediated apoptosis), swainsonine (a glycosidase inhibitor resulting in the accumulation of glycoproteins, hence causing ERS and initiating the UPR and apoptosis),  $\beta$ -D-xyloside (disrupts glycoprotein assembly and reversibly inhibits cell proliferation by arresting cells in the  $G_1$  phase of the cell cycle).

To evaluate the effects of these compounds on SK-N-BE(2) cells, distinctive characteristics previously reported on the cells such as morphological characteristics and their expression of P-gp were retrieved and integrated with experimental data obtained in this study, using tissue methodologies to evaluate cell growth and proliferation, multiplex fluorescence and luminescence assays for cell proliferation, viability and apoptosis, microscopic visualization of annexin-based fluorescence apoptosis and the Calcein-AM P-gp drug efflux assay.

Statistical analyses were performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)) or SigmaPlot version 13 (<https://systatsoftware.com/products/sigmaplot/>).

### 3.2 Morphology of SK-N-BE(2) Neuroblastoma Cells

The continuous SK-N-BE(2) neuroblastoma (NB) cell line was derived from a bone marrow biopsy taken from a 2-year old boy with disseminated (metastatic) NB after repeated courses of chemotherapy and radiotherapy.<sup>766-769</sup> SK-N-BE(2) cells can achieve a saturation density (confluence) in excess of  $1 \times 10^6$  cells/cm<sup>2</sup>. The morphology of the cells fluctuates with some cells exhibiting long processes and others assuming an epithelioid organization (Figure 3.1). In culture, the cells often aggregate and form dense clusters or, at high density, detach from the culture substratum to form floating clumps or suspensions.



Source: <https://www.lgcstandards-atcc.org/~media/45C32586E3974C539A8D3F5579EC5920.ashx>

**Figure 3.1:** Morphology of SK-N-BE(2) neuroblastoma cells

### 3.3 Expression of P-Glycoprotein in SK-N-BE(2) Neuroblastoma Cells

SK-N-BE(2) NB cells are known to overexpress the multidrug transporter, P-glycoprotein (P-gp) (Figure 3.2A).<sup>785</sup> Therefore, of particular relevance to the work presented in this thesis is that P-gp is indeed expressed in SK-N-BE(2) NB cells.<sup>786</sup> A recent study compared the expression of various members of the ATP-Binding Cassette (ABC) family of drug

transporters, including ABCC1 (*MRP1*), ABCC2 (*MRP2*), ABCC6 (*mrp6*), ABCC8 (*mrp8*), ABCC10 (*mrp10*), ABCC11 (*mrp11*), ABCC12 (*mrp12*) and ABCC13 (*mrp13*), and found that they were expressed either at similar or elevated levels in drug-resistant NB cell lines compared to parental controls (Table 3.1).<sup>786</sup>

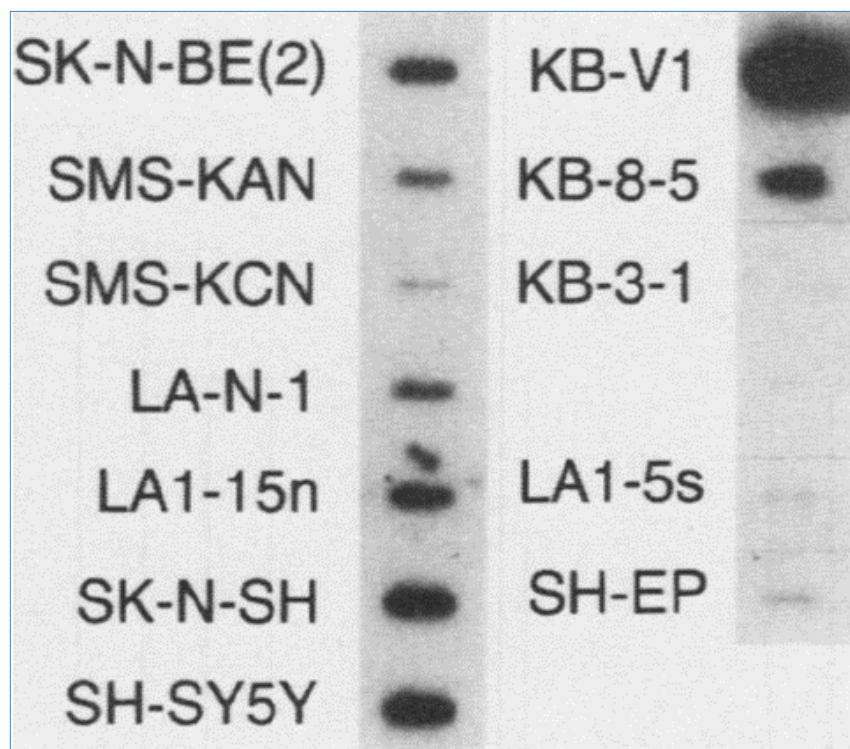
**Table 3.1:** Relative expression of known drug-resistance genes in neuroblastoma cell lines

Gene Symbol	Gene Entrez	DoxR		DoxR-v	
		SK-N-SH	SK-N-Be(2)C	SK-N-SH	SK-N-Be(2)C
ABCB1 ( <i>mdr1</i> )	5243	4.63	4.23	1.98	2.04
ABCB6 ( <i>prp</i> )	10058	1.68	1.81	1.57	1.81
ABCC3 ( <i>mrp3</i> )	8714	N.D.	-2.39	N.D.	N.D.
ABCC4 ( <i>mrp4</i> )	10257	2.17	2.12	N.D.	N.D.
ABCC5 ( <i>mrp5</i> )	10057	-1.63	N.D.	-2.26	-2.68
ABCC9 ( <i>mrp9</i> )	10060	-2.76	-4.15	-2.22	-2.89
BCL-2	596	1.53	N.D.	N.D.	N.D.
SIRT1	23411	1.75	2.02	N.D.	N.D.
BDNF	627	3.69	5.17	2.56	3.60
TH	7054	-1.87	-2.47	25.5	31.0

Relative expression of known drug-resistance genes in doxorubicin resistant (DoxR) and vorinostat-treated doxorubicin-resistant (DoxR-v) cells compared to the parental lines. Results are expressed as a fold-change (all  $p < 0.1$ ). N.D. indicates no difference in gene expression (fold-change  $< 1.5$  and/or  $p > 0.1$ ). The following genes had no significant difference in any comparison: ABCC1 (*MRP1*), ABCC2 (*MRP2*), ABCC6 (*mrp6*), ABCC8 (*mrp8*), ABCC10 (*mrp10*), ABCC11 (*mrp11*), ABCC12 (*mrp12*), ABCC13 (*mrp13*), MGMT, SOD, HDAC1-8. doi:10.1371/journal.pone.0040816.t001.

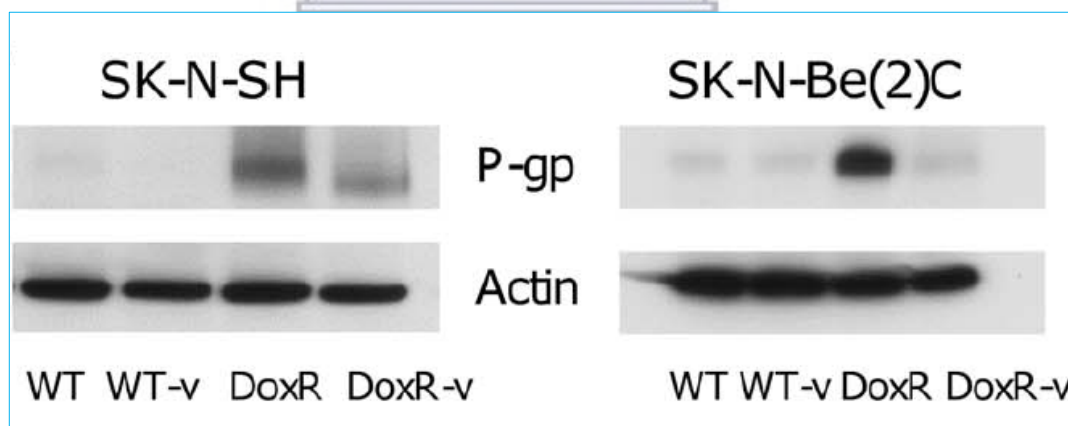
**Source:**<sup>764</sup> Lautz TB, Jie C, Clark S, Naiditch JA, Jafari N, Qiu YY, Zheng X, Chu F, Madonna MB. The effect of vorinostat on the development of resistance to doxorubicin in neuroblastoma. *PLoS One* 2012;7(7):e40816, with permission (This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3400660/>).

Western immunoblotting demonstrated significantly greater upregulation of P-gp in the SK-N-BE(2) subset doxorubicin-resistant (DoxR) SK-N-BE(2)C cells than the vorinostat-treated doxorubicin-resistant (DoxR-v) cells, relative to wild-type (WT) parental cells (Figure 3.2B).<sup>764</sup>



A. Expression of *mdrl/Pgp* in nine human neuroblastoma cell lines.

**Source:**<sup>786</sup> Bates SE, Mickley LA, Chen YN, Richert N, Rudick J, Biedler JL, Fojo AT. Expression of a drug resistance gene in human neuroblastoma cell lines: Modulation by retinoic acid-induced differentiation. *Molecular and Cellular Biology* 1989;9(10):4337-4344, with permission from the American Society for Microbiology (ASM), under the Creative Commons license and ASM Journals Public Access Policy ([http://journals.asm.org/site/misc/index\\_compliance.xhtml](http://journals.asm.org/site/misc/index_compliance.xhtml)).



B. Western immunoblotting of P-gp in doxorubicin-resistant (DoxR) and vorinostat-treated doxorubicin-resistant (DoxR-v) cells compared to wild-type (WT) cells.

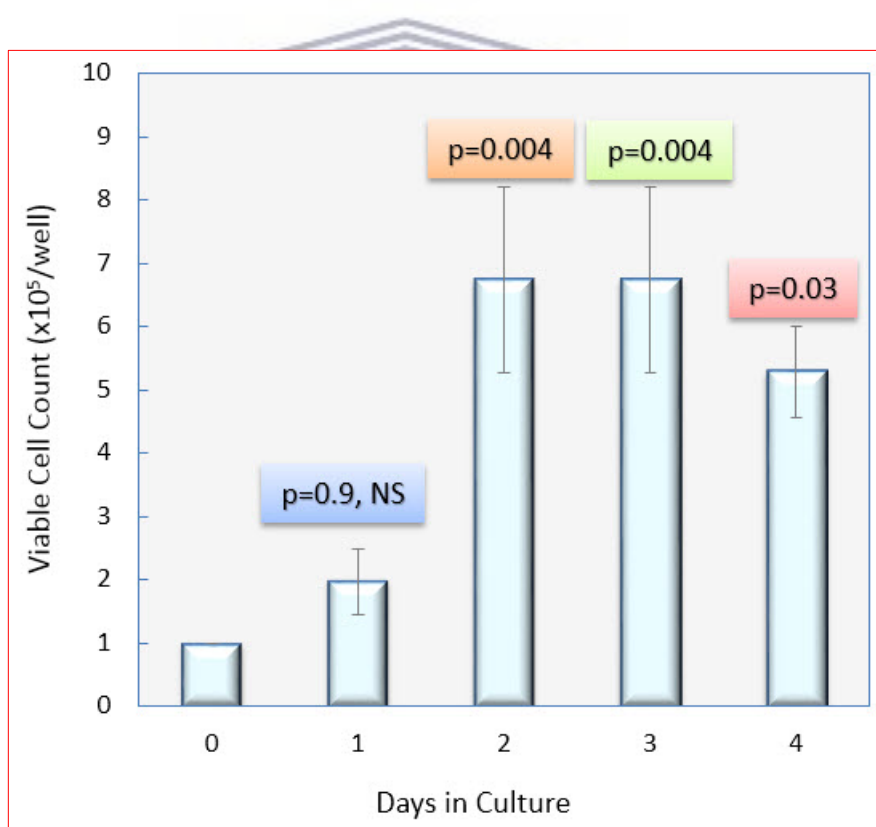
**Source:**<sup>764</sup> Lantz TB, Jie C, Clark S, Naiditch JA, Jafari N, Qiu YY, Zheng X, Chu F, Madonna MB. The effect of vorinostat on the development of resistance to doxorubicin in neuroblastoma. *PLoS One* 2012;7(7):e40816, with permission (This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3400660/>).

**Figure 3.2:** Upregulation of P-glycoprotein expression in neuroblastoma cell lines



### 3.4 Growth Curve Analysis of SK-N-BE(2) Neuroblastoma Cells

A cell viability growth curve for SK-N-BE(2) cells is shown in Figure 3.3. SK-N-BE(2) NB cells were seeded at a density of 100,000 cells per well onto 96-well plates and subsequently monitored over a 4-day period. Cell growth progressed through an initial log phase and then reached a plateau by days 2 and 3, after which time the viability began to decline. Viable cell counts were compared by one-way ANOVA with Dunnett's multiple comparisons post-hoc test using SigmaPlot version 13 (<https://systatsoftware.com/products/sigmaplot/>). The one-way ANOVA tests yielded p values as indicated for control vs days 1, 2, 3 and 4, respectively. On days 2, 3 and 4, the viable cell count of SK-N-BE(2) NB cells was significantly greater ( $p < 0.05$ ) than that of day 0 (Figure 3.3).



Values are means  $\pm$  SEM (n=4) of a representative experiment. One-way ANOVA (Dunnett's multiple comparison test) yielded p values as indicated for control vs days 1, 2, 3 and 4, respectively.

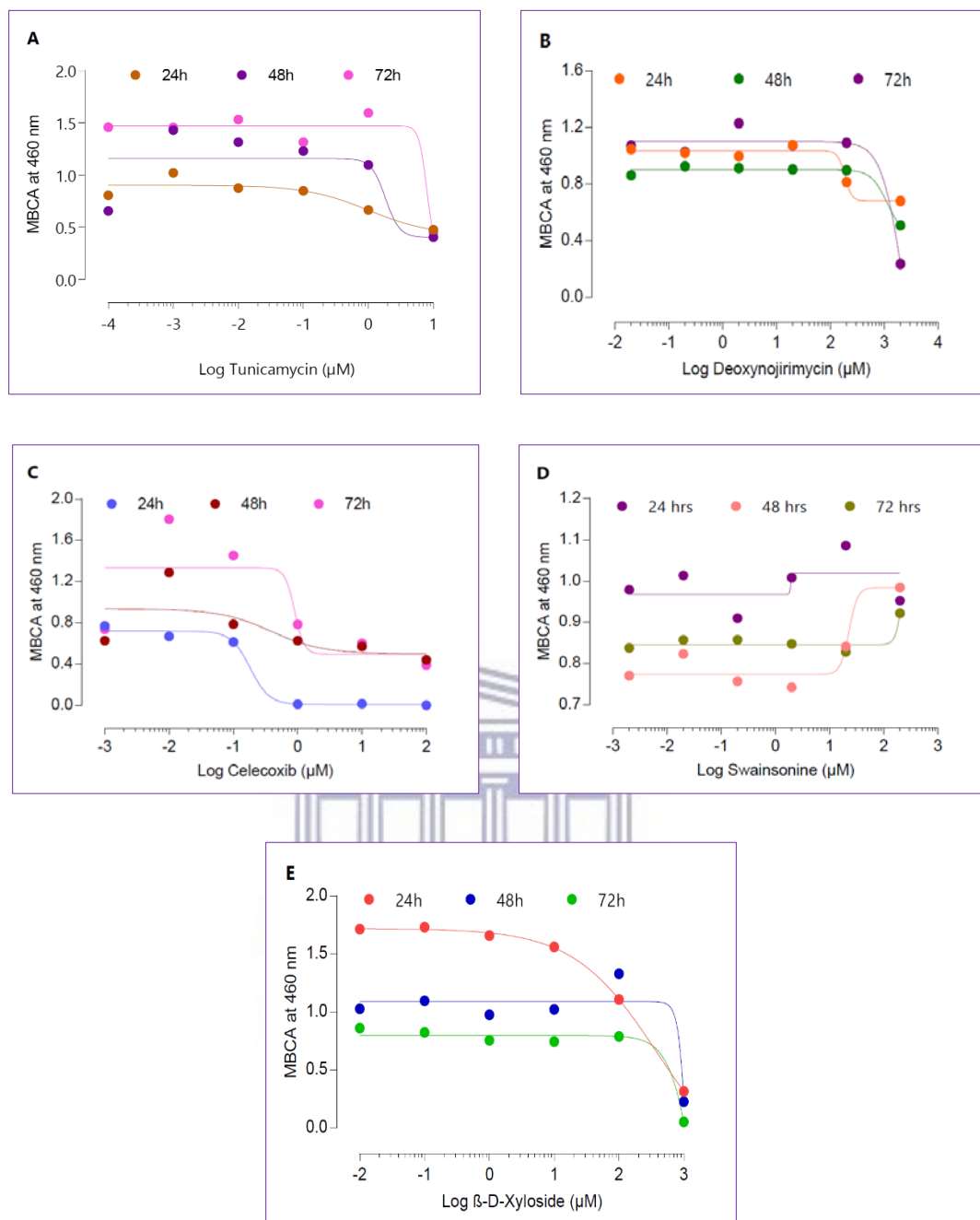
**Figure 3.3:** Growth curve analysis of SK-N-BE(2) neuroblastoma cells in culture

### 3.5 Cell Counting Kit-8 (CCK-8) Cell Viability Assays

SK-N-BE(2) NB cell proliferation was assessed by the CCK-8 cell viability assay as described in the research methodology (Chapter 2). Cells ( $5 \times 10^4$  cells/ml) were seeded in 96-well plates and incubated for 24h, 48h and 72h with incremental  $\log_{10}$  concentrations of tunicamycin, deoxynojirimycin, celecoxib, swainsonine and  $\beta$ -D-xyloside. Cell viability in the presence of different concentrations of these ERS inducers or glycoprotein processing inhibitors was determined by comparison with untreated control cells, i.e., the data points represent blank-corrected absorbances at 460 nm. Figure 3.4 shows the CCK-8 dose-response curves for test compounds assessed at varying concentrations using the GraphPad Prism 7 ([www.graphpad.com](http://www.graphpad.com)) four-parameter non-linear regression model with variable Hill slope. Table 3.2 summarizes the non-linear regression analysis data and validation parameters of the respective dose-response curves for the test compounds.

The half maximal inhibitory concentrations ( $IC_{50}$ ) of tunicamycin for SK-N-BE(2) NB cells after 24h, 48h and 72h exposure times were  $1.02 \mu\text{M}$  (95% CI: 0.0001 to 9181;  $R^2=0.87$ ),  $1.88 \mu\text{M}$  (95% CI: 0 to  $+\infty$ ;  $R^2=0.56$ ) and  $7.89 \mu\text{M}$  (95% CI: 0 to  $+\infty$ ;  $R^2=0.95$ ), respectively (Figure 3.4A and Table 3.2). A 95% CI of 0 to  $\infty$ , in the case of the 48h and 72h exposure times of SK-N-BE(2) NB cells to tunicamycin, implies that the non-linear dose-response curve did not completely converge in terms of the four-parameter logistic model (Figure 3.4A and Table 3.2). Judged by the estimated  $IC_{50}$ s, tunicamycin seems to be more potent at shorter exposure times (24h and 48h) in SK-N-BE(2) NB cells compared to the longer 72h exposure.

Deoxynojirimycin was also more potent after 24h ( $IC_{50}=186 \mu\text{M}$ , 95% CI: 0 to  $+\infty$ ;  $R^2=0.97$ ) compared to the 6-fold and 12-fold increases in  $IC_{50}$  values ( $1139 \mu\text{M}$  and  $2237 \mu\text{M}$ ) obtained for 48h and 72h, respectively (Figure 3.4B and Table 3.2).



SK-N-BE(2) neuroblastoma cells were exposed to log<sub>10</sub> increments of ERS inducer or glycoprotein processing inhibitor for the times indicated. Data points (blank-corrected absorbance at 460 nm) are the means of quadruplicate measurements (n=4), representing one of 3 independent experiments. The non-linear regression model used does not assume a standard slope, but rather fits the Hill slope from the data, and so is called a Variable slope model or a four-parameter dose-response curve, or four-parameter logistic curve, abbreviated as 4PL. MBCA, mean blank-corrected absorbance.

**Figure 3.4:** CCK-8 dose-response curves for test compounds assessed at varying concentrations

**Table 3.2:** Regression analysis data and summary of dose-response parameters

Drug ( $\mu\text{M}$ )	Exposure Time (Hours)	IC <sub>50</sub> ( $\mu\text{M}$ )	95% CI ( $\mu\text{M}$ )	R <sup>2</sup>
<b>Tunicamycin</b>	24	1.02	0.0001 to 9181	0.87
	48	1.88	0 to + $\infty$	0.56
	72	7.89	0 to + $\infty$	0.95
<b>Deoxynojirimycin</b>	24	186	0 to + $\infty$	0.97
	48	1139	0 to + $\infty$	0.98
	72	2237	0 to + $\infty$	0.96
<b>Celecoxib</b>	24	0.18	0.001 to 29.07	0.99
	48	0.39	2.43e-008 to 6.36e+006	0.47
	72	0.90	0 to + $\infty$	0.59
<b>Swainsonine</b>	24	1.93	0 to + $\infty$	0.20
	48	22.93	0 to + $\infty$	0.91
	72	227	0 to + $\infty$	0.88
<b><math>\beta</math>-D-Xyloside</b>	24	403	33.74 to 4819	0.99
	48	1334	0 to + $\infty$	0.89
	72	1914	0 to + $\infty$	0.98

IC<sub>50</sub>, half maximal inhibitory concentration of a drug estimated by the non-linear four-parameter logistic regression model;  $\infty$ , infinity symbol; 95% CI, 95 percent confidence interval; R<sup>2</sup>, regression coefficient (goodness of fit). All regression plots passed the test for homoscedasticity (equal variances, homogeneity of variance, i.e., same scatter across the independent variable around the regression line).

The estimated IC<sub>50</sub> of 0.18  $\mu\text{M}$  (95% CI: 0.001 to 29.07; R<sup>2</sup>=0.99) following 24h exposure of cells to celecoxib was also 2-fold (0.39  $\mu\text{M}$ , 95% CI: 2.43e-008 to 6.36e+006; R<sup>2</sup>=0.47, very poor non-linear regression convergence) and 5-fold (0.90  $\mu\text{M}$ , 95% CI: 0 to + $\infty$ ; R<sup>2</sup>=0.59), very poor non-linear regression convergence) lower than those obtained for the 48h and 72h exposure times (Figure 3.4C and Table 3.2). The potency of swainsonine at 24h (IC<sub>50</sub> of 1.93  $\mu\text{M}$ , 95% CI: 0 to + $\infty$ ; R<sup>2</sup>=0.20, very poor non-linear regression convergence) was also 12 times greater than at 48h (IC<sub>50</sub> of 22.93  $\mu\text{M}$ , 95% CI: 0 to + $\infty$ ; R<sup>2</sup>=0.91, very good non-linear regression convergence), and 118 times greater than at 72h (IC<sub>50</sub> of 227  $\mu\text{M}$ , 95% CI: 0 to + $\infty$ ; R<sup>2</sup>=0.88, moderate non-linear regression convergence; Figure 3.4D and Table 3.2). Likewise,

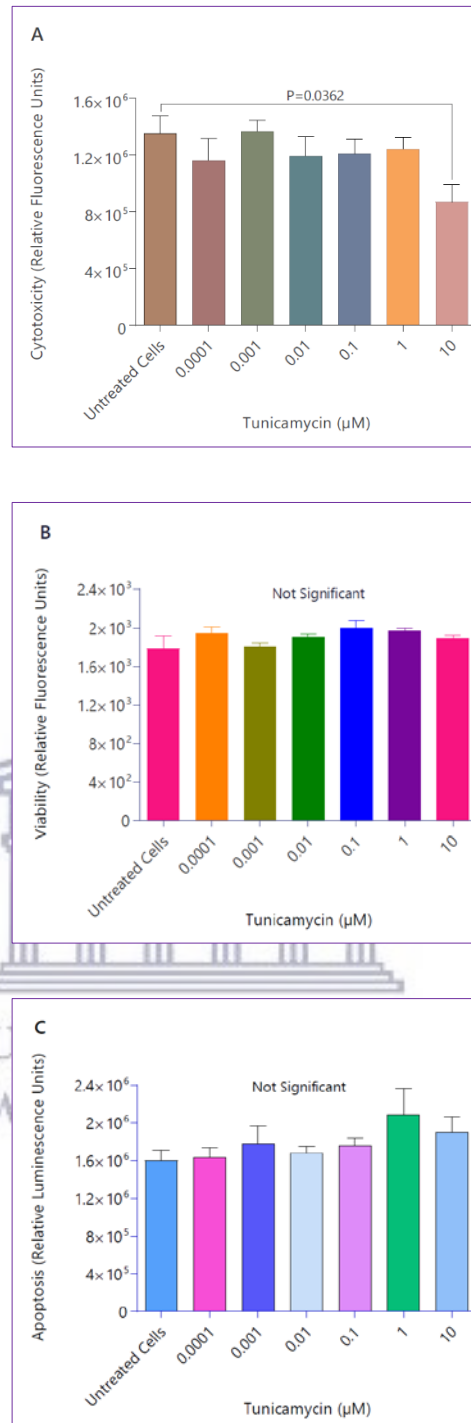
at 24h (IC<sub>50</sub> of 403 μM, 95% CI: 33.74 to 4819; R<sup>2</sup>=0.99, excellent non-linear regression convergence), β-D-xyloside exhibited a 3-fold greater potency than at 48h (IC<sub>50</sub> of 1334 μM, 95% CI: 0 to +∞; R<sup>2</sup>=0.89, good non-linear regression convergence) and a 5-fold greater potency than at 72h (IC<sub>50</sub> of 1914 μM, 95% CI: 0 to +∞; R<sup>2</sup>=0.98, excellent non-linear regression convergence; Figure 3.4E and Table 3.2).

### 3.6 Apotox-Glo™ Triplex Cell Cytotoxicity, Viability and Apoptosis Assays

The effects of tunicamycin, deoxynojirimycin, celecoxib, swainsonine and β-D-xyloside on SK-N-BE(2) neuroblastoma cell cytotoxicity, viability and apoptosis were measured using the Promega MultiTox-Fluor™ and Caspase-Glo® 3/7 Triplex assay kit according to the manufacturer's protocol. The results are presented in Figures 3.5 to 3.9 below and interpreted as follows:

The Triplex cell-based assay simultaneously measures three parameters—cell viability, cytotoxicity, and apoptosis. The method combines two fluorescent and one luminescent assay chemistries offered by Promega (Caspase-Glo® 3/7 and MultiTox-Fluor™ Assays) in the same assay well to extract information about viability, cytotoxicity and caspase activation events. These parameters are particularly useful to define mechanisms associated with a cytotoxic profile of an investigative compound.

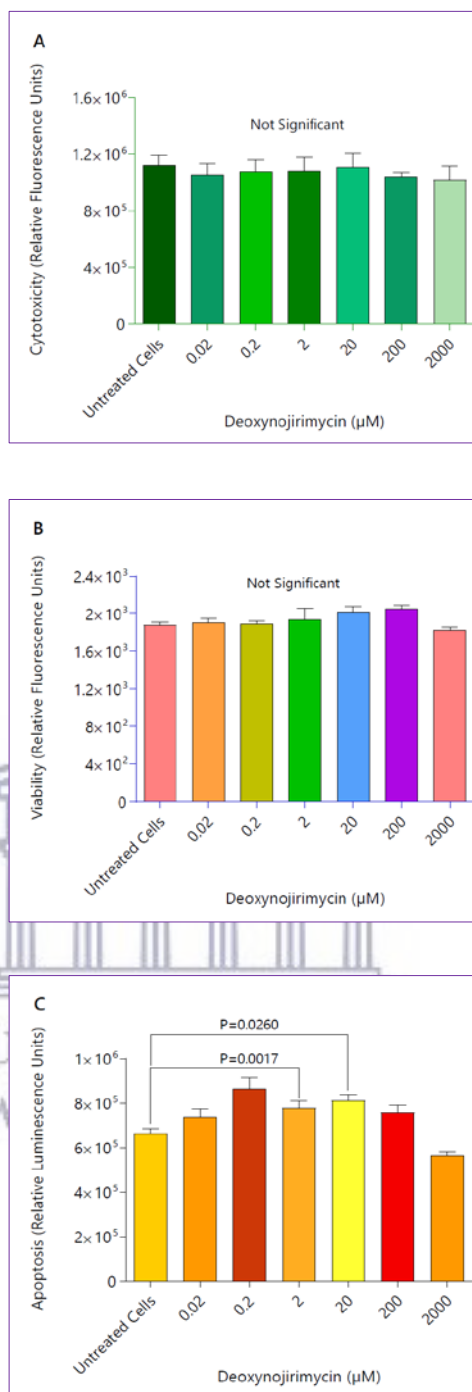
Tunicamycin at log<sub>10</sub> incremental concentrations of 0.0001 to 1 μM had no effect on cytotoxicity fluorescence in SK-N-BE(2) neuroblastoma cells, but at 10 μM the cytotoxicity fluorescence was decreased compared to untreated controls (p=0.0362, Figure 3.5A). No significant changes in viability fluorescence (Figure 3.5B) and apoptosis luminescence (Figure 3.5C) was demonstrated for tunicamycin in the log<sub>10</sub> concentration range of 0.0001 to 10 μM.



Values are means  $\pm$  SEM ( $n=4$ ) of a representative experiment. One-way ANOVA followed by Dunnett's multiple comparisons test yielded P values for comparison of treated vs untreated SK-N-BE(2) cells (vehicle-treated controls).

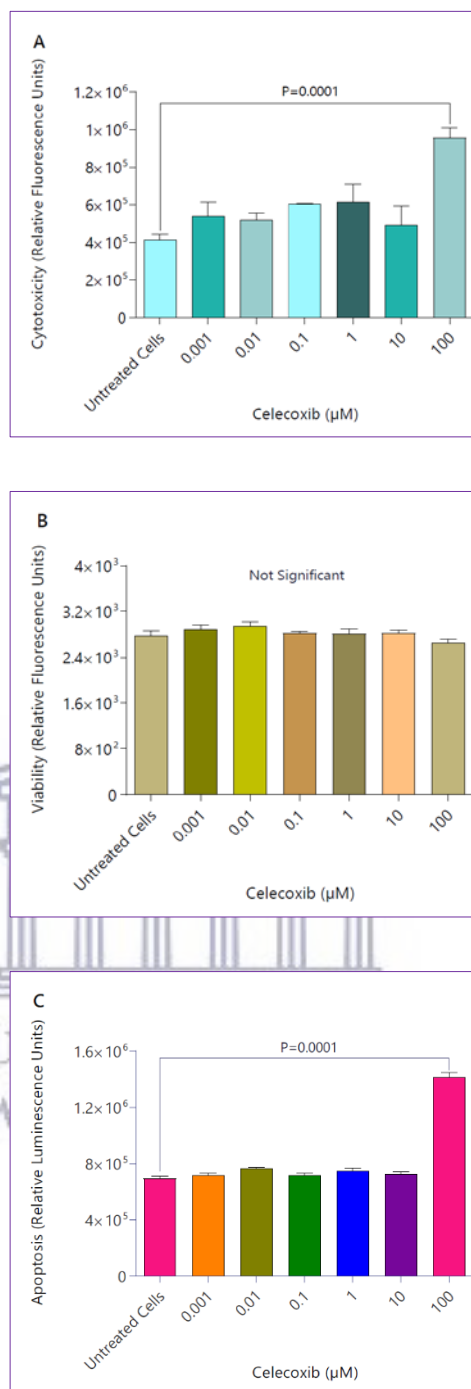
**Figure 3.5:** Effects of tunicamycin on SK-N-BE(2) cell cytotoxicity, viability and apoptosis





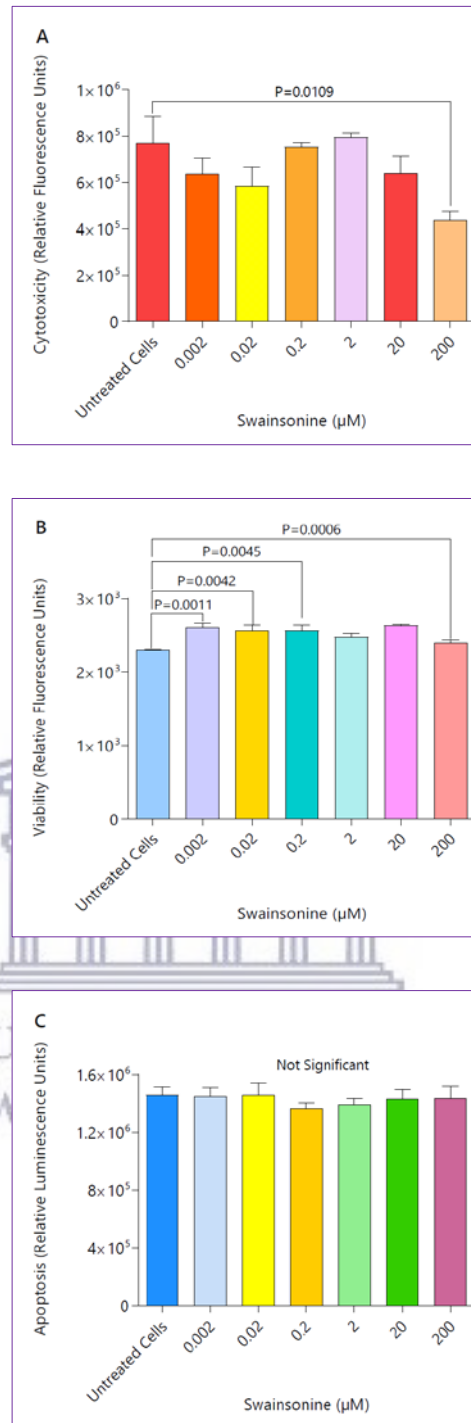
Values are means ± SEM (n=4) of a representative experiment. One-way ANOVA followed by Dunnett’s multiple comparisons test yielded P values for comparison of treated vs untreated SK-N-BE(2) cells (vehicle-treated controls).

**Figure 3.6:** Effects of deoxynojirimycin on SK-N-BE(2) cell cytotoxicity, viability and apoptosis



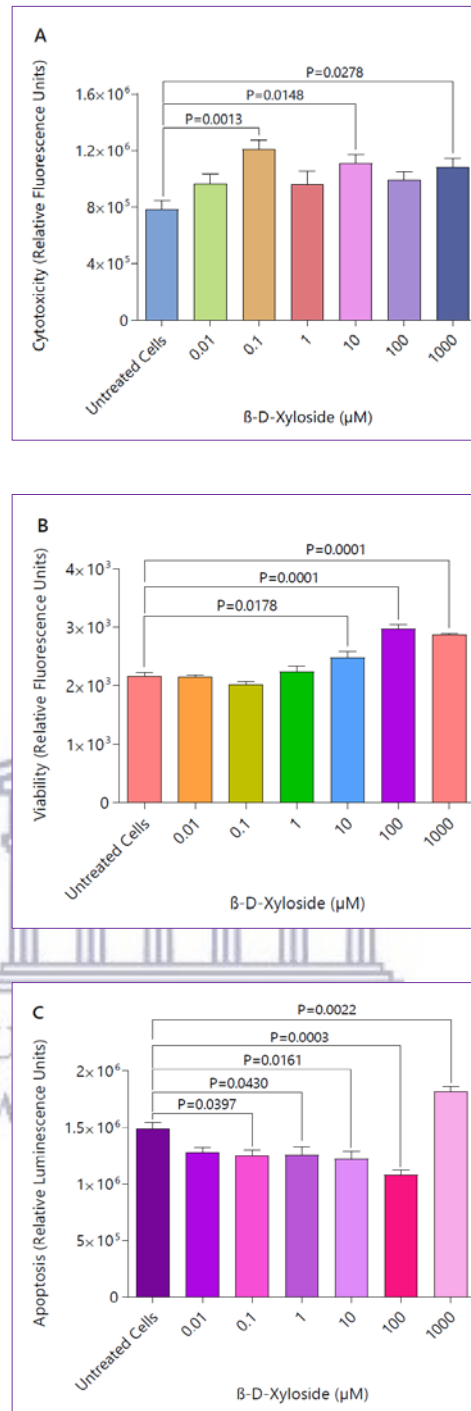
Values are means  $\pm$  SEM ( $n=4$ ) of a representative experiment. One-way ANOVA followed by Dunnett's multiple comparisons test yielded P values for comparison of treated vs untreated SK-N-BE(2) cells (vehicle-treated controls).

**Figure 3.7:** Effects of celecoxib on SK-N-BE(2) cell cytotoxicity, viability and apoptosis



Values are means  $\pm$  SEM ( $n=4$ ) of a representative experiment. One-way ANOVA followed by Dunnett's multiple comparisons test yielded P values for comparison of treated vs untreated SK-N-BE(2) cells (vehicle-treated controls).

**Figure 3.8:** Effects of swainsonine on SK-N-BE(2) cell cytotoxicity, viability and apoptosis



Values are means  $\pm$  SEM (n=4) of a representative experiment. One-way ANOVA followed by Dunnett's multiple comparisons test yielded P values for comparison of treated vs untreated SK-N-BE(2) cells (vehicle-treated controls).

**Figure 3.9:** Effects of  $\beta$ -D-xyloside on SK-N-BE(2) cell cytotoxicity, viability and apoptosis

Deoxynojirimycin in the  $\log_{10}$  concentration range 0.02 to 2000  $\mu\text{M}$  did not affect cytotoxicity and viability fluorescence in SK-N-BE(2) cells as compared with untreated controls (Figure 3.6A and B). However, deoxynojirimycin significantly increased apoptosis luminescence (Figure 3.6C) at concentrations of 2  $\mu\text{M}$  ( $p=0.0017$ ) and 20  $\mu\text{M}$  ( $p=0.0260$ ).

Likewise, celecoxib in the  $\log_{10}$  concentration range 0.001 to 10  $\mu\text{M}$  did not affect cytotoxicity fluorescence, but significantly increased it at 100  $\mu\text{M}$  ( $p=0.0001$ ) relative to untreated controls (Figure 3.7A).  $\log_{10}$  incremental concentrations of celecoxib between 0.001 and 100  $\mu\text{M}$  did not change cell viability fluorescence (Figure 3.7B), but at 100  $\mu\text{M}$ , apoptosis luminescence increased ( $p=0.0001$ ), most likely a reflection of the increased cytotoxicity seen at this concentration (Figure 3.7C).

According to Figure 3.8A, swainsonine in the  $\log_{10}$  concentration range 0.002 to 20  $\mu\text{M}$  had no cytotoxic effects on SK-N-BE(2) cells as compared with untreated controls, but at 200  $\mu\text{M}$  a drop in cytotoxicity fluorescence occurred ( $p=0.0109$ ). Swainsonine concentrations of 0.002  $\mu\text{M}$  ( $p=0.0011$ ), 0.02  $\mu\text{M}$  ( $p=0.0042$ ), 0.2  $\mu\text{M}$  ( $p=0.0045$ ) and 200  $\mu\text{M}$  ( $p=0.0006$ ) significantly increased viability fluorescence over that of untreated controls (Figure 3.8B), whereas swainsonine at all concentrations tested failed to induce apoptosis (Figure 3.8C).

In the case of  $\beta$ -D-xyloside, concentrations of 0.1  $\mu\text{M}$  ( $p=0.0013$ ), 10  $\mu\text{M}$  ( $p=0.0148$ ) and 1000  $\mu\text{M}$  ( $p=0.0278$ ) significantly increased cytotoxicity fluorescence in SK-N-BE(2) cells over that of untreated controls (Figure 3.9A).  $\beta$ -D-xyloside concentrations of 10  $\mu\text{M}$  ( $p=0.0178$ ), 100  $\mu\text{M}$  ( $p=0.0001$ ) and 1000  $\mu\text{M}$  ( $p=0.0001$ ) significantly increased viability fluorescence (Figure 3.9B) whereas at 0.1  $\mu\text{M}$  ( $p=0.0397$ ), 1  $\mu\text{M}$  ( $p=0.043$ ), 10  $\mu\text{M}$  ( $p=0.00161$ ) and 100  $\mu\text{M}$  ( $p=0.0003$ ) it decreased apoptosis luminescence (Figure 3.9C). However, at 1000  $\mu\text{M}$  ( $p=0.0022$ ), it increased apoptosis luminescence (Figure 3.9C).

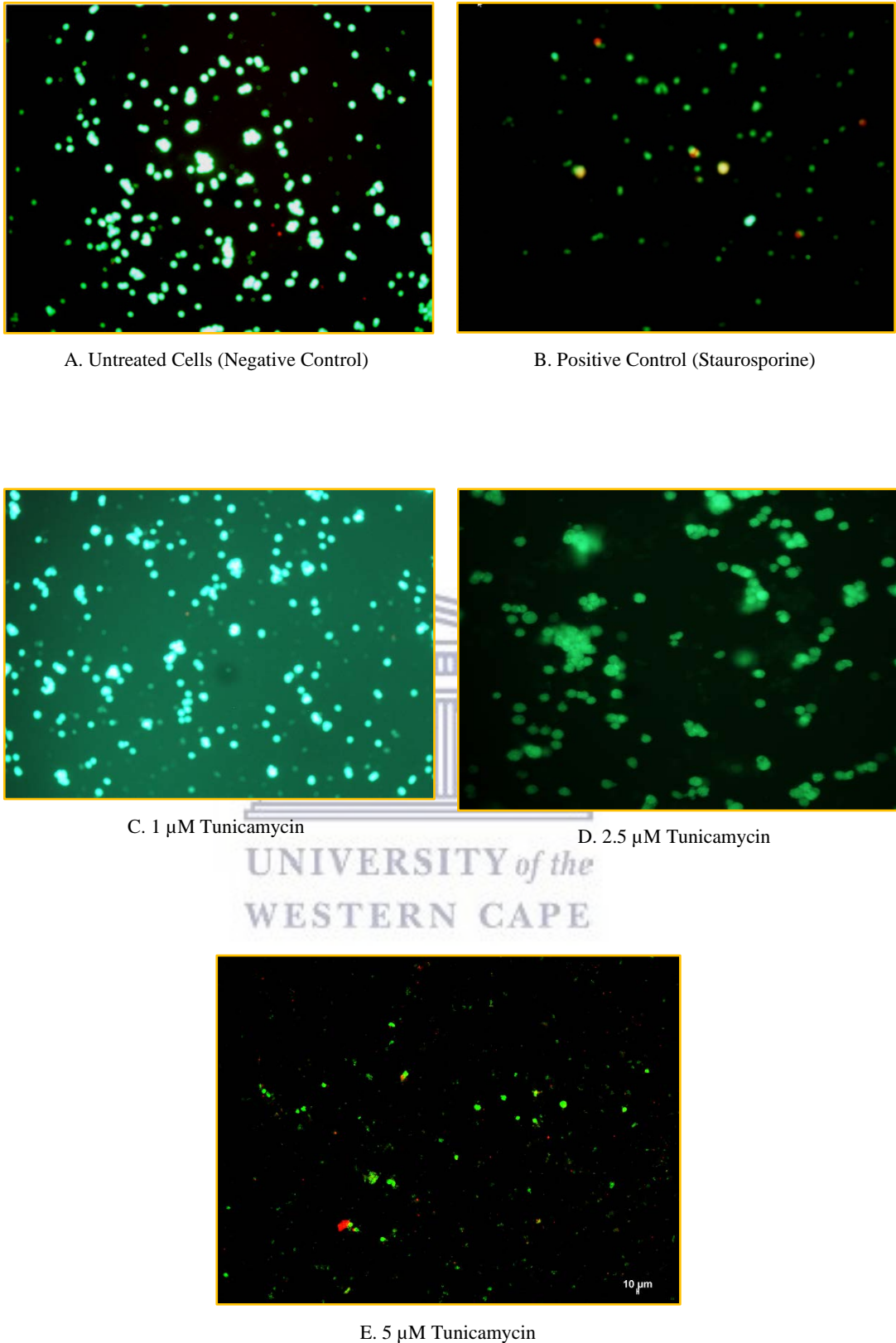
### 3.7 Annexin-V Cy3™ Apoptosis Assays

The Annexin-V Cy3™ Apoptosis Assay kit provides a rapid and convenient assay for apoptosis in cells. Annexin-Cy3 binds to phosphatidylserine (PS) in apoptotic cells and is visualized as red fluorescence. 6-Carboxyfluorescein diacetate (6-CFDA) is used to measure cell viability. Upon entering of 6-CFDA (non-fluorescent compound) into living cells, esterases cleave it, producing the fluorescent compound, 6-carboxyfluorescein (6-CF). This appears as green fluorescence. Cells are incubated either with Annexin-Cy3 or 6-CFDA separately or simultaneously. After labelling at room temperature, the cells are immediately observed by fluorescence microscopy. Live cells will be labelled only with 6-CF (*green*), while necrotic cells will label only with Annexin-Cy3 (*red*). Cells in the early stage of apoptosis, however, will be labelled with both red and green.

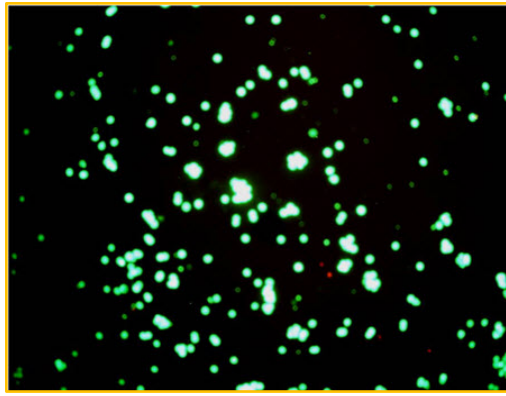
To induce apoptosis, SK-N-BE(2) cells were exposed for 24h to selected concentrations of tunicamycin (1, 2.5, 5  $\mu\text{M}$ ), deoxynojirimycin (200, 1200, 2400  $\mu\text{M}$ ), celecoxib (0.1, 0.4, 0.8  $\mu\text{M}$ ), swainsonine (2, 20, 200  $\mu\text{M}$ ) and  $\beta$ -D-xyloside (400, 1200, 2000  $\mu\text{M}$ ), using staurosporine as a positive control for apoptosis, as described in the research methodology (Chapter 2). Morphological observation of apoptosis induced by these compounds in SK-N-BE(2) cells was done by fluorescence microscopy and images were processed using Nikon Eclipse 50i software (IMP, Cape Town, South Africa, <http://www.imp.co.za/>).

Untreated SK-N-BE(2) cells displayed live cells (green fluorescence) as well as canonical apoptotic transformation as evidenced by various morphological changes, including apoptotic bodies, nuclear condensation and cell shrinkage, which were also observed under fluorescence microscopy, but very few necrotic (red fluorescence) cells (Figures 3.10A to 3.14A). The staurosporine apoptosis-positive control micrograph shows mostly live cells and very few green-and-red fluorescing cells (cells in the process of undergoing apoptosis) and some fully apoptosed cells (Figure 3.10B to 3.14B).

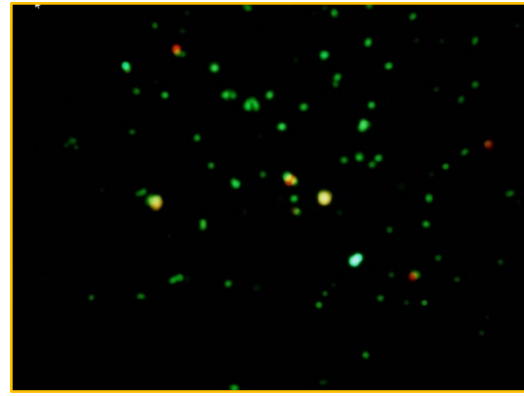




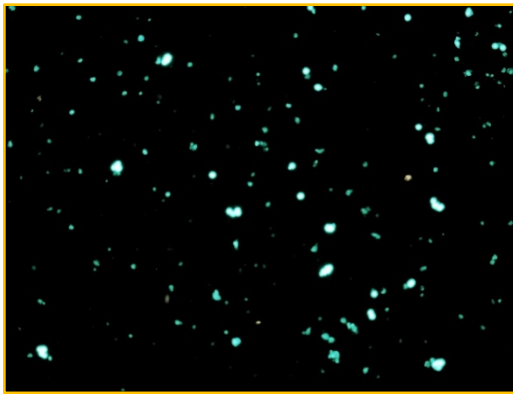
**Figure 3.10:** Fluorescence micrographs of the effects of tunicamycin on SK-N-BE(2) cell apoptosis



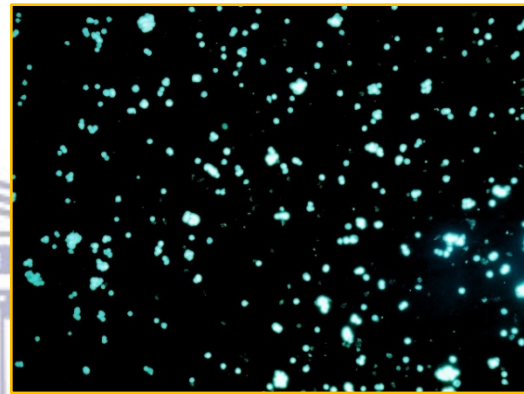
A. Untreated Cells (Negative Control)



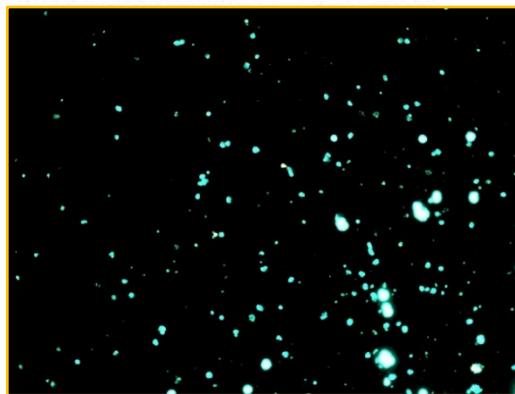
B. Positive Control (Staurosporine)



C. 200  $\mu$ M Deoxynojirimycin

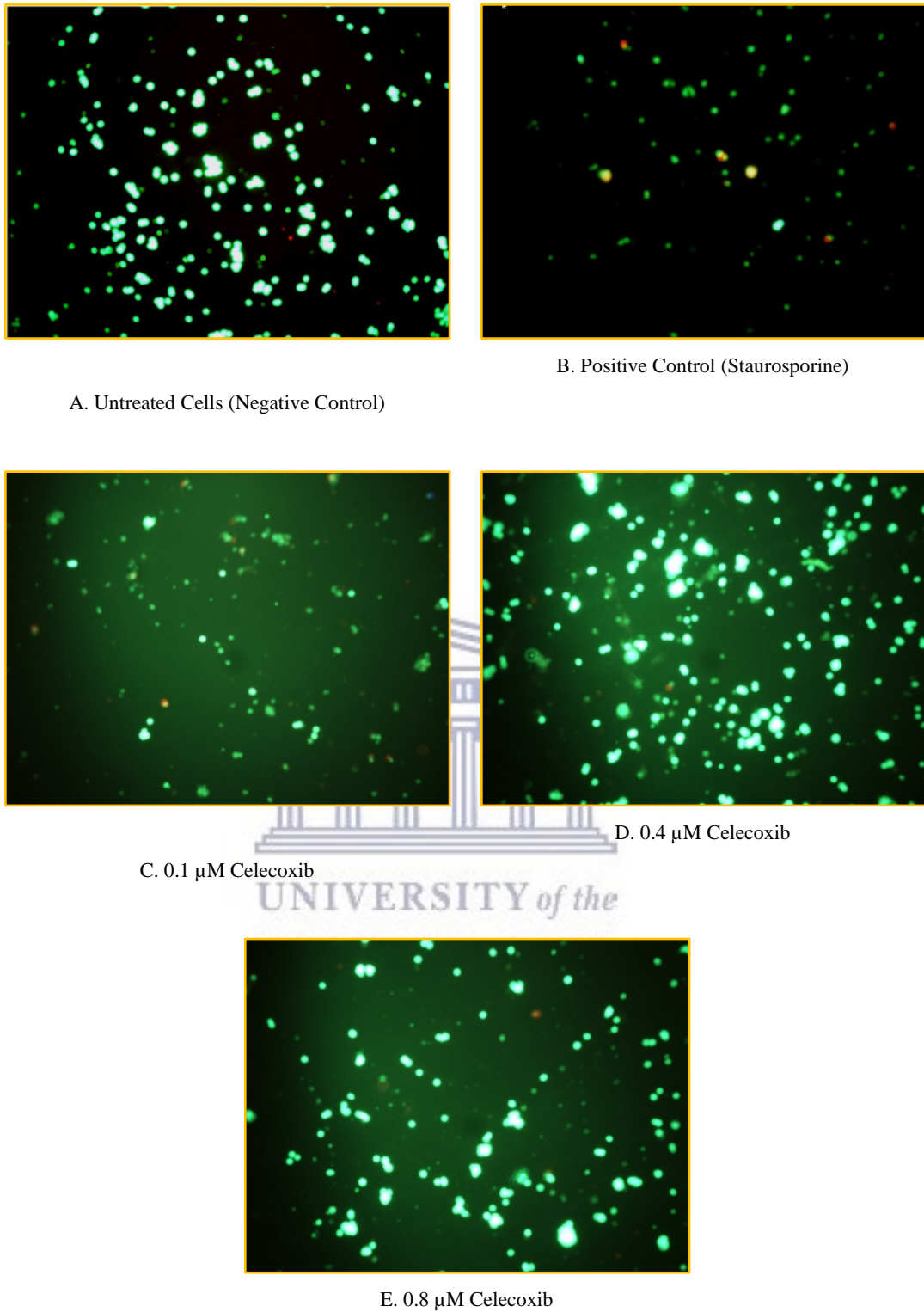


D. 1200  $\mu$ M Deoxynojirimycin

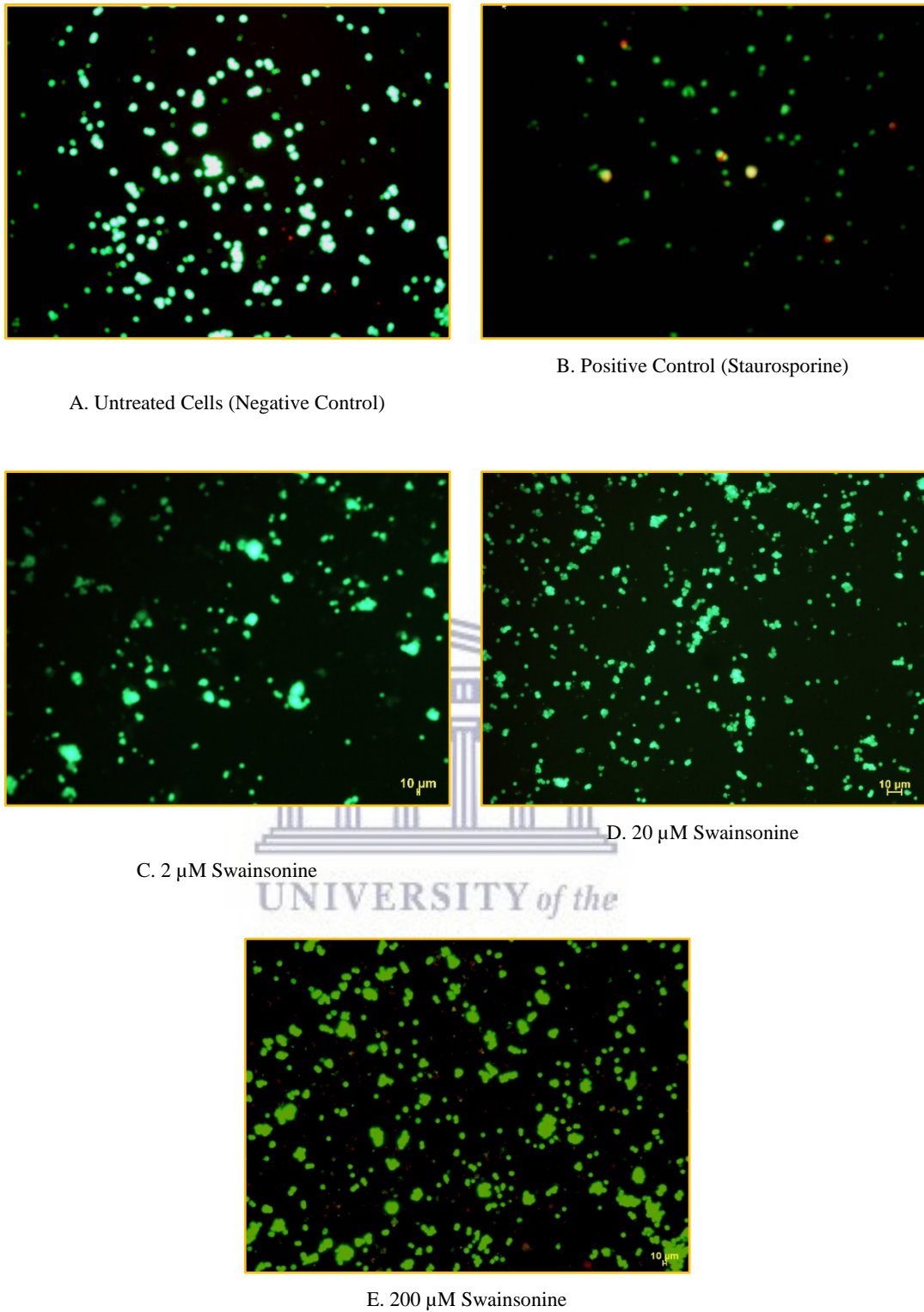


E. 2400  $\mu$ M Deoxynojirimycin

**Figure 3.11:** Fluorescence micrographs of the effects of deoxynojirimycin on SK-N-BE(2) cell apoptosis

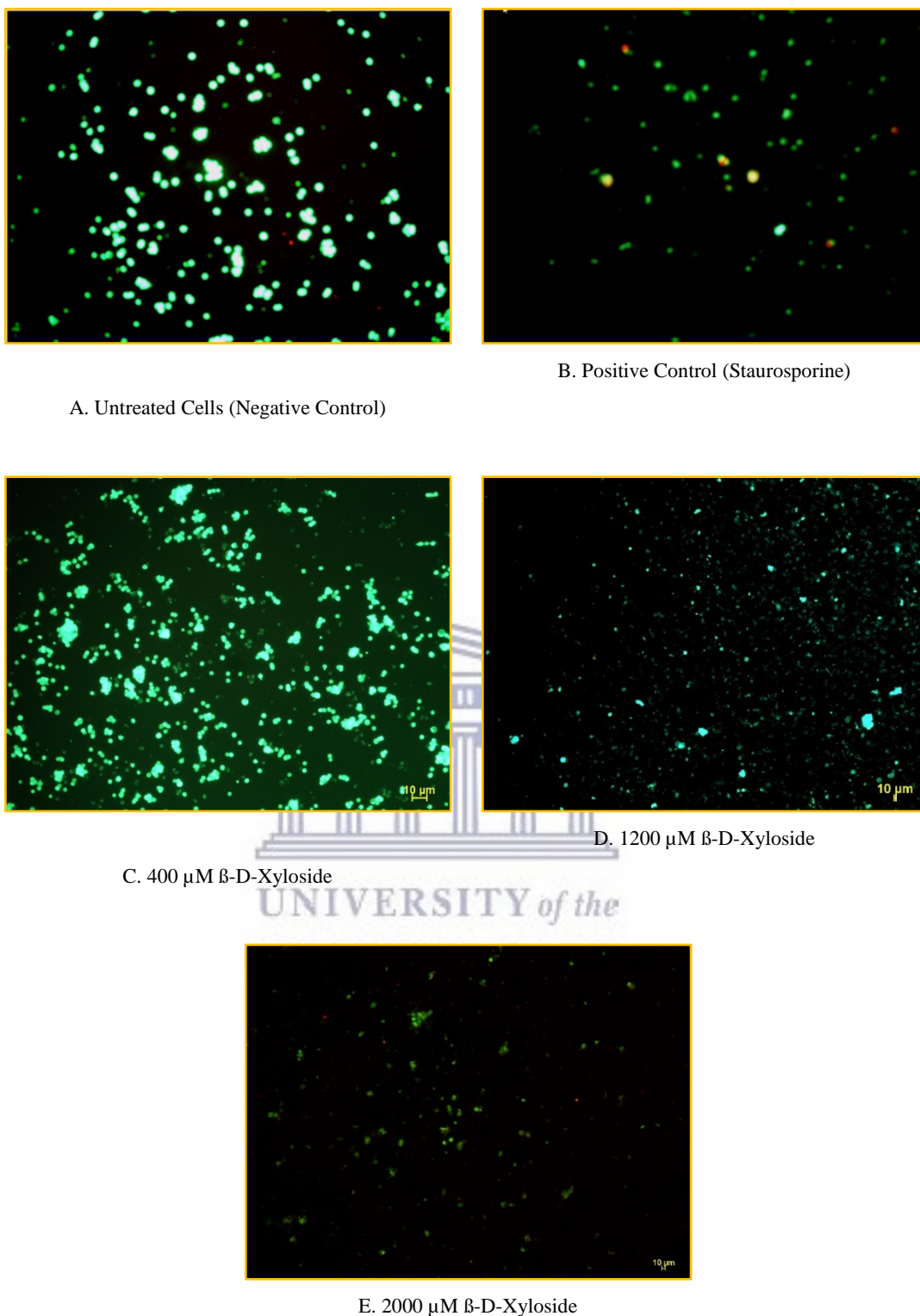


**Figure 3.12:** Fluorescence micrographs of the effects of celecoxib on SK-N-BE(2) cell apoptosis



**Figure 3.13:** Fluorescence micrographs of the effects of swainsonine on SK-N-BE(2) cell apoptosis





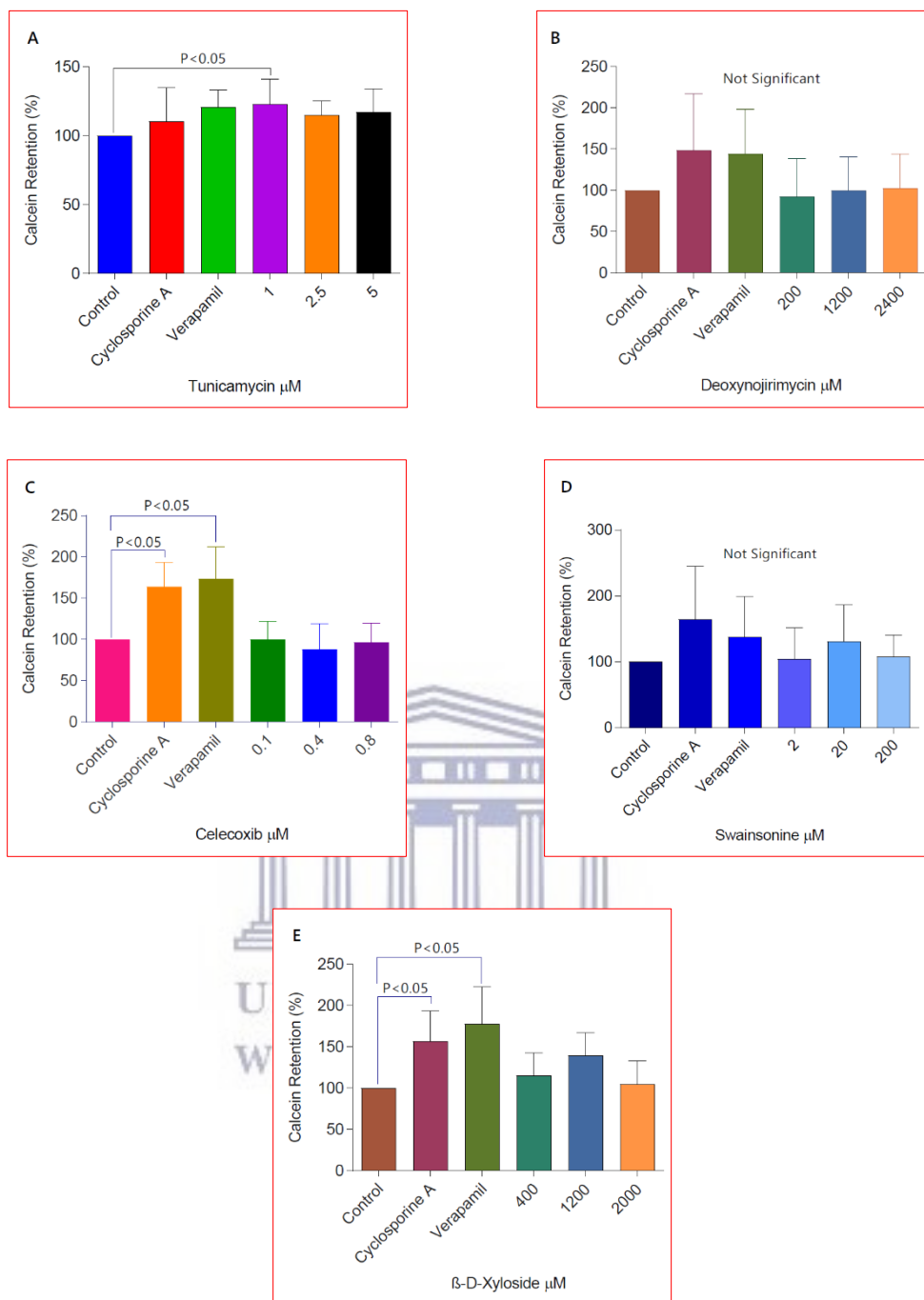
**Figure 3.14:** Fluorescence micrographs of the effects of  $\beta$ -D-xyloside on SK-N-BE(2) cell apoptosis

Figure 3.10 shows the fluorescence micrographs of the effects of tunicamycin on SK-N-BE(2) cell apoptosis. Cells treated with 1 and 2.5  $\mu\text{M}$  tunicamycin exhibited predominantly live cells (Figure 3.10C and D), but at 5  $\mu\text{M}$ , few apoptotic cells were visible (Figure 3.10E). Exposure of cells to 200, 1200 and 2400  $\mu\text{M}$  deoxynojirimycin yielded some distinguishing features of apoptosis, but did not show red fluorescence (Figure 3.11C-E). Celecoxib at 0.1  $\mu\text{M}$  did not cause significant apoptosis (Figure 3.12C), but at 0.4 and 0.8  $\mu\text{M}$  some signs of mitosis and sparse apoptosis was evident (Figure 3.12D and E). Swainsonine at 2, 20 and 2000  $\mu\text{M}$  seemed to induce a concentration-dependent cytoplasmic vacuolation (Figure 3.13C to E), a signature of paraptosis (a cell adaptation or death pathway independent of caspase activation and inhibition which further lacks major hallmark characteristics of apoptosis), but no visible red fluorescence (apoptosis). Similarly,  $\beta$ -D-xyloside at 400, 1200 and 2000  $\mu\text{M}$  did not produce any clear evidence of apoptosis in SK-N-BE(2) cells (Figure 3.14 C to E).

### 3.8 Measurement of P-Glycoprotein-Mediated Efflux Function

Calcein-acetoxymethylester (calcein-AM) was used as a neutral substrate to determine ABC transporter activity, i.e., P-glycoprotein (P-gp) efflux function on the basis of fluorescence.<sup>778-781,787</sup> The Cayman's Multi-Drug Resistance Assay Kit (Calcein-AM) was used as described in the research methodology (Chapter2). The rate of calcein accumulation in human MDR1-expressing cells is significantly lower relative to control cells, while various drug-resistance reversing agents (verapamil, vinblastine, oligomycin, cyclosporin A and MDR1-specific monoclonal antibodies) greatly increase calcein trapping only in the MDR1-expressing cells.<sup>779</sup> In this study, cyclosporin A—a competitive inhibitor—and verapamil—a non-competitive inhibitor—of P-gp, were included as positive controls. Representative results of calcein retention in SK-N-BE(2) cells treated for 24h with selected concentrations of tunicamycin (1, 2.5, 5  $\mu\text{M}$ ), deoxynojirimycin (200, 1200, 2400  $\mu\text{M}$ ), celecoxib (0.1, 0.4, 0.8  $\mu\text{M}$ ), swainsonine (2, 20, 200  $\mu\text{M}$ ) and  $\beta$ -D-xyloside (400, 1200, 2000  $\mu\text{M}$ ), relative to control, are summarized in Figure 3.15A to E).





The extent of calcein-AM retention was determined by fluorescence measurements using a plate reader. Data were transformed to percentages, with the control set as 100%. Values are means  $\pm$  95% CI ( $n=4$ ) of a representative experiment. One-way ANOVA followed by Tukey's multiple comparisons test yielded P values for comparison of treated vs control SK-N-BE(2) cells (vehicle-treated controls). Cyclosporin A, a competitive inhibitor, and verapamil, a non-competitive inhibitor of P-gp, were included as positive controls.

**Figure 3.15:** Effects of test compounds on P-glycoprotein function in SK-N-BE(2) cells

Exposure of SK-N-BE(2) cells to 1  $\mu$ M tunicamycin significantly increased the retention of calcein ( $p < 0.05$ ), i.e., it inhibited P-gp-mediated efflux of calcein from cells whereas cyclosporine A and verapamil (inhibitors of P-gp-mediated efflux function) did not (Figure 3.15A). In the case of deoxynojirimycin and swainsonine, none of the concentrations tested increased calcein retention and neither verapamil nor cyclosporine altered, as expected, the retention of this natural substrate probe of P-gp (Figure 3.15B and D). However, cyclosporine A and verapamil significantly ( $p < 0.05$ ) increased calcein accumulation in SK-N-BE(2) cells compared to untreated controls (Figure 3.15C and E), but celecoxib (Figure 3.15C) and  $\beta$ -D-xyloside (Figure 3.15E) at all concentrations failed to do so.

### 3.9 Summary

This chapter presented the results obtained in this study. The SK-N-BE(2) is a model for a continuous neuroblastoma (NB) cell line. In culture, these cells grow as epithelial-like monolayers, exhibiting cellular elongation processes. The cells form dense aggregates and, when overgrown or post-confluent, lift from the culture substratum to form floating clusters. SK-N-BE(2) NB cells, like most drug-resistant NB cell lines, overexpress the multidrug transporter, P-gp and various other members of the ATP-Binding Cassette (ABC) family of drug efflux pumps. Growth curve analysis of SK-N-BE(2) cells indicated that the cells conformed to canonical log-plateau-decline cell proliferation kinetics.

CCK-8 cell proliferation  $\log_{10}$  incremental dose-response assays for the N-glycosylation inhibitors and pharmacologic modulators of ERS and UPR signalling, were used to estimate their time-dependent (24, 48, 72h) half maximal inhibitory concentrations ( $IC_{50}$ ) or potencies according to the non-linear four-parameter logistic regression model for variable Hill slopes. These  $IC_{50}$  values were used to intuitively select concentrations of the test compounds for further assays of Annexin-V Cy3 apoptosis and measurement of P-gp-mediated efflux function in SK-N-BE(2) NB cells.

The Apotox-Glo™ Triplex (cell cytotoxicity, viability and apoptosis) assays showed that tunicamycin significantly reduced cytotoxicity fluorescence at 10  $\mu\text{M}$ , but at all concentrations of tunicamycin tested no effect on viability fluorescence and apoptosis luminescence could be demonstrated. Deoxynojirimycin also did not cause any changes in cytotoxicity and viability fluorescence in SK-N-BE(2) cells as compared with untreated controls, but intensified apoptosis luminescence at concentrations of 2 and 20  $\mu\text{M}$ . Celecoxib at 100  $\mu\text{M}$  increased cytotoxicity fluorescence and apoptosis luminescence, but concentrations of celecoxib in the range of 0.001 to 100  $\mu\text{M}$  did not alter cell viability fluorescence. At 200  $\mu\text{M}$ , swainsonine decreased cytotoxicity fluorescence and, remarkably over the entire concentration range of 0.002 to 200  $\mu\text{M}$ , it increased viability fluorescence compared to control, but no effect on apoptosis was noted.  $\beta$ -D-xyloside, concentrations of 0.1, 10 and 1000  $\mu\text{M}$  significantly increased cytotoxicity fluorescence in SK-N-BE(2) cells over that of untreated controls, while concentrations of 10, 100 and 1000  $\mu\text{M}$  significantly increased viability fluorescence whereas at 0.1, 1, 10 and 100  $\mu\text{M}$  decreased apoptosis luminescence. However, at 1000  $\mu\text{M}$ , it increased apoptosis luminescence.

Annexin-V Cy3 apoptosis assays revealed mostly live cells and occasional apoptotic cells for all the test compounds. Remarkably, swainsonine at 2, 20 and 200  $\mu\text{M}$  appeared to have caused a concentration-dependent cytoplasmic vacuolation, a sign of paraptosis (a physiological cell adaptation separate of caspase activation and inhibition and absence of the hallmarks of apoptosis).

In some cases, cyclosporin A and verapamil (inhibitors of P-gp-mediated drug efflux) did not produce the expected increased calcein retention in SK-N-BE(2) cells, and neither did deoxynojirimycin, celecoxib, swainsonine and  $\beta$ -D-xyloside at their selected concentrations. Only tunicamycin at 1  $\mu\text{M}$  significantly enhanced calcein uptake and retention in SK-N-BE(2) cells compared to untreated controls, i.e., at this concentration, P-gp-mediated efflux of calcein was blocked.

# CHAPTER 4

## CONCLUSION AND FUTURE DIRECTIONS

### 4.1 Introduction

Among all childhood cancers diagnosed, neuroblastoma (NB) is the most prevalent during infancy. In patients younger than 15 years, it accounts for more than 7% of malignancies and about 15% of all paediatric mortalities.<sup>16,64,788</sup> A family history of NB is considered a genetic predisposition and risk factor.<sup>104</sup> In 1-2% of inherited cases, the molecular causative factors linked to the disease are germline mutations, including anaplastic lymphoma kinase (*ALK*, gain of function),<sup>155,179</sup> *PHOX2B* (loss of function)<sup>156,163,167</sup> and *MYCN* (gain of function).<sup>4,154,789,790</sup>

NB differs from most solid tumours because of its heterogeneity in both pathobiologic and clinical behaviour,<sup>9</sup> ranging from spontaneous regression<sup>12</sup> to highly-aggressive metastatic disease resistant to conventional and investigational anticancer drugs.<sup>64</sup> Hence, NB embodies a spectrum of disease.<sup>6,791,792</sup> Moreover, disease progression (uncontrolled cellular proliferation), a recognizable signature of NB, may be precisely set off by inadequate targeting of resistant subpopulations (e.g., side-population cells or cancer stem cells/tumour-initiating cells).<sup>793-798</sup>

Accordingly, the complex molecular heterogeneity responsible for the disparate responses to treatment of NB provides a platform for discovering molecules and pathways for specific biologically-targeted interventions.<sup>111,398,799,800</sup> In recent years, the importance of targeting the NB glycome,<sup>496,527</sup> particularly glycosylation, has grown in intensity since glycans participate in nearly all physiological processes<sup>520,801</sup> and variations in glycome composition are associated with disease risk, disease course and responses to therapy.<sup>494,544,646,759,763</sup> This study focused on cell proliferation, viability, apoptosis and drug resistance.

## 4.2 Research Hypothesis and Objectives of the Study

In this study, the N-glycosylation inhibitors and ERS inducers—tunicamycin, deoxynojirimycin, celecoxib, swainsonine and  $\beta$ -D-xyloside—were evaluated for their effects on SK-N-BE(2) NB cell growth, viability, apoptosis and P-gp efflux function. It was hypothesized that N-glycosylation inhibitors and ERS inducers will alter the expression of SK-N-BE(2) cancer cell signatures evaluated, namely, cell survival (proliferation, viability and apoptosis) and P-gp-mediated drug efflux function. Based on the results of this study it can be accepted that, indeed, these investigative compounds altered the proliferation, viability, apoptosis and P-gp function in SK-N-BE(2) NB cells, but the conflicting and duality of effects imply that additional refinements are necessary in the conceptualization and execution of future studies.

## 4.3 Context and Significance of the Study

### 4.3.1 Neuroblastoma Multidrug Resistance, Endoplasmic Reticulum Stress and Glycosylation

In spite of contemporary improvements in intensive multimodal therapeutic approaches to NB (surgery, local radiation and differentiation therapy with 13-*cis*-retinoic acid, and myeloablative cytotoxic therapy with autologous haematopoietic stem cell transplantation),<sup>3,8,151</sup> the overall survival of high risk patients has persistently been less than 40%.<sup>260,802</sup> NB is a heterogeneous paediatric tumour that arises from the embryonic neural crest and it is therefore not surprising that cancer stem cells (CSCs) or tumour initiating cells (TICs) have been implicated in tumour relapse and resistance to chemotherapy in high-risk neuroblastoma (HR-NB) patients.<sup>7,131,294,792,793,803</sup> In 40-50% of the HR-NB cases, amplification of the *MYCN* gene remains the major key predictor of poor clinical outcomes. HR-NB patients with *MYCN* amplification generally show emergence of resistance to induction chemotherapy.<sup>6,260,392</sup> Thus, novel effective therapies are urgently needed to improve clinical outcomes of HR-NB patients. Remarkably, coordinated expression of the *MYC* gene and those

that encode for the ABC multidrug resistance transporters<sup>311,329,804,805</sup> has been confirmed in a larger number of NB patient samples, experimental NB models and NB cell lines, but more work is needed to clarify this poorly understood phenomenon.<sup>294,591,806,807</sup> Earlier work has found that even though high levels of ABCB1(P-glycoprotein/P-gp/MDR1) RNA are frequently associated with resistance to chemotherapy in NBs, many of the NB samples evaluated for *N-myc* amplification showed no correlation between *N-myc* copy number and the level of MDR1 mRNA expression.<sup>329,335</sup> However, a later study unequivocally provided evidence that *MYCN* enhances *P-gp/MDR1* gene expression in the human metastatic NB IGR-N-91 model.<sup>591</sup> Moreover, MDR in NB might be caused by multiple resistance mechanisms and a higher proliferation rate of NB cells presumably also implicate altered *MYCN* gene expression.<sup>808</sup>

CSCs in the heterogeneous NB tumour microenvironment (TME)<sup>147</sup> exhibit high resistance to standard therapy, a cancer hallmark<sup>144,146,148</sup> that correlates with overexpression of some members of the ABC family of MDR transporters, increased expression of anti-apoptotic molecules, increased DNA repair capacity, activation of specific stem cell survival signals (unlimited proliferative potential and prosurvival signalling, in particular, Notch, Hedgehog, Wnt, JAK/STAT, and others).<sup>809,810</sup> In recent years, the NB glycome (set of all glycans in an organism/tissue/cell, or even of a single glycoprotein)<sup>544,763</sup> or its study (glycomics)<sup>495,496</sup> has emerged as an important research paradigm that aims to show the significant impact of NB glycans in the TME on tumour progression, MDR and patient clinical responses to anticancer therapy.<sup>527,561,584</sup>

Thus, the glycome constitutes the entire array of free carbohydrates and glycoconjugates expressed on whole cells or tissues.<sup>811</sup> The glycans of the human N-glycome (i.e., proteins that are N-glycosylated), for instance, are connected to nearly all physiological processes and inter-individual variations in glycome composition have been associated with disease risk, disease course and responses to therapy.<sup>491,492,544,576,812-818</sup> The role of protein N-glycosylation in neural



transmission is widely recognized, but less well understood.<sup>819</sup> Glycosylation is the multifaceted post-translational modification of proteins and offers a landscape for developing novel targeted approaches to CSC eradication and anticancer chemotherapy.<sup>494,646,759,815,820</sup> Altered glycans on tumour- and host-cell surfaces and constituents of the extracellular matrix in the TME mediate key events in cancer pathogenesis, progression and survival.<sup>494,646,821,822</sup>

In addition, expression of tumour-associated glycans is involved in the dysregulation of glycozymes, which mainly comprise glycosyltransferases and glycosidases resident in the endoplasmic reticulum (ER) and Golgi apparatus.<sup>823</sup> Glycan-based therapeutic rationales have been applied to cancers of the liver, pancreas, breast, lung, gastrointestinal system, melanomas, and lymphomas, but rarely to NBs.<sup>494,814,818,820,824-826</sup> The success of anti-disialoganglioside (GD2, a glycolipid antigen) antibodies support glycan-based therapies for NB and also implies a proof-of-concept for protein glycosylation-based therapies for NB.<sup>11,151,296,306,527</sup>

Many membrane-resident and secreted proteins, including growth factors and their receptors are N-glycosylated.<sup>497</sup> The precursor N-glycan structure of 14 sugar residues (Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>) is initially synthesized in the ER as a branched structure on a lipid anchor (dolicholpyrophosphate) and then co-translationally en bloc transferred and linked via N-acetylglucosamine (GlcNAc) to asparagine (Asn) within a specific N-glycosylation acceptor sequence (Asn-X-Ser/Thr) of the nascent acceptor protein. In the ER and then the Golgi apparatus, the N-linked glycan structure is modified by hydrolytic trimming of sugar residues followed by re-glycosylation with additional sugar residues (processing) such as galactose, fucose or sialic acid in complex N-glycoproteins.<sup>497,643,671,827</sup>

N-glycosylation determines protein conformation, stability and function, degradation of misfolded proteins, programmed cell death (apoptosis), cell survival and immunity.<sup>666</sup> N-glycans are critical for proper protein folding and quality control by chaperones in the ER, a process now known as the calnexin/calreticulin cycle in the unfolded protein response (UPR)

and ER-assisted degradation (ERAD). The quality control system of the ER protein processing and folding capacity is vital for cellular homeostasis and survival.<sup>650,679</sup> Any disruption of this function of the ER to coordinate and maintain proper protein folding can lead to the ER stress (ERS) response.<sup>828</sup> Typical TME cellular imbalances such as nutrient deprivation, perturbations in Ca<sup>2+</sup>-flux, changes in redox status, aberrant post-translational processing (e.g., N-glycosylation) and abnormal synthesis and accumulation of secretory proteins can cause protein misfolding and trigger the ERS.<sup>684,829</sup> The ERS can be induced physiologically or experimentally by various agents.<sup>594,666,684,691</sup> Prolonged ERS triggers the UPR and eventually apoptosis.<sup>666,684,691</sup> However, cancer cells and their prosurvival TME usually adapt through various mechanisms, including epithelial-mesenchymal transition (EMT),<sup>148,830</sup> to evade ERS-induced apoptosis,<sup>684,831</sup> and so continue to proliferate disproportionately.

As indicated earlier, P-gp and many other ABC drug transporters are associated with resistance to chemotherapy in NB.<sup>589</sup> P-gp protects drug-resistant tumour cells from multiple forms of caspase-dependent apoptosis.<sup>832</sup> As a glycoprotein, P-gp is also subject to protein N-glycosylation. It has been reported that inhibition of N-linked glycosylation of P-gp by tunicamycin results in a reduced MDR phenotype.<sup>593,596</sup> Inhibitors of N-glycosylation and oligosaccharide-processing have been widely used to delineate the significance of glycosylation patterns of cell surface glycoproteins and glycolipids in the malignant phenotype.<sup>594,638</sup> Consequently, targeting of the ERS and UPR has been the subject of intense investigation in recent years.<sup>661,662,664,676,693-698</sup>

Interestingly, primary patient-derived NB cell lines, e.g., the SK-N-SH cell line, have been shown to be quite heterogeneous in terms of their constituent cell types. This cell line is composed of side population cells with cancer stem cell-like properties.<sup>798</sup> SK-N-BE(2) NB cells, selected for this study, also overexpress P-gp.<sup>785,786</sup> Various members of the ABC family of drug transporters, including ABCC1 (*MRP1*), ABCC2 (*MPR2*), ABCC6 (*mrp6*), ABCC8 (*mrp8*), ABCC10 (*mrp10*), ABCC11 (*mrp11*), ABCC12 (*mrp12*) and ABCC13 (*mrp13*), were

found to be expressed either at equal or raised levels in drug-resistant NB cell lines compared to parental controls.<sup>786</sup> Another recent study demonstrated significantly greater upregulation of P-gp in the SK-N-BE(2) subset doxorubicin-resistant (DoxR) SK-N-BE(2)C cells than the vorinostat-treated doxorubicin-resistant (DoxR-v) cells, relative to wild-type (WT) parental cells.<sup>764</sup> A fundamental tenet of the present study is that, transcriptionally, MYCN (a hallmark of NB) directly regulates the ABC transporters,<sup>328</sup> and their upregulation correlates with poor prognosis.<sup>327,329</sup> Most aggressive NBs exhibit MDR,<sup>311</sup> attributable to p53 mutations and/or a loss of function induced during chemotherapy,<sup>331</sup> which promote relapse.<sup>333,334</sup> p53 is a stress protein activated in response to various stresses and, in different contexts, the activation of p53 can either keep cells alive (the survival pathway) or kill the cells (the death pathway).<sup>833</sup>

In the current study, selected glycosylation inhibitors and ERS inducers—tunicamycin (an antibiotic that blocks the rate-limiting step of protein N-glycosylation and induces ERS and apoptosis in cells), deoxynojirimycin (inhibits Golgi mannosidases and thus maturation of glycoproteins, causing ERS), celecoxib (a potent cyclooxygenase 2 inhibitor, known to impede sarcoplasmic/endoplasmic reticulum calcium ATPase/SERCA) and so triggering relentless Ca<sup>2+</sup> dysregulation that can induce ERS-mediated apoptosis), swainsonine (a glycosidase inhibitor resulting in the accumulation of glycoproteins, hence causing ERS and initiating the UPR and apoptosis), β-D-xyloside (disrupts glycoprotein assembly and reversibly inhibits cell proliferation by arresting cells in the G<sub>1</sub> phase of the cell cycle)—were tested for their potential to inhibit P-gp-mediated drug transport function and drug resistance as well as their efficacy to induce apoptosis in the SK-N-BE(2) NB cell line. The effects of the compounds are discussed below in the context of work that had already been done in this field.

### 4.3.2 Tunicamycin

Tunicamycin is a potent inhibitor of N-linked glycosylation and blocks the initial step of glycoprotein synthesis, thus inhibiting global cellular synthesis of N-linked glycoproteins.<sup>704</sup> Since N-linked glycosylation occurs in the ER,<sup>755,834</sup> perturbation of this highly regulated post-

translational modification, which is involved in several biological processes such as protein folding and conformation, oligomerization, sorting, cell-cell interactions, results in the accumulation of aberrant or misfolded proteins which can lead to ERS and induce the UPR to activate apoptosis.<sup>685,835,836</sup> Glycosylation regulates functions of frequently mutated oncogenes and tumour suppressors.<sup>710</sup> Inhibition of protein glycosylation reverses the MDR phenotype of cancer cell lines.<sup>593</sup> In this study, the CCK-8 assay and the estimated IC<sub>50</sub>s for tunicamycin revealed greater potency at shorter exposure times (24h and 48h) in SK-N-BE(2) NB cells compared to the longer 72h exposure.

However, using the Promega Caspase-Glo® 3/7 and MultiTox-Fluor™ Triplex assay which simultaneously measures three parameters—cell viability, cytotoxicity and apoptosis—tunicamycin did not produce significant changes in viability and apoptosis in SK-N-BE(2) NB cells relative to untreated controls. Exposure of SK-N-BE(2) cells to 1 µM tunicamycin significantly increased the retention of calcein, i.e., it inhibited P-gp-mediated efflux of calcein from cells.

Classical studies have shown that tunicamycin inhibited glycosylation by 80-90%, but reduced protein biosynthesis by only 10-20% in cultured NB cells and that glycosylation is required for maintenance of functional sodium channels in NB cells.<sup>837</sup> Glycosylation inhibition by tunicamycin in mouse neuroblastoma N18 cells correlated with a marked decrease in glycoprotein degradation.<sup>838</sup>

Tunicamycin has been demonstrated to potentiate drug cytotoxicity and vincristine retention in multidrug resistant cancer cell lines.<sup>597,839</sup> Similarly, inhibition of N-linked glycosylation of P-gp by tunicamycin downregulated the MDR phenotype<sup>596,840</sup> and enhanced sensitivity to cisplatin in human head-and-neck carcinoma cells.<sup>626</sup> Tunicamycin specifically enhances the antitumour activity of trastuzumab on breast cancer *in vitro* and *in vivo*.<sup>708</sup> Impairment of N-linked glycosylation by tunicamycin impedes ALK phosphorylation and pro-survival

signalling in NB cell lines.<sup>556</sup> From these studies it can be concluded that N-glycosylation of P-gp is required to establish and maintain the cellular MDR phenotype. However, tunicamycin has also been reported to reduce P-gp glycosylation without an effect on its membrane localization and drug efflux activity in L1210 mouse lymphocytic leukaemia cells.<sup>599</sup>

A current study confirmed that upon treatment of human lung adenocarcinoma cell lines (A549 and H358) with 2-deoxyglucose, ionomycin, bortezomib and tunicamycin, strong activation of ERS and EMT was induced.<sup>830</sup> ERS and EMT are concomitant events that confer a prosurvival advantage on cancer cells, i.e., providing cancer stemness, MDR and invasiveness.<sup>144,293,830</sup> EMT is a process in which cells lose epithelial characteristics and gain mesenchymal (migratory, metastatic) properties.<sup>796</sup>

The acquisition of EMT capability underscores the aggressive behaviour of many cancers, including NB,<sup>7</sup> and explains the high relapse and progression rates presented by first-line chemotherapy agents.<sup>841</sup> Emerging evidence implies complex links between EMT-type cells and MDR in tumours.<sup>842,843</sup> In recent years, it has been acknowledged that upon EMT, alterations in cell surface glycosylation occur in malignant and premalignant epithelia and that these glycomic changes can direct the signals elicited by endogenous carbohydrate binding proteins such as galectins, selectins or siglecs.<sup>817,844</sup> Moreover, galectin-1 (Gal-1) has gained distinction as an emerging target in NB translational therapeutics.<sup>494,527</sup>

Aberrant expression of glycans has been observed in several EMT models, and the functional roles of such glycans in cancer development and progression are presently being investigated.<sup>845</sup> Alterations in protein glycosylation are thus key elements of oncogenesis and have been shown to influence cancer cell behaviour by perturbing cell adhesion, promoting cell migration and metastasis.<sup>554,846</sup> Finally, the neural crest is a transient embryonic cell population characterized by its multipotency and migratory ability—and NB cells fit the classical neural crest model of EMT and its sequelae.<sup>139,494</sup>

### 4.3.3 Deoxynojirimycin

N-linked glycans extrinsically boost glycoprotein folding capacity by utilizing the glycoprotein homeostasis or “glycoproteostasis” network, comprising numerous glycan binding and/or modification enzymes or proteins that synthesize, transfer, trim and utilize N-linked glycans to direct folding vs degradation, and trafficking of nascent N-glycoproteins through the cellular secretory pathway. If protein maturation is perturbed by misfolding and/or aggregation, ERS pathways are activated, resulting in transcriptional remodelling of the secretory pathway, in an attempt to alleviate the insult(s).<sup>847</sup> Deoxynojirimycin is an inhibitor of  $\alpha$ -glucosidases and mannosidases involved in glycoprotein processing and can thus induce ERS, the UPR and apoptosis if the glycoproteostasis network is disrupted.<sup>497,602,848-850</sup>

In this study, deoxynojirimycin significantly increased apoptosis in SK-N-BE(2) cells, but did not alter P-gp function. Recently, a derivative of deoxynojirimycin has been shown to inhibit angiogenesis, reduce secretion of matrix metalloproteinases-2 (MMP-2) from bovine aortic endothelial cells and diminish cell motility.<sup>621</sup> Likewise, deoxynojirimycin also inhibited metastasis of B16F10 melanoma cells by attenuating the activity and expression of MMP-2 and -9 and altering cell surface glycosylation.<sup>623</sup> These effects may have direct applications in targeting aggressive NB metastases.

### 4.3.4 Celecoxib

Abundant evidence suggests that the widely prescribed nonsteroidal anti-inflammatory drug (NSAID) and cyclo-oxygenase-2 (COX-2) inhibitor, celecoxib, possesses antitumour activity, in particular, by sensitizing cancer cells to apoptosis, possibly by perturbing intracellular calcium via inhibition of ER  $\text{Ca}^{2+}$ -ATPases and inducing ERS.<sup>666,676,726,851,852</sup> In this study, certain concentrations of celecoxib significantly increased cytotoxicity and apoptosis in SK-N-BE(2) cells compared with untreated controls. It has been shown that celecoxib or its analogue, 2,5-dimethyl-celecoxib,<sup>722</sup> in combination with bortezomib, induces aggravated ERS (by inducing CHOP and GRP78 expression)<sup>726</sup> and apoptosis,<sup>723</sup> as a basis for enhanced



glioblastoma cell killing.<sup>701</sup> In HepG2 hepatoma cells, celecoxib increased the accumulation of doxorubicin and enhanced the sensitivity of the cells to doxorubicin by tenfold. The induction of MDR1 (P-gp) expression by PGE<sub>2</sub> and its downregulation by COX-2 knockdown imply that the enhanced sensitivity of HepG2 cells to doxorubicin by celecoxib is mediated by the downregulation of MDR1 expression and AP-1 (activator protein-1), a transcription factor involved in cellular proliferation, transformation and death.<sup>853</sup> BRAF is known to induce ABCB1/P-gp expression in cells and AP-1 activity is crucial in this mechanism, which may be targeted by celecoxib to impede disease progression and occurrence of resistance in cancers.<sup>854,855</sup> Targeting of COX-2 was confirmed as an efficacious approach to control P-gp.<sup>856</sup>

Also, combination of celecoxib with other anticancer drugs might lead to ERS aggravation and parallel increases in cell death because of the inability of cells to alleviate the proteotoxic insult of accumulated misfolded or unfolded proteins.<sup>687,726</sup> A recent multi-institutional phase II study that evaluated the efficacy of a 5-drug oral regimen (celecoxib, thalidomide and fenofibrate, with alternating 21-day cycles of low-dose cyclophosphamide and etoposide) in children with recurrent or progressive cancers reported that oral celecoxib prevented the progression of NB.<sup>857</sup> Additionally, a previous study demonstrated that celecoxib combined with irinotecan inhibits the growth of neuroblastoma xenografts through induction of apoptosis and suppression of angiogenesis with decreased VEGF expression,<sup>858</sup> effects corroborated in recent studies.<sup>278</sup>

However, it should be noted that NSAIDs like aspirin, celecoxib, diclofenac, ibuprofen, indomethacin, naproxen, nimesulide, piroxicam, and sulindac have been reported to cause stomach damage<sup>859</sup> and hepatotoxicity with separate characteristics, varying from elevation of transaminases, cholestasis and hepatitis, to acute liver failure in some cases.<sup>687,860</sup> Clearly, celecoxib is a paradigmatic selective inhibitor of COX-2 with potent antitumour activity in a wide variety of human epithelial cancers and may be of specific value for the treatment of apoptosis-resistant tumours, although special caution is necessary in its clinical use.<sup>721</sup>

### 4.3.5 Swainsonine

Swainsonine, an indolizidine alkaloid, hinders the processing of N-linked glycoproteins in both cell-free extracts and mammalian cells in culture.<sup>861,862</sup> In the present study, swainsonine in the concentration range used had no cytotoxic or apoptotic effects on SK-N-BE(2) cells as compared with untreated controls. Likewise, swainsonine did not affect P-gp function (as measured by calcein retention). Interestingly, swainsonine at 2, 20 and 200  $\mu\text{M}$  induced a concentration-dependent cytoplasmic vacuolation,<sup>863,864</sup> an indication of paraptosis (a cell adaptation or death pathway independent of caspase activation and inhibition which further lacks major hallmark characteristics of apoptosis), but no detectable apoptosis in SK-N-BE(2) cells.

In support of this notion, it has been reported that high-dose swainsonine-treated mice developed neurologic disease with neurovisceral vacuolation.<sup>618</sup> Swainsonine has been shown to decrease the growth rate of human melanoma cells *in vitro* and human melanoma tumour xenografts in athymic nude mice.<sup>865,866</sup> In another study, swainsonine reduced 5-fluorouracil (5-FU)-tolerance in 5-FU-resistant murine colorectal cells, whereas the sensitivity of their parental counterparts remained unchanged.<sup>867</sup> A phase IB clinical trial of the oligosaccharide processing inhibitor, swainsonine, in patients with advanced malignancies has revealed its potential tolerability<sup>868</sup> and supported the role for glycosylation inhibitors as combination therapies for cancer<sup>497,594,646,869</sup>

### 4.3.6 $\beta$ -D-Xyloside

Glycoside primers are compounds which impede the process of glycoprotein assembly, i.e., instead of the endogenous glycoprotein core, subsequent sugar moieties elongate the chain formed by exogenous primers, which results in premature inhibition of glycan synthesis. The best known compound of this class, discovered more than 40 years ago, is  $\beta$ -D-xyloside.<sup>594,615</sup>  $\beta$ -D-xyloside also inhibits proliferation of different cell types because it reversibly arrests cells in the G<sub>1</sub> phase of the cell cycle.<sup>870</sup> Such primers represent highly valuable chemical biology

tools to probe the functions of glycoproteins and glycosaminoglycans in cell systems and model organisms and as a starting point toward the design of anticancer compounds.<sup>503,617</sup>

#### 4.4 Limitations of the Study

This study was limited to SK-N-BE(2) NB cells. Considerable effort was made to study a range of concentrations of the investigative compounds in all assays, but funding constraints hindered such objectives.

#### 4.5 Conclusion and Future Directions

Human NB is an embryonic cancer of the neural crest. Cellular heterogeneity is a characteristic feature of both tumours and derived cell lines. Recent studies have revealed that both cell lines and tumours contain CSCs that are self-renewing, multipotent and highly malignant. In tumours their frequency correlates with a worse prognosis, therefore, their identification and characterization should permit a targeted approach to more effective treatment of this fatal childhood cancer.<sup>793,803</sup> A better understanding of the mechanisms underlying aberrant glycan patterns in EMT and cancer will facilitate the development of glycans as cancer biomarkers or as targets in the design and synthesis of antitumour drugs.<sup>845</sup> Targeting the hallmarks of NB with therapy-induced ERS, especially in the conducive pro-survival TME should be a focus of future studies.<sup>676,687,871,872</sup>

In the past decade, very few drugs have been introduced as frontline therapy for NB. There is a dire need to improve the response rate of induction chemotherapy, which is not efficacious in a third of NB patients. Topotecan, irinotecan and temozolomide are anticipated to improve the response in HR-NB, but their impact on OS and EFS is unknown.<sup>398</sup> Anti-GD2 antibodies combined with other immunomodulators (IL-2, GM-CSF) and maintenance with 13-cis-RA for the treatment of minimal residual disease, represent major advances in the treatment of paediatric NB patients,<sup>873</sup> but still hope is pinned on novel drugs directed to molecular targets of NB.<sup>8,151,345</sup> The identification and characterization of genes elevated in highly malignant NB

stem cells, could provide the basis for developing novel therapies for treatment of this lethal childhood cancer.<sup>376,797</sup> Anti-angiogenic drugs, ALK<sup>173</sup> and MYCN<sup>165,198,399</sup> antagonists and PI3K/Akt/mTOR inhibitors<sup>399</sup> are among the most promising,<sup>392,874</sup> especially in view of chemotherapy-associated enhanced vessel formation in tumours from NB patients.<sup>459</sup>

MDR and relapse is still a major concern in the treatment of NB patients.<sup>260,311</sup> More efforts need to be directed at unravelling the mechanisms of MDR, including the role of N-glycosylation in P-gp-mediated drug efflux function. A nobler perception of NB glycomics<sup>494</sup> and potential of glycosylation inhibitors may yield impressive translational therapeutic promise in this regard.<sup>594,664,797,875</sup> Several efficient P-gp transport blockers have been tested with the objective of enhancing the bioavailability of vital anticancer drugs which are also substrates of P-gp (i.e., they can be effluxed, which reduces their anticancer efficacy), but undesirable side effects and toxicities that impede their clinical utility need to be tackled.<sup>736</sup>

Chemical tools for perturbing, profiling, and perceiving glycans should reduce such barriers, e.g., developing a glycomics database has begun to facilitate bioinformatics studies on glycans (<http://www.functionalglycomics.org/fg/>) and profiles of glycans from tissues and body fluids have produced new biomarker candidates.<sup>503</sup> NSAIDs perturb ER homeostasis by eliciting hallmark events,<sup>726</sup> including mostly the expression of GRP78 and CHOP, and the activation of PERK and ATF6 and, therefore, ERS and apoptosis. However, comprehensive mechanistic studies are needed to facilitate a reliable understanding of how such effects are initially triggered and how NSAID-associated adverse events can be prevented.<sup>687,859,860</sup> The future therapeutic landscape for NB looks challenging, but also promising.

## REFERENCES

1. Colon NC, Chung DH. Neuroblastoma. *Advances in Pediatrics* 2011;58(1):297-311.
2. Deyell RJ, Attiyeh EF. Advances in the understanding of constitutional and somatic genomic alterations in neuroblastoma. *Cancer Genetics* 2011;204(3):113-121.
3. Maris JM. Recent advances in neuroblastoma. *New England Journal of Medicine* 2010;362(23):2202-2211.
4. Huang M, Weiss WA. Neuroblastoma and MYCN. *Cold Spring Harbor Perspectives in Medicine* 2013;3(10):a014415.
5. Dupin E, Calloni G, Real C, Goncalves-Trentin A, Le Douarin NM. Neural crest progenitors and stem cells. *Comptes Rendus Biologies* 2007;330(6-7):521-529.
6. Louis CU, Shohet JM. Neuroblastoma: Molecular pathogenesis and therapy. *Annual Review of Medicine* 2015;66:49-63.
7. Garner EF, Beierle EA. Cancer stem cells and their interaction with the tumor microenvironment in neuroblastoma. *Cancers* 2015;8(1).
8. Moreno L, Marshall LV, Pearson AD. At the frontier of progress for paediatric oncology: The neuroblastoma paradigm. *British Medical Bulletin* 2013;108:173-188.
9. Schleiermacher G, Janoueix-Lerosey I, Delattre O. Recent insights into the biology of neuroblastoma. *International Journal of Cancer* 2014;135(10):2249-2261.
10. Sridhar S, Al-Moallem B, Kamal H, Terrile M, Stallings RL. New insights into the genetics of neuroblastoma. *Molecular Diagnosis & Therapy* 2013;17(2):63-69.
11. Davidoff AM. Neuroblastoma. *Seminars in Pediatric Surgery* 2012;21(1):2-14.
12. Brodeur GM, Bagatell R. Mechanisms of neuroblastoma regression. *Nature Reviews: Clinical Oncology* 2014;11(12):704-713.
13. Glebova NO, Ginty DD. Growth and survival signals controlling sympathetic nervous system development. *Annual Review of Neuroscience* 2005;28:191-222.
14. Heck JE, Ritz B, Hung RJ, Hashibe M, Boffetta P. The epidemiology of neuroblastoma: A review. *Paediatric and Perinatal Epidemiology* 2009;23(2):125-143.
15. Tortora GJ, Derrickson B. Principles of Anatomy & Physiology. 14 ed. Hoboken, NJ: John Wiley & Sons; 2014.
16. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians* 2016;66(1):7-30.
17. Kajanti M. Neuroblastoma in 88 children. Clinical features, prognostic factors, results and late effects of therapy. *Annals of Clinical Research* 1982;15:1-68.
18. Irwin MS, Park JR. Neuroblastoma: Paradigm for precision medicine. *Pediatric Clinics of North America* 2015;62(1):225-256.
19. Stigliani S, Coco S, Moretti S, Oberthuer A, Fischer M, Theissen J, Gallo F, Garavento A, Berthold F, Bonassi S, Tonini GP, Scaruffi P. High genomic instability predicts survival in metastatic high-risk neuroblastoma. *Neoplasia* 2012;14(9):823-832.
20. London WB, Castleberry RP, Matthay KK, Look AT, Seeger RC, Shimada H, Thorner P, Brodeur G, Maris JM, Reynolds CP, Cohn SL. Evidence for an age cutoff greater than 365 days for Neuroblastoma risk Group stratification in the Children's Oncology Group. *Journal of Clinical Oncology* 2005;23(27):6459-6465.
21. Nguyen LB, Diskin SJ, Capasso M, Wang K, Diamond MA, Glessner J, Kim C, Attiyeh EF, Mosse YP, Cole K. Phenotype restricted genome-wide association study using a gene-centric approach identifies three low-risk neuroblastoma susceptibility loci. *PLoS Genetics* 2011;7(3).
22. Carén H, Kryh H, Nethander M, Sjöberg R-M, Träger C, Nilsson S, Abrahamsson J, Kogner P, Martinsson T. High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset. *Proceedings of the National Academy of Sciences* 2010;107(9):4323-4328.



23. Bown N, Cotterill S, Łastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *New England Journal of Medicine* 1999;340(25):1954-1961.
24. Attiyeh EF, London WB, Mossé YP, Wang Q, Winter C, Khazi D, McGrady PW, Seeger RC, Look AT, Shimada H. Chromosome 1p and 11q deletions and outcome in neuroblastoma. *New England Journal of Medicine* 2005;353(21):2243-2253.
25. Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, Kaneko M, London WB, Matthay KK, Nuchtern JG, von Schweinitz D, Simon T, Cohn SL, Pearson AD, Force IT. The International Neuroblastoma Risk Group (INRG) staging system: An INRG task force report. *Journal of Clinical Oncology* 2009;27(2):298-303.
26. Moroz V, Machin D, Faldum A, Hero B, Iehara T, Mosseri V, Ladenstein R, De Bernardi B, Rubie H, Berthold F, Matthay KK, Monclair T, Ambros PF, Pearson AD, Cohn SL, London WB. Changes over three decades in outcome and the prognostic influence of age-at-diagnosis in young patients with neuroblastoma: A report from the International Neuroblastoma Risk Group project. *European Journal of Cancer* 2011;47(4):561-571.
27. Aydın GB, Kutluk MT, Yalçın B, Büyükpamukçu M, Kale G, Varan A, Akyüz C, Senocak ME, Büyükpamukçu N. Neuroblastoma in Turkish children: Experience of a single center. *Journal of Pediatric Hematology/Oncology* 2009;31(7):471-480.
28. Carlsen NL. Neuroblastomas in Denmark 1943-80. Epidemiological and clinical studies. *Acta Paediatrica Supplement* 1994;403(s403):1-27.
29. Cotterill S, Pearson A, Pritchard J, Foot A, Roald B, Kohler J, Imeson J, Group ENS, Group UKCsCS. Clinical prognostic factors in 1277 patients with neuroblastoma: Results of the European Neuroblastoma Study Group 'survey' 1982-1992. *European Journal of Cancer* 2000;36(7):901-908.
30. Gutierrez JC, Fischer AC, Sola JE, Perez EA, Koniaris LG. Markedly improving survival of neuroblastoma: A 30-year analysis of 1,646 patients. *Pediatric Surgery International* 2007;23(7):637-646.
31. Esiashvili N, Goodman M, Ward K, Marcus RB, Johnstone PAS. Neuroblastoma in adults: Incidence and survival analysis based on SEER data. *Pediatric Blood & Cancer* 2007;49(1):41-46.
32. Gloeckler Ries LA, Reichman ME, Lewis DR, Hankey BF, Edwards BK. Cancer survival and incidence from the surveillance, epidemiology, and end results (SEER) Program. *The Oncologist* 2003;8(6):541-552.
33. Bordbar M, Tasbihi M, Kamfiroozi R, Haghpanah S. Epidemiological and clinical characteristics of neuroblastoma in southern Iran. *Iranian Journal of Pediatric Hematology and Oncology* 2014;4(3):89-96.
34. Mehdiabadi GB, Arab E, Rafsanjani KA, Ansari S, Moinzadeh AM. Neuroblastoma in Iran: An experience of 32 years at a referral childrens hospital. *Asian Pacific Journal of Cancer Prevention* 2013;14(5):2739-2742.
35. Juárez-Ocaña S, Palma-Padilla V, González-Miranda G, Siordia-Reyes AG, López-Aguilar E, Aguilar-Martínez M, Mejía-Aranguré JM, Carreón-Cruz R, Rendón-Macías ME, Fajardo-Gutiérrez A. Epidemiological and some clinical characteristics of neuroblastoma in Mexican children (1996-2005). *BMC Cancer* 2009;9(1):266.
36. Haupt R, Garaventa A, Gambini C, Parodi S, Cangemi G, Casale F, Viscardi E, Bianchi M, Prete A, Jenkner A. Improved survival of children with neuroblastoma between 1979 and 2005: A report of the Italian Neuroblastoma Registry. *Journal of Clinical Oncology* 2010;28(14):2331-2338.
37. Bosse KR, Maris JM. Advances in the translational genomics of neuroblastoma: From improving risk stratification and revealing novel biology to identifying actionable genomic alterations. *Cancer* 2016;122(1):20-33.
38. Jebi NY, Iqbal CW, Joliat GR, Sebo TJ, Farley DR. Review of our experience with neuroblastoma and ganglioneuroblastoma in adults. *World Journal of Surgery* 2014;38(11):2871-2874.
39. Okamatsu C, London WB, Naranjo A, Hogarty MD, Gastier-Foster JM, Look AT, LaQuaglia M, Maris JM, Cohn SL, Matthay KK, Seeger RC, Saji T, Shimada H. Clinicopathological characteristics of ganglioneuroma and ganglioneuroblastoma: A report from the CCG and COG. *Pediatric Blood & Cancer* 2009;53(4):563-569.
40. Squillaci S. Olfactory neuroblastoma with focal ganglioneuroblastic differentiation: A case report with literature review. *Pathologica* 2014;106(2):61-66.



41. Teshiba R, Kawano S, Wang LL, He L, Naranjo A, London WB, Seeger RC, Gastier-Foster JM, Look AT, Hogarty MD, Cohn SL, Maris JM, Park JR, Shimada H. Age-dependent prognostic effect by mitosis-karyorrhexis index in neuroblastoma: A report from the Children's Oncology Group. *Pediatric and Developmental Pathology* 2014;17(6):441-449.
42. Megison ML, Gillory LA, Beierle EA. Cell survival signaling in neuroblastoma. *Anti-Cancer Agents in Medicinal Chemistry* 2013;13(4):563-575.
43. Bagatell R, Cohn SL. Genetic discoveries and treatment advances in neuroblastoma. *Current Opinion in Pediatrics* 2016;28(1):19-25.
44. Pinto NR, Applebaum MA, Volchenbom SL, Matthay KK, London WB, Ambros PF, Nakagawara A, Berthold F, Schleiermacher G, Park JR, Valteau-Couanet D, Pearson AD, Cohn SL. Advances in risk classification and treatment Strategies for Neuroblastoma. *Journal of Clinical Oncology* 2015;33(27):3008-3017.
45. Bhatia S, Armenian SH, Armstrong GT, van Dulmen-den Broeder E, Hawkins MM, Kremer LCM, Kuehni CE, Olsen JH, Robison LL, Hudson MM. Collaborative research in childhood cancer survivorship: The current landscape. *Journal of Clinical Oncology* 2015;33(27):3055-3064.
46. Charron M. Contemporary approach to diagnosis and treatment of neuroblastoma. *Quarterly Journal of Nuclear Medicine and Molecular Imaging* 2013;57(1):40-52.
47. Kembhavi SA, Shah S, Rangarajan V, Qureshi S, Popat P, Kurkure P. Imaging in neuroblastoma: An update. *Indian Journal of Radiology & Imaging* 2015;25(2):129-136.
48. Liu W, Zheng J, Li Q. Application of imaging modalities for evaluating neuroblastoma. *Journal of Pediatric Endocrinology and Metabolism* 2013;26(11-12):1015-1020.
49. Matthay KK, Shulkin B, Ladenstein R, Michon J, Giammarile F, Lewington V, Pearson ADJ, Cohn SL. Criteria for evaluation of disease extent by 123I-metaiodobenzylguanidine scans in neuroblastoma: A report for the International Neuroblastoma Risk Group (INRG) task force. *British Journal of Cancer* 2010;102(9):1319-1326.
50. Piccardo A, Lopci E, Conte M, Foppiani L, Garaventa A, Cabria M, Villavecchia G, Fanti S, Cistaro A. PET/CT imaging in neuroblastoma. *Quarterly Journal of Nuclear Medicine and Molecular Imaging* 2013;57(1):29-39.
51. Piccardo A, Puntoni M, Lopci E, Conte M, Foppiani L, Sorrentino S, Morana G, Naseri M, Cistaro A, Villavecchia G, Fanti S, Garaventa A. Prognostic value of 18F-DOPA PET/CT at the time of recurrence in patients affected by neuroblastoma. *European Journal of Nuclear Medicine and Molecular Imaging* 2014;41(6):1046-1056.
52. Papaioannou G, McHugh K. Neuroblastoma in childhood: Review and radiological findings. *Cancer Imaging* 2005;5:116-127.
53. Berger M, Hurtado MF, Bertolez SP, Fernandez-Pineda I. Primary paratesticular neuroblastoma: An important differential diagnosis. *Journal of Pediatric Urology* 2013;9(2):e117-122.
54. Bleeker G, Tytgat GAM, Adam JA, Caron HN, Kremer LCM, Hooft L, van Dalen EC. 123I-MIBG scintigraphy and 18F-FDG-PET imaging for diagnosing neuroblastoma. *Cochrane Database of Systematic Reviews* 2015(9).
55. Bleeker G, van Eck-Smit BL, Zwinderman KH, Versteeg R, van Noesel MM, Kam BL, Kaspers GJ, van Schie A, Kreissman SG, Yanik G, Hero B, Schmidt M, Laureys G, Lambert B, Øra I, Schulte JH, Caron HN, Tytgat GA. MIBG scans in patients with stage 4 neuroblastoma reveal two metastatic patterns, one is associated with MYCN amplification and in MYCN-amplified tumours correlates with a better prognosis. *European Journal of Nuclear Medicine and Molecular Imaging* 2015;42:222-230.
56. Delbeke D. Oncological applications of FDG PET imaging. *Journal of Nuclear Medicine* 1999;40(10):1706-1715.
57. Franzius C, Hermann K, Weckesser M, Kopka K, Juergens KU, Vormoor J, Schober O. Whole-body PET/CT with 11C-meta-hydroxyephedrine in tumors of the sympathetic nervous system: Feasibility Study and comparison with 123I-MIBG SPECT/CT. *Journal of Nuclear Medicine* 2006;47(10):1635-1642.
58. Piccardo A, Morana G, Massollo M, Pescetto M, Conte M, Garaventa A. Brain metastasis from Neuroblastoma depicted by 18F-DOPA PET/CT. *Nuclear Medicine and Molecular Imaging* 2015;49(3):241-242.
59. Rufini V, Calcagni ML, Baum RP. Imaging of neuroendocrine tumors. *Seminars in Nuclear Medicine* 2006;36(3):228-247.

60. Zhou Y, Li K, Zheng S, Chen L. Retrospective study of neuroblastoma in Chinese neonates from 1994 to 2011: An evaluation of diagnosis, treatments, and prognosis: A 10-year retrospective study of neonatal neuroblastoma. *Journal of Cancer Research and Clinical Oncology* 2014;140(1):83-87.
61. Hayat MA. *Pediatric Cancer: Neuroblastoma: Diagnosis, therapy, and prognosis*: Springer Netherlands; 2012.
62. Weinstein JL, Katzenstein HM, Cohn SL. Advances in the diagnosis and treatment of neuroblastoma. *The Oncologist* 2003;8(3):278-292.
63. Barco S, Gennai I, Reggiardo G, Galleni B, Barbagallo L, Maffia A, Viscardi E, De Leonardi F, Cecinati V, Sorrentino S, Garaventa A, Conte M, Cangemi G. Urinary homovanillic and vanillylmandelic acid in the diagnosis of neuroblastoma: Report from the Italian cooperative Group for Neuroblastoma. *Clinical Biochemistry* 2014;47(9):848-852.
64. Mullasery D, Losty PD. Neuroblastoma. *Paediatrics and Child Health* 2016;26(2):68-72.
65. Joshi VV, Larkin EW, Holbrook CT, Silverman JF, Norris HT, Cantor AB, Shuster JJ, Brodeur GM, Look AT, Hayes FA, Altshuler G, Smith EI, Castleberry RP. Correlation between morphologic and other prognostic markers of neuroblastoma a study of histologic grade, DNA index, N-myc gene copy number, and lactic dehydrogenase in patients in the pediatric oncology group. *Cancer* 1993;71(10):3173-3181.
66. Silber JH, Evans AE, Fridman M. Models to predict outcome from childhood neuroblastoma: The role of serum ferritin and tumor histology. *Cancer Research* 1991;51(5):1426-1433.
67. Hann H-WL, Evans AE, Siegel SE, Wong KY, Sather H, Dalton A, Hammond D, Seeger RC. Prognostic importance of serum ferritin in patients with stages III and IV neuroblastoma: The Children's Cancer Study Group experience. *Cancer Research* 1985;45(6):2843-2848.
68. Zeltzer PM, Marangos PJ, Evans AE, Schneider SL. Serum neuron-specific enolase in children with neuroblastoma. Relationship to stage and disease course. *Cancer* 1986;57(6):1230-1234.
69. Shuster JJ, McWilliams NB, Castleberry R, Nitschke R, Smith EI, Altshuler G, Kun L, Brodeur G, Joshi V, Vietti T. Serum lactate dehydrogenase in childhood Neuroblastoma a Pediatric Oncology Group recursive partitioning Study. *American Journal of Clinical Oncology* 1992;15(4):295-303.
70. Cabanillas Stanchi KM, Bruchelt G, Handgretinger R, Holzer U. Nifurtimox reduces N-Myc expression and aerobic glycolysis in neuroblastoma. *Cancer Biology & Therapy* 2015;16(9):1353-1363.
71. Shuster JJ, McWilliams NB, Castleberry R, Nitschke R, Smith EI, Altshuler G, Kun L, Brodeur G, Joshi V, Vietti T. Serum lactate dehydrogenase in childhood neuroblastoma. A Pediatric Oncology Group recursive partitioning study. *American Journal of Clinical Oncology* 1992;15(4):295-303.
72. De Campo M. Ultrasound diagnosis of abdomino-pelvic neuroblastoma. *Pediatric Radiology* 1985;15(5):324-328.
73. Dumba M, Jawad N, McHugh K. Neuroblastoma and nephroblastoma: A radiological review. *Cancer Imaging* 2015;15:5.
74. Kilani M, Hammami S, Darmoul M, Haddad S, Ben Nsir A, Mnari W, Hattab MN. Congenital neuroblastoma presenting with paraplegia following spinal puncture in a neonate. Case report and review of the literature. *Archives de Pédiatrie* 2016;23(3):279-282.
75. Ren AJ, Ning HY, Lin E. Serial diffusion-weighted and conventional MR imaging in primary cerebral Neuroblastoma treated with radiotherapy and chemotherapy. A case report and literature review. *Neuroradiology Journal* 2014;27(4):417-421.
76. Liu B, Zhuang H, Servaes S. Comparison of [123I]MIBG and [131I]MIBG for imaging of neuroblastoma and other neural crest tumors. *Quarterly Journal of Nuclear Medicine and Molecular Imaging* 2013;57(1):21-28.
77. French S, DuBois SG, Horn B, Granger M, Hawkins R, Pass A, Plummer E, Matthay K. 131I-MIBG followed by consolidation with busulfan, melphalan and autologous stem cell transplantation for refractory neuroblastoma. *Pediatric Blood & Cancer* 2013;60(5):879-884.
78. Weyl Ben-Arush M, Ben Barak A, Bar-Deroma R, Ash S, Goldstein G, Golan H, Houry H, Waldman D, Nevo N, Bar Shalom R, Berniger A, Nevelsky A, Toren A, Yaniv I, Kuten A. Targeted therapy with low doses of 131I-MIBG is effective for disease palliation in highly refractory neuroblastoma. *Israel Medical Association Journal* 2013;15(1):31-34.
79. Wong T, Matthay KK, Boscardin WJ, Hawkins RA, Brakeman PR, DuBois SG. Acute changes in blood pressure in patients with neuroblastoma treated with 131I-metaiodobenzylguanidine (MIBG). *Pediatric Blood & Cancer* 2013;60(9):1424-1430.

80. Zhou MJ, Doral MY, DuBois SG, Villablanca JG, Yanik GA, Matthay KK. Different outcomes for relapsed versus refractory neuroblastoma after therapy with 131I-metaiodobenzylguanidine (131I-MIBG). *European Journal of Cancer* 2015;51(16):2465-2472.
81. Souzaki R, Tajiri T, Teshiba R, Higashi M, Kinoshita Y, Tanaka S, Taguchi T. The genetic and clinical significance of MYCN gain as detected by FISH in neuroblastoma. *Pediatric Surgery International* 2011;27(3):231-236.
82. Van Roy N, Van Limbergen H, Vandesompele J, Van Gele M, Poppe B, Salwen H, Laureys G, Manoel N, De Paepe A, Speleman F. Combined M-FISH and CGH analysis allows comprehensive description of genetic alterations in neuroblastoma cell lines. *Genes, Chromosomes and Cancer* 2001;32(2):126-135.
83. Bell JL, Turlapati R, Liu T, Schulte JH, Hüttelmaier S. IGF2BP1 harbors prognostic significance by gene gain and diverse expression in neuroblastoma. *Journal of Clinical Oncology* 2015;33(11):1285-1293.
84. Bell JL, Raseswari T, Liu T, Atmadibrata B, Carter D, Marshall G, Krohn K, Hüttelmaier S. IGF2BP1 and MYCN cooperate in an oncogenic feedback loop, in high-risk neuroblastoma. *Cancer Research* 2014;74(19 Supplement):3103-3103.
85. Shimada H. International neuroblastoma pathology classification. *Pathology* 2012;44:S11-S12.
86. Joshi VV, Silverman JF. Pathology of neuroblastic tumors. *Seminars in Diagnostic Pathology* 1994;11(2):107-117.
87. Navarro S, Amann G, Beiske K, Cullinane CJ, d'Amore ES, Gambini C, Mosseri V, De Bernardi B, Michon J, Peuchmaur M, European Study Group T, Protocol. Prognostic value of International Neuroblastoma Pathology Classification in localized resectable peripheral neuroblastic tumors: A histopathologic study of localized neuroblastoma European Study Group 94.01 trial and protocol. *Journal of Clinical Oncology* 2006;24(4):695-699.
88. Goto S, Umehara S, Gerbing RB, Stram DO, Brodeur GM, Seeger RC, Lukens JN, Matthay KK, Shimada H. Histopathology (International Neuroblastoma Pathology Classification) and MYCN status in patients with peripheral neuroblastic tumors: A report from the Children's Cancer Group. *Cancer* 2001;92(10):2699-2708.
89. Marachelian A, Shimada H, Sano H, Jackson H, Stein J, Sposto R, Matthay KK, Baker D, Villablanca JG. The significance of serial histopathology in a residual mass for outcome of intermediate risk stage 3 neuroblastoma. *Pediatric Blood & Cancer* 2012;58(5):675-681.
90. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B. Terminology and morphologic criteria of neuroblastic tumors: Recommendations by the International Neuroblastoma Pathology Committee. *Cancer* 1999;86(2):349-363.
91. Shimada H, Nakagawa A. Pathology of the peripheral neuroblastic tumors. *Laboratory Medicine* 2006;37(11):684-689.
92. Munchar MJ, Sharifah NA, Jamal R, Looi LM. Cd44s expression correlated with the International Neuroblastoma Pathology Classification (Shimada system) for neuroblastic tumours. *Pathology* 2003;35(2):125-129.
93. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B, Stram DO, Gerbing RB, Lukens JN, Matthay KK, Castleberry RP. The International Neuroblastoma Pathology Classification (the Shimada system). *Cancer* 1999;86(2):364-372.
94. Joshi VV, Cantor AB, Altshuler G, Larkin EW, Neill JS, Shuster JJ, Holbrook CT, Hayes FA, Castleberry RP. Recommendations for modification of terminology of neuroblastic tumors and prognostic significance of Shimada classification. A clinicopathologic study of 213 cases from the Pediatric Oncology Group. *Cancer* 1992;69(8):2183-2196.
95. Ambros IM, Hata J, Joshi VV, Roald B, Dehner LP, Tuchler H, Potschger U, Shimada H. Morphologic features of neuroblastoma (Schwannian stroma-poor tumors) in clinically favorable and unfavorable groups. *Cancer* 2002;94(5):1574-1583.
96. Sano H, Bonadio J, Gerbing RB, London WB, Matthay KK, Lukens JN, Shimada H. International neuroblastoma pathology classification adds independent prognostic information beyond the prognostic contribution of age. *European Journal of Cancer* 2006;42(8):1113-1119.
97. Chan EL, Harris RE, Emery KH, Gelfand MJ, Collins MH, Gruppo RA. Favorable histology, MYCN-amplified 4S neonatal neuroblastoma. *Pediatric Blood & Cancer* 2007;48(4):479-482.
98. Tornoczky T, Semjen D, Shimada H, Ambros IM. Pathology of peripheral neuroblastic tumors: Significance of prominent nucleoli in undifferentiated/poorly differentiated neuroblastoma. *Pathology Oncology Research* 2007;13(4):269-275.

99. Das D, Bhattacharjee K, Barthakur SS, Tahiliani PS, Deka P, Bhattacharjee H, Deka A, Paul R. A new rosette in retinoblastoma. *Indian Journal of Ophthalmology* 2014;62(5):638-641.
100. Wippold FJ, Perry A. Neuropathology for the neuroradiologist: Rosettes and pseudorosettes. *American Journal of Neuroradiology* 2006;27(3):488-492.
101. Acosta S, Mayol G, Rodriguez E, Lavarino C, de Preter K, Kumps C, Garcia I, de Torres C, Mora J. Identification of tumoral glial precursor cells in neuroblastoma. *Cancer Letters* 2011;312(1):73-81.
102. Moon SB. Childhood neuroblastoma masquerading as pheochromocytoma: Case report. *International Medical Case Reports Journal* 2016;9:65-67.
103. Aminzadeh S, Vidali S, Sperl W, Kofler B, Feichtinger RG. Energy metabolism in neuroblastoma and Wilms tumor. *Translational Pediatrics* 2015;4(1):20-32.
104. Marshall GM, Carter DR, Cheung BB, Liu T, Mateos MK, Meyerowitz JG, Weiss WA. The prenatal origins of cancer. *Nature Reviews: Cancer* 2014;14(4):277-289.
105. Szychot E, Apps J, Pritchard-Jones K. Wilms' tumor: Biology, diagnosis and treatment. *Translational Pediatrics* 2014;3(1):12-24.
106. Davidoff AM. Wilms tumor. *Advances in Pediatrics* 2012;59(1):247-267.
107. Kieran K, Anderson JR, Dome JS, Ehrlich PF, Ritchey ML, Shamberger RC, Perlman EJ, Green DM, Davidoff AM. Lymph node involvement in Wilms tumor: Results from National Wilms tumor studies 4 and 5. *Journal of Pediatric Surgery* 2012;47(4):700-706.
108. Strenger V, Kerbl R, Dornbusch HJ, Ladenstein R, Ambros PF, Ambros IM, Urban C. Diagnostic and prognostic impact of urinary catecholamines in neuroblastoma patients. *Pediatric Blood & Cancer* 2007;48(5):504-509.
109. Keikhaei B, Pedram M, Popak B, Heidari M, Hadadi N, Samadi B. Signs and symptoms of Neuroblastoma. *Journal of Medicine and Medical Science* 2012;3(4):243-246.
110. Zhang YT, Feng LH, Zhang Z, Zhong XD, Chang J. Different kinds of paraneoplastic syndromes in childhood Neuroblastoma. *Iranian Journal of Pediatrics* 2015;25(1):e266.
111. Kiyonari S, Kadomatsu K. Neuroblastoma models for insights into tumorigenesis and new therapies. *Expert Opinion on Drug Discovery* 2015;10(1):53-62.
112. Stewart E, Shelat A, Bradley C, Chen X, Federico S, Thiagarajan S, Shirinifard A, Bahrami A, Pappo A, Qu C, Finkelstein D, Sablauer A, Dyer MA. Development and characterization of a human orthotopic neuroblastoma xenograft. *Developmental Biology* 2015;407(2):344-355.
113. Tatekawa Y. Unusual differentiation to pheochromocytoma-like cells in an adrenal Neuroblastoma after chemotherapy: A case report and literature review. *Fetal and Pediatric Pathology* 2015;34(5):322-327.
114. Vo KT, Matthay KK, Neuhaus J, London WB, Hero B, Ambros PF, Nakagawara A, Miniati D, Wheeler K, Pearson AD, Cohn SL, DuBois SG. Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: A report from the international neuroblastoma risk group project. *Journal of Clinical Oncology* 2014;32(28):3169-3176.
115. PDQ Pediatric treatment editorial board. Neuroblastoma treatment (PDQ®): Health professional version. 2016 Jan 14. In: PDQ Cancer information summaries [internet]. Bethesda (MD): National Cancer Institute (US); 2002-. Available from: <http://europepmc.org/books/nbk65747>.
116. Cowie S, Gunn L, Madhavan P. Horner's syndrome secondary to epidural anaesthesia following posterior instrumented scoliosis correction. *Asian Spine Journal* 2015;9(1):121-126.
117. Krstacic A, Krstacic G, Zupetic I. Horner's syndrome due to cervical myelopathy. *Acta Neurologica Belgica* 2015;115(3):435-437.
118. Morandi F, Corrias MV, Pistoia V. Evaluation of bone marrow as a metastatic site of human neuroblastoma. *Annals of the New York Academy of Sciences* 2015;1335:23-31.
119. De Bernardi B, Quaglietta L, Haupt R, Castellano A, Tirtei E, Luksch R, Mastrangelo S, Viscardi E, Indolfi P, Cellini M, Tamburini A, Erminio G, Gandolfo C, Sorrentino S, Vetrella S, Gigliotti AR. Neuroblastoma with symptomatic epidural compression in the infant: The AIEOP experience. *Pediatric Blood & Cancer* 2014;61(8):1369-1375.
120. Matthew J. McGirt, Kaisorn L. Chaichana, April Atiba, Ali Bydon, Timothy F. Witham, Kevin C. Yao, George I. Jallo. Incidence of spinal deformity after resection of intramedullary spinal cord tumors in children who underwent laminectomy compared with laminoplasty. *Journal of Neurosurgery: Pediatrics* 2008;1(1):57-62.



121. Angelini P, Plantaz D, De Bernardi B, Passagia JG, Rubie H, Pastore G. Late sequelae of symptomatic epidural compression in children with localized neuroblastoma. *Pediatric Blood & Cancer* 2011;57(3):473-480.
122. Kunc M, Gabrych A, Czapiewski P, Sworczak K. Paraneoplastic syndromes in olfactory neuroblastoma. *Contemporary Oncology* 2015;19(1):6-16.
123. Bourdeaut F, de Carli E, Timsit S, Coze C, Chastagner P, Sarnacki S, Delattre O, Peuchmaur M, Rubie H, Michon J. VIP hypersecretion as primary or secondary syndrome in neuroblastoma: A retrospective study by the Société Française des Cancers de l'Enfant (SFCE). *Pediatric Blood & Cancer* 2009;52(5):585-590.
124. Brunklaus A, Pohl K, Zuberi SM, de Sousa C. Investigating neuroblastoma in childhood opsoclonus-myoclonus syndrome. *Archives of Disease in Childhood* 2012;97(5):461-463.
125. Sadeghian H, Vernino S. Review: Progress in the management of paraneoplastic neurological disorders. *Therapeutic Advances in Neurological Disorders* 2010;3(1):43-52.
126. Ishola TA, Chung DH. Neuroblastoma. *Surgical Oncology* 2007;16(3):149-156.
127. Aagre SV, Patel A, Choudhary M, Kataria P, Baldaniya K. Paraneoplastic encephalitis as a first evidence of recurrent neuroblastoma: A rare case entity. *Journal of Pediatric Neurosciences* 2015;10(4):404-407.
128. Darnell RB, Posner JB. Paraneoplastic syndromes involving the nervous system. *New England Journal of Medicine* 2003;349(16):1543-1554.
129. Aldaqal SM, Turki AM. Clinico-pathological patterns of a rare presentation of abdominal neuroblastoma in children. *African Journal of Paediatric Surgery* 2013;10(2):100-107.
130. Taggart DR, London WB, Schmidt ML, DuBois SG, Monclair TF, Nakagawara A, De Bernardi B, Ambros PF, Pearson AD, Cohn SL. Prognostic value of the stage 4S metastatic pattern and tumor biology in patients with metastatic neuroblastoma diagnosed between birth and 18 months of age. *Journal of Clinical Oncology* 2011;29(33):4358-4364.
131. Buhagiar A, Ayers D. Chemoresistance, cancer stem cells, and miRNA influences: The case for neuroblastoma. *Analytical Cellular Pathology (Amsterdam)* 2015;2015:150634.
132. Brodeur GM. Neuroblastoma: Biological insights into a clinical enigma. *Nature Reviews: Cancer* 2003;3(3):203-216.
133. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet* 2007;369(9579):2106-2120.
134. Betters E, Liu Y, Kjaeldgaard A, Sundstrom E, Garcia-Castro MI. Analysis of early human neural crest development. *Developmental Biology* 2010;344(2):578-592.
135. Le Douarin N, Kalcheim C. *The neural crest*: Cambridge University Press; 1999.
136. Morales AV, Barbas JA, Nieto MA. How to become neural crest: From segregation to delamination. *Seminars in Cell & Developmental Biology* 2005;16(6):655-662.
137. Jiang M, Stanke J, Lahti JM. The connections between neural crest development and neuroblastoma. *Current Topics in Developmental Biology* 2011;94:77.
138. Mayanil CS. Transcriptional and epigenetic regulation of neural crest induction during neurulation. *Developmental Neuroscience* 2013;35(5):361-372.
139. Strobl-Mazzulla PH, Bronner ME. Epithelial to mesenchymal transition: New and old insights from the classical neural crest model. *Seminars in Cancer Biology* 2012;22(5-6):411-416.
140. Sauka-Spengler T, Bronner-Fraser M. A gene regulatory network orchestrates neural crest formation. *Nature Reviews: Molecular Cell Biology* 2008;9(7):557-568.
141. Rogers CD, Saxena A, Bronner ME. Sip1 mediates an E-cadherin-to-N-cadherin switch during cranial neural crest EMT. *Journal of Cell Biology* 2013;203(5):835-847.
142. Beck B, Blanpain C. Unravelling cancer stem cell potential. *Nature Reviews: Cancer* 2013;13(10):727-738.
143. Howk CL, Voller Z, Beck BB, Dai D. Genetic diversity in normal cell populations is the earliest stage of oncogenesis leading to intra-tumor heterogeneity. *Frontiers in Oncology* 2013;3:61.
144. Mimeault M, Hauke R, Mehta PP, Batra SK. Recent advances in cancer stem/progenitor cell research: Therapeutic implications for overcoming resistance to the most aggressive cancers. *Journal of Cellular and Molecular Medicine* 2007;11(5):981-1011.
145. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57-70.

146. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011;144(5):646-674.
147. Borriello L, Seeger RC, Asgharzadeh S, DeClerck YA. More than the genes, the tumor microenvironment in neuroblastoma. *Cancer Letters* 2016;380(1):304-314.
148. Hanahan D, Coussens LM. Accessories to the crime: Functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;21(3):309-322.
149. Capasso M, Diskin SJ. Genetics and genomics of neuroblastoma. In: Pasche B, ed. *Cancer Genetics*. Berlin: Springer; 2010:65-84.
150. Van Roy N, De Preter K, Hoebeeck J, Van Maerken T, Pattyn F, Mestdagh P, Vermeulen J, Vandesompele J, Speleman F. The emerging molecular pathogenesis of neuroblastoma: Implications for improved risk assessment and targeted therapy. *Genome Medicine* 2009;1(7):74.
151. Cheung NK, Dyer MA. Neuroblastoma: Developmental biology, cancer genomics and immunotherapy. *Nature Reviews: Cancer* 2013;13(6):397-411.
152. Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, Swift P, Shimada H, Black CT, Brodeur GM, Gerbing RB, Reynolds CP. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *New England Journal of Medicine* 1999;341(16):1165-1173.
153. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, Smith M, Anderson B, Villablanca JG, Matthay KK, Shimada H, Grupp SA, Seeger R, Reynolds CP, Buxton A, Reisfeld RA, Gillies SD, Cohn SL, Maris JM, Sondel PM. Children's Oncology G. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *New England Journal of Medicine* 2010;363(14):1324-1334.
154. Schwab M, Alitalo K, Klempnauer K-H, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 1983;305(5931):245-248.
155. Janoueix-Lerosey I, Lequin D, Brugieres L, Ribeiro A, de Pontual L, Combaret V, Raynal V, Puisieux A, Schleiermacher G, Pierron G, Valteau-Couanet D, Frebourg T, Michon J, Lyonnet S, Amiel J, Delattre O. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008;455(7215):967-970.
156. Trochet D, Bourdeaut F, Janoueix-Lerosey I, Deville A, de Pontual L, Schleiermacher G, Coze C, Philip N, Frebourg T, Munnich A, Lyonnet S, Delattre O, Amiel J. Germline mutations of the paired-like homeobox 2b (PHOX2B) gene in neuroblastoma. *American Journal of Human Genetics* 2004;74(4):761-764.
157. Nguyen le B, Diskin SJ, Capasso M, Wang K, Diamond MA, Glessner J, Kim C, Attiyeh EF, Mosse YP, Cole K, Iolascon A, Devoto M, Hakonarson H, Li HK, Maris JM. Phenotype restricted genome-wide association study using a gene-centric approach identifies three low-risk neuroblastoma susceptibility loci. *PLoS Genetics* 2011;7(3):e1002026.
158. Pugh TJ, Morozova O, Attiyeh EF, Asgharzadeh S, Wei JS, Auclair D, Carter SL, Cibulskis K, Hanna M, Kiezun A, Kim J, Lawrence MS, Lichtenstein L, McKenna A, Pedamallu CS, Ramos AH, Shefler E, Sivachenko A, Sougnez C, Stewart C, Ally A, Birol I, Chiu R, Corbett RD, Hirst M, Jackman SD, Kamoh B, Khodabakshi AH, Krzywinski M, Lo A, Moore RA, Mungall KL, Qian J, Tam A, Thiessen N, Zhao Y, Cole KA, Diamond M, Diskin SJ, Mosse YP, Wood AC, Ji L, Spoto R, Badgett T, London WB, Moyer Y, Gastier-Foster JM, Smith MA, Guidry Auvil JM, Gerhard DS, Hogarty MD, Jones SJ, Lander ES, Gabriel SB, Getz G, Seeger RC, Khan J, Marra MA, Meyerson M, Maris JM. The genetic landscape of high-risk neuroblastoma. *Nature Genetics* 2013;45(3):279-284.
159. Cheung NK, Zhang J, Lu C, Parker M, Bahrami A, Tickoo SK, Heguy A, Pappo AS, Federico S, Dalton J, Cheung IY, Ding L, Fulton R, Wang J, Chen X, Becksfort J, Wu J, Billups CA, Ellison D, Mardis ER, Wilson RK, Downing JR, Dyer MA, St Jude Children's Research Hospital-Washington University Pediatric Cancer Genome P. Association of age at diagnosis and genetic mutations in patients with neuroblastoma. *Journal of the American Medical Association* 2012;307(10):1062-1071.
160. Valentijn LJ, Koster J, Haneveld F, Aissa RA, van Sluis P, Broekmans MEC, Molenaar JJ, van Nes J, Versteeg R. Functional MYCN signature predicts outcome of neuroblastoma irrespective of MYCN amplification. *Proceedings of the National Academy of Sciences* 2012;109(47):19190-19195.
161. Asgharzadeh S, Salo JA, Ji L, Oberthuer A, Fischer M, Berthold F, Hadjidanil M, Liu CW, Metelitsa LS, Pique-Regi R, Wakamatsu P, Villablanca JG, Kreissman SG, Matthay KK, Shimada H, London WB, Spoto R, Seeger RC. Clinical significance of tumor-associated inflammatory cells in metastatic neuroblastoma. *Journal of Clinical Oncology* 2012;30(28):3525-3532.



162. Larsson K, Kock A, Idborg H, Arsenian Henriksson M, Martinsson T, Johnsen JI, Korotkova M, Kogner P, Jakobsson P-J. COX/mPGES-1/PGE2 pathway depicts an inflammatory-dependent high-risk neuroblastoma subset. *Proceedings of the National Academy of Sciences* 2005;112(26):8070-8075.
163. Mosse YP, Laudenslager M, Khazi D, Carlisle AJ, Winter CL, Rappaport E, Maris JM. Germline PHOX2B mutation in hereditary neuroblastoma. *American Journal of Human Genetics* 2004;75(4):727-730.
164. Bourdeaut F, Trochet D, Janoueix-Lerosey I, Ribeiro A, Deville A, Coz C, Michiels JF, Lyonnet S, Amiel J, Delattre O. Germline mutations of the paired-like homeobox 2b (PHOX2B) gene in neuroblastoma. *Cancer Letters* 2005;228(1-2):51-58.
165. Zhang JT, Weng ZH, Tsang KS, Tsang LL, Chan HC, Jiang XH. MycN is critical for the maintenance of human embryonic stem cell-derived neural crest stem cells. *PLoS One* 2016;11(1):e0148062.
166. Trochet D, Hong SJ, Lim JK, Brunet J-F, Munnich A, Kim K-S, Lyonnet S, Goridis C, Amiel J. Molecular consequences of PHOX2B missense, frameshift and alanine expansion mutations leading to autonomic dysfunction. *Human Molecular Genetics* 2005;14(23):3697-3708.
167. Raabe EH, Laudenslager M, Winter C, Wasserman N, Cole K, LaQuaglia M, Maris DJ, Mosse YP, Maris JM. Prevalence and functional consequence of PHOX2B mutations in neuroblastoma. *Oncogene* 2008;27(4):469-476.
168. Morris SW, Naeve C, Mathew P, James PL, Kirstein MN, Cui X, Witte DP. ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene* 1997;14(18):2175-2188.
169. Hurley SP, Clary DO, Copié V, Lefcort F. Anaplastic lymphoma kinase is dynamically expressed on subsets of motor neurons and in the peripheral nervous system. *Journal of Comparative Neurology* 2006;495(2):202-212.
170. Degoutin J, Brunet-de Carvalho N, Cifuentes-Diaz C, Vigny M. ALK (anaplastic lymphoma kinase) expression in DRG neurons and its involvement in neuron-Schwann cells interaction. *European Journal of Neuroscience* 2009;29(2):275-286.
171. Palmer RH, Vernersson E, Grabbe C, Hallberg B. Anaplastic lymphoma kinase: Signalling in development and disease. *Biochemical Journal* 2009;420(3):345-361.
172. Cheng M, R Ott G. Anaplastic lymphoma kinase as a therapeutic target in anaplastic large cell lymphoma, non-small cell lung cancer and neuroblastoma. *Anti-Cancer Agents in Medicinal Chemistry* 2010;10(3):236-249.
173. Kruczynski A, Delsol G, Laurent C, Brousset P, Lamant L. Anaplastic lymphoma kinase as a therapeutic target. *Expert Opinion on Therapeutic Targets* 2012;16(11):1127-1138.
174. Carén H, Abel F, Kogner P, Martinsson T. High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. *Biochemical Journal* 2008;416(2):153-159.
175. George RE, Sanda T, Hanna M, Fröhling S, Luther II W, Zhang J, Ahn Y, Zhou W, London WB, McGrady P. Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008;455(7215):975-978.
176. Janoueix-Lerosey I, Lequin D, Brugières L, Ribeiro A, de Pontual L, Combaret V, Raynal V, Puisieux A, Schleiermacher G, Pierron G. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008;455(7215):967-970.
177. Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008;455(7215):930-935.
178. Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, Wang L, Soda M, Kikuchi A, Igarashi T. Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008;455(7215):971-974.
179. Passoni L, Longo L, Collini P, Coluccia AML, Bozzi F, Podda M, Gregorio A, Gambini C, Garaventa A, Pistoia V. Mutation-independent anaplastic lymphoma kinase overexpression in poor prognosis neuroblastoma patients. *Cancer Research* 2009;69(18):7338-7346.
180. Motegi A, Fujimoto J, Kotani M, Sakuraba H, Yamamoto T. ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth. *Journal of Cell Science* 2004;117(15):3319-3329.
181. Osajima-Hakomori Y, Miyake I, Ohira M, Nakagawara A, Nakagawa A, Sakai R. Biological role of anaplastic lymphoma kinase in neuroblastoma. *American Journal of Pathology* 2005;167(1):213-222.

182. Schönherr C, Yang H, Vigny M, Palmer RH, Hallberg B. Anaplastic lymphoma kinase activates the small GTPase Rap1 via the Rap1-specific GEF C3G in both neuroblastoma and PC12 cells. *Oncogene* 2010;29(19):2817-2830.
183. van Noesel MM, Versteeg R. Pediatric neuroblastomas: Genetic and epigenetic 'danse macabre'. *Gene* 2004;325:1-15.
184. Thompson D, Vo KT, London WB, Fischer M, Ambros PF, Nakagawara A, Brodeur GM, Matthay KK, DuBois SG. Identification of patient subgroups with markedly disparate rates of MYCN amplification in neuroblastoma: A report from the International Neuroblastoma Risk Group project. *Cancer* 2016;122(6):935-945.
185. Schwab M, Varmus H, Bishop J, Grzeschik K, Naylor S, Sakaguchi A, Brodeur G, Trent J. Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc. *Nature* 1984;308(5956):288.
186. Pandey GK, Kanduri C. Long noncoding rnas and neuroblastoma. *Oncotarget* 2015;6(21):18265-18275.
187. Pelengaris S, Khan M, Evan G. C-MYC: More than just a matter of life and death. *Nature Reviews Cancer* 2002;2(10):764-776.
188. Reiter JL, Brodeur GM. MYCN is the only highly expressed gene from the core amplified domain in human neuroblastomas. *Genes, Chromosomes and Cancer* 1998;23(2):134-140.
189. Reiter JL, Brodeur GM. High-resolution mapping of a 130-kb core region of the MYCN amplicon in neuroblastomas. *Genomics* 1996;32(1):97-103.
190. Emanuel BS, Balaban G, Boyd JP, Grossman A, Negishi M, Parmiter A, Glick MC. N-myc amplification in multiple homogeneously staining regions in two human neuroblastomas. *Proceedings of the National Academy of Sciences of the United States of America* 1985;82(11):3736-3740.
191. Hurlin PJ. N-Myc functions in transcription and development. *Birth Defects Research Part C Embryo Today Reviews* 2005;75(4):340-352.
192. Strieder V, Lutz W. E2f proteins regulate MYCN expression in neuroblastomas. *Journal of Biological Chemistry* 2003;278(5):2983-2989.
193. Thompson EB. The many roles of c-Myc in apoptosis. *Annual Review of Physiology* 1998;60:575-600.
194. Gherardi S, Valli E, Enriquez D, Perini G. MYCN-mediated transcriptional repression in neuroblastoma: The other side of the coin. *Frontiers in Oncology* 2013;3:42.
195. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nature Reviews: Molecular Cell Biology* 2005;6(8):635-645.
196. Slack A, Chen Z, Tonelli R, Pule M, Hunt L, Pession A, Shohet JM. The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102(3):731-736.
197. Stafman LL, Beierle EA. Cell proliferation in Neuroblastoma. *Cancers* 2016;8(1).
198. Ham J, Costa C, Sano R, Lochmann TL, Sennott EM, Patel NU, Dastur A, Gomez-Caraballo M, Krytska K, Hata AN, Floros KV, Hughes MT, Jakubik CT, Heisey DA, Ferrell JT, Bristol ML, March RJ, Yates C, Hicks MA, Nakajima W, Gowda M, Windle BE, Dozmorov MG, Garnett MJ, McDermott U, Harada H, Taylor SM, Morgan IM, Benes CH, Engelman JA, Mosse YP, Faber AC. Exploitation of the apoptosis-primed state of MYCN-amplified neuroblastoma to develop a potent and specific targeted therapy combination. *Cancer Cell* 2016;29(2):159-172.
199. Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop JM. Targeted expression of MYCN causes neuroblastoma in transgenic mice. *EMBO Journal* 1997;16(11):2985-2995.
200. Althoff K, Beckers A, Bell E, Nortmeyer M, Thor T, Sprüssel A, Lindner S, De Preter K, Florin A, Heukamp LC, Klein-Hitpass L, Astrahantseff K, Kumps C, Speleman F, Eggert A, Westermann F, Schramm A, Schulte JH. A cre-conditional MYCN-driven neuroblastoma mouse model as an improved tool for preclinical studies. *Oncogene* 2015;34(26):3357-3368.
201. Chesler L, Weiss WA. Genetically engineered murine models - contribution to our understanding of the genetics, molecular pathology and therapeutic targeting of neuroblastoma. *Seminars in Cancer Biology* 2011;21(4):245-255.
202. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 1998;351(9106):882-883.
203. Samkari A, Cooper ZA, Holloway MP, Liu J, Altura RA. Rapamycin induces the anti-apoptotic protein survivin in neuroblastoma. *International Journal of Biochemistry and Molecular Biology* 2012;3(1):28-35.

204. Fiscella M, Zhang H, Fan S, Sakaguchi K, Shen S, Mercer WE, Woude GFV, O'Connor PM, Appella E. Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. *Proceedings of the National Academy of Sciences* 1997;94(12):6048-6053.
205. Fujimoto H, Onishi N, Kato N, Takekawa M, Xu XZ, Kosugi A, Kondo T, Imamura M, Oishi I, Yoda A, Minami Y. Regulation of the antioncogenic chk2 kinase by the oncogenic wip1 phosphatase. *Cell Death and Differentiation* 2006;13(7):1170-1180.
206. Ogasawara S, Kiyota Y, Chuman Y, Kowata A, Yoshimura F, Tanino K, Kamada R, Sakaguchi K. Novel inhibitors targeting ppm1d phosphatase potently suppress cancer cell proliferation. *Bioorganic and Medicinal Chemistry* 2015;23(19):6246-6249.
207. Yagi H, Chuman Y, Kozakai Y, Imagawa T, Takahashi Y, Yoshimura F, Tanino K, Sakaguchi K. A small molecule inhibitor of p53-inducible protein phosphatase ppm1d. *Bioorganic and Medicinal Chemistry Letters* 2012;22(1):729-732.
208. Hartsough MT, Steeg PS. Nm23/nucleoside diphosphate kinase in human cancers. *Journal of Bioenergetics and Biomembranes* 2000;32(3):301-308.
209. Hiyama E, Hiyama K, Yamaoka H, Sueda T, Reynolds CP, Yokoyama T. Expression profiling of favorable and unfavorable neuroblastomas. *Pediatric Surgery International* 2004;20(1):33-38.
210. Matthay KK, Stram D. Is adjuvant therapy ever warranted in localized neuroblastoma. *Journal of Pediatric Hematology/Oncology* 2000;22(5):399-402.
211. Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, Laureys G, Speleman F, Nicholson J, Bernheim A, Betts DR, Vandesompele J, Van Roy N. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *New England Journal of Medicine* 1999;340(25):1954-1961.
212. Lastowska M, Nacheva E, McGuckin A, Curtis A, Grace C, Pearson A, Bown N. Comparative genomic hybridization study of primary neuroblastoma tumors. United Kingdom Children's Cancer Study Group. *Genes, Chromosomes and Cancer* 1997;18(3):162-169.
213. Meddeb M, Danglot G, Chudoba I, Venuat AM, Benard J, Avet-Loiseau H, Vasseur B, Le Paslier D, Terrier-Lacombe MJ, Hartmann O, Bernheim A. Additional copies of a 25 mb chromosomal region originating from 17q23.1-17qter are present in 90% of high-grade neuroblastomas. *Genes, Chromosomes and Cancer* 1996;17(3):156-165.
214. Corvi R, Savelyeva L, Schwab M. Duplication of n-MYC at its resident site 2p24 may be a mechanism of activation alternative to amplification in human neuroblastoma cells. *Cancer Research* 1995;55(16):3471-3474.
215. Corvi R, Savelyeva L, Amler L, Handgretinger R, Schwab M. Cytogenetic evolution of MYCN and MDM2 amplification in the neuroblastoma ls tumour and its cell line. *European Journal of Cancer* 1995;31(4):520-523.
216. George RE, Kenyon RM, McGuckin AG, Malcolm AJ, Pearson AD, Lunec J. Investigation of co-amplification of the candidate genes ornithine decarboxylase, ribonucleotide reductase, syndecan-1 and a dead box gene, DDX1, with N-myc in neuroblastoma. United Kingdom Children's Cancer Study Group. *Oncogene* 1996;12(7):1583-1587.
217. Jinbo T, Iwamura Y, Kaneko M, Sawaguchi S. Coamplification of the l-myc and N-myc oncogenes in a neuroblastoma cell line. *Japanese Journal of Cancer Research* 1989;80(4):299-301.
218. Bader SA, Fasching C, Brodeur GM, Stanbridge EJ. Dissociation of suppression of tumorigenicity and differentiation *in vitro* effected by transfer of single human chromosomes into human neuroblastoma cells. *Cell Growth and Differentiation* 1991;2(5):245-255.
219. Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D, Bredel M, Vogel H, Mills AA. CHD5 is a tumor suppressor at human 1p36. *Cell* 2007;128(3):459-475.
220. Munirajan AK, Ando K, Mukai A, Takahashi M, Suenaga Y, Ohira M, Koda T, Hirota T, Ozaki T, Nakagawara A. KIF1B $\beta$  functions as a haploinsufficient tumor suppressor gene mapped to chromosome 1p36. 2 by inducing apoptotic cell death. *Journal of Biological Chemistry* 2008;283(36):24426-24434.
221. Welch C, Chen Y, Stallings R. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* 2007;26(34):5017-5022.
222. Chen Y, Stallings RL. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Research* 2007;67(3):976-983.
223. Schlisio S, Kenchappa RS, Vredevelde LC, George RE, Stewart R, Greulich H, Shahriari K, Nguyen NV, Pigny P, Dahia PL. The kinesin KIF1B $\beta$  acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. *Genes & Development* 2008;22(7):884-893.

224. Guo J, Dong Q, Fang Z, Chen X, Lu H, Wang K, Yin Y, Cai X, Zhao N, Chen J. Identification of miRNAs that are associated with tumor metastasis in neuroblastoma. *Cancer Biology & Therapy* 2010;9(6):446-452.
225. Maris JM, Matthay KK. Molecular biology of neuroblastoma. *Journal of Clinical Oncology* 1999;17(7):2264-2279.
226. Gomyo H, Arai Y, Tanigami A, Murakami Y, Hattori M, Hosoda F, Arai K, Aikawa Y, Tsuda H, Hirohashi S. A 2-Mb sequence-ready contig map and a novel immunoglobulin superfamily gene IGSF4 in the LOH region of chromosome 11q23. *Genomics* 1999;62(2):139-146.
227. Ando K, Ohira M, Ozaki T, Nakagawa A, Akazawa K, Suenaga Y, Nakamura Y, Koda T, Kamijo T, Murakami Y. Expression of TSLC1, a candidate tumor suppressor gene mapped to chromosome 11q23, is downregulated in unfavorable neuroblastoma without promoter hypermethylation. *International Journal of Cancer* 2008;123(9):2087-2094.
228. Ochiai H, Takenobu H, Nakagawa A, Yamaguchi Y, Kimura M, Ohira M, Okimoto Y, Fujimura Y, Koseki H, Kohno Y. Bmi1 is a MYCN target gene that regulates tumorigenesis through repression of KIF1B $\beta$  and TSLC1 in neuroblastoma. *Oncogene* 2010;29(18):2681-2690.
229. Lehalle D, Sanlaville D, Guimier A, Plouvier E, Leblanc T, Galmiche L, Radford I, Romana S, Colleaux L, de Pontual L, Lyonnet S, Amiel J. Multiple congenital anomalies-intellectual disability (MCA-ID) and neuroblastoma in a patient harboring a de novo 14q23.1q23.3 deletion. *American Journal of Medical Genetics Part A* 2014;164A(5):1310-1317.
230. Thompson PM, Seifried BA, Kyemba SK, Jensen SJ, Guo C, Maris JM, Brodeur GM, Stram DO, Seeger RC, Gerbing R, Matthay KK, Matisse TC, White PS. Loss of heterozygosity for chromosome 14q in neuroblastoma. *Medical and Pediatric Oncology* 2001;36(1):28-31.
231. Suzuki T, Yokota J, Mugishima H, Okabe I, Ookuni M, Sugimura T, Terada M. Frequent loss of heterozygosity on chromosome 14q in neuroblastoma. *Cancer Research* 1989;49(5):1095-1098.
232. Saulnier Sholler GL, Bond JP, Bergendahl G, Dutta A, Dragon J, Neville K, Ferguson W, Roberts W, Eslin L, Kraveka J, Kaplan J, Mitchell D, Parikh N, Merchant M, Ashikaga T, Hanna G, Lescault PJ, Siniard A, Corneveaux J, Huentelman M, Trent J. Feasibility of implementing molecular-guided therapy for the treatment of patients with relapsed or refractory neuroblastoma. *Cancer Medicine* 2015;4(6):871-886.
233. Saletta F, Wadham C, Ziegler DS, Marshall GM, Haber M, McCowage G, Norris MD, Byrne JA. Molecular profiling of childhood cancer: Biomarkers and novel therapies. *BBA Clinical* 2014;1:59-77.
234. Domingo-Fernandez R, Watters K, Piskareva O, Stallings RL, Bray I. The role of genetic and epigenetic alterations in neuroblastoma disease pathogenesis. *Pediatric Surgery International* 2013;29(2):101-119.
235. Everson TC, Cole WH. Spontaneous regression of cancer: Preliminary report. *Annals of Surgery* 1956;144(3):366.
236. Stewart FW. Experiences in spontaneous regression of neoplastic disease in man. *Texas Reports on Biology and Medicine* 1951;10(1):239-253.
237. Cushing H, Wolbach SB. The transformation of a malignant paravertebral sympatheticoblastoma into a benign ganglioneuroma. *American Journal of Pathology* 1927;3(3):203-216 207.
238. Nickerson HJ, Matthay KK, Seeger RC, Brodeur GM, Shimada H, Perez C, Atkinson JB, Selch M, Gerbing RB, Stram DO, Lukens J. Favorable biology and outcome of stage IV-S neuroblastoma with supportive care or minimal therapy: A Children's Cancer Group study. *Journal of Clinical Oncology* 2000;18(3):477-486.
239. Ambros PF, Brodeur GM. Concept of tumorigenesis and regression. In: Brodeur GM, Sawada T, Tsuchida Y, eds. *Neuroblastoma*. Amsterdam: Elsevier Science; 2000:21-29.
240. Carlsen N. How frequent is spontaneous remission of neuroblastomas? Implications for screening. *British Journal of Cancer* 1990;61(3):441.
241. Benard J, Raguenez G, Kauffmann A, Valent A, Ripoché H, Joulin V, Job B, Danglot G, Cantais S, Robert T, Terrier-Lacombe MJ, Chassevent A, Koscielny S, Fischer M, Berthold F, Lipinski M, Tursz T, Dessen P, Lazar V, Valteau-Couanet D. MYCN-non-amplified metastatic neuroblastoma with good prognosis and spontaneous regression: A molecular portrait of stage 4S. *Molecular Oncology* 2008;2(3):261-271.
242. Westermann F, Muth D, Benner A, Bauer T, Henrich KO, Oberthuer A, Brors B, Beissbarth T, Vandesompele J, Pattyn F, Hero B, König R, Fischer M, Schwab M. Distinct transcriptional MYCN/c-MYC activities are associated with spontaneous regression or malignant progression in neuroblastomas. *Genome Biology* 2008;9(10):R150.



243. Jiang M, Stanke J, Lahti JM. The connections between neural crest development and neuroblastoma. *Current Topics in Developmental Biology* 2011;94:77-127.
244. Hero B, Simon T, Spitz R, Ernestus K, Gnekow AK, Scheel-Walter H-G, Schwabe D, Schilling FH, Benz-Bohm G, Berthold F. Localized infant neuroblastomas often show spontaneous regression: Results of the prospective trials NB95-S and NB97. *Journal of Clinical Oncology* 2008;26(9):1504-1510.
245. Kocak H, Ackermann S, Hero B, Kahlert Y, Oberthuer A, Juraeva D, Roels F, Theissen J, Westermann F, Deubzer H. Hox-C9 activates the intrinsic pathway of apoptosis and is associated with spontaneous regression in neuroblastoma. *Cell Death & Disease* 2013;4(4):e586.
246. Mao L, Ding J, Zha Y, Yang L, McCarthy BA, King W, Cui H, Ding H-F. HOXC9 links cell-cycle exit and neuronal differentiation and is a prognostic marker in neuroblastoma. *Cancer Research* 2011;71(12):4314-4324.
247. Nakagawara A. Molecular basis of spontaneous regression of neuroblastoma: Role of neurotrophic signals and genetic abnormalities. *Human Cell* 1998;11(3):115-124.
248. Hoehner JC, Olsen L, Sandstedt B, Kaplan DR, Pahlman S. Association of neurotrophin receptor expression and differentiation in human neuroblastoma. *American Journal of Pathology* 1995;147(1):102-113.
249. Yamashiro D, Liu X-G, Lee C, Nakagawara A, Ikegaki N, McGregor L, Baylin S, Brodeur G. Expression and function of Trk-c in favourable human neuroblastomas. *European Journal of Cancer* 1997;33(12):2054-2057.
250. Schor NF. Aiming at neuroblastoma and hitting other worthy targets. *Journal of Child Neurology* 2013;28(6):768-773.
251. Kushner BH, Cohn SL, Matthay KK, Cheung N-KV, La Quaglia MP, Haas-Kogan DA, Wolden SL, Grupp SA, Cheung IY, Ambros PF. Treatment of Neuroblastoma. In: Cheung N-KV, Cohn SL, eds. *Neuroblastoma*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010:123-192.
252. Kubota M. The role of surgery in the treatment of neuroblastoma. *Surgery Today* 2010;40(6):526-532.
253. Jackson JR, Kim ES. The Surgical management of high-risk Neuroblastoma. *Pediatric Neonatal Care* 2016;4(4):00145.
254. Yeung F, Chung PH, Tam PK, Wong KK. Is complete resection of high-risk stage IV neuroblastoma associated with better survival? *Journal of Pediatric Surgery* 2015;50(12):2107-2111.
255. Simon T, Haberle B, Hero B, von Schweinitz D, Berthold F. Role of surgery in the treatment of patients with stage 4 neuroblastoma age 18 months or older at diagnosis. *Journal of Clinical Oncology* 2013;31(6):752-758.
256. Cañete A, Jovani C, Lopez A, Costa E, Segarra V, Fernández JM, Verdeguer A, Velázquez J, Castel V. Surgical treatment for neuroblastoma: Complications during 15 years' experience. *Journal of Pediatric Surgery* 1998;33(10):1526-1530.
257. Kiely EM. The surgical challenge of neuroblastoma. *Journal of Pediatric Surgery* 1994;29(2):128-133.
258. Kiely E. Surgery for neuroblastoma. In: Spitz L, Wurnig P, Angerpointner TA, eds. *Pediatric Surgical Oncology*. Berlin: Springer-Verlag; 1989;22:140-145.
259. Platek ME, Merzianu M, Mashtare TL, Popat SR, Rigual NR, Warren GW, Singh AK. Improved survival following surgery and radiation therapy for olfactory neuroblastoma: Analysis of the SEER database. *Radiation Oncology* 2011;6:41.
260. Morgenstern DA, Baruchel S, Irwin MS. Current and future strategies for relapsed neuroblastoma: Challenges on the road to precision therapy. *Journal of Pediatric Hematology/Oncology* 2013;35(5):337-347.
261. Park JR, Eggert A, Caron H. Neuroblastoma: Biology, prognosis, and treatment. *Hematology/Oncology Clinics of North America* 2010;24(1):65-86.
262. Weber A. Minimal residual disease in Neuroblastoma. In: Christiansen H, Christiansen NM, eds. *Progressive Neuroblastoma: Innovation and Novel Therapeutic Strategies*. Basel: Karger Publishers; 2015;20:178-186.
263. Beiske K, Ambros PF, Burchill SA, Cheung IY, Swerts K. Detecting minimal residual disease in neuroblastoma patients-the present state of the art. *Cancer Letters* 2005;228(1-2):229-240.
264. Choi YB, Bae GE, Lee NH, Kim JS, Lee SH, Yoo KH, Sung KW, Koo HH. Clinical significance of persistent tumor in bone marrow during treatment of high-risk neuroblastoma. *Journal of Korean Medical Science* 2015;30(8):1062-1067.

265. O'Leary M, Reaman GH. Principles of pediatric oncology. In: Hong WK, Bast RC, Jr., Hait WN, Kufe DW, Pollock RE, Weichselbaum RR, Holland JF, Frei E, III., eds. *Cancer Medicine*. Shelton, Connecticut: BC Decker, People's Medical Publishing House-USA; 2010:1723-1739.
266. Bernard JL, Philip T, Zucker JM, Frappaz D, Robert A, Marguerite G, Boilletot A, Philippe N, Lutz P, Roche H. Sequential cisplatin/VM-26 and vincristine/cyclophosphamide/doxorubicin in metastatic neuroblastoma: An effective alternating non-cross-resistant regimen? *Journal of Clinical Oncology* 1987;5(12):1952-1959.
267. Guglielmi M, De Bernardi B, Rizzo A, Federici S, Boglino C, Siracusa F, Leggio A, Cozzi F, Cecchetto G, Musi L. Resection of primary tumor at diagnosis in stage IV-S neuroblastoma: Does it affect the clinical course? *Journal of Clinical Oncology* 1996;14(5):1537-1544.
268. Bowman LC, Hancock ML, Santana VM, Hayes FA, Kun L, Parham DM, Furman WL, Rao BN, Green AA, Crist WM. Impact of intensified therapy on clinical outcome in infants and children with neuroblastoma: The St Jude Children's Research Hospital experience, 1962 to 1988. *Journal of Clinical Oncology* 1991;9(9):1599-1608.
269. Garaventa A, Boni L, Lo Piccolo MS, Tonini GP, Gambini C, Mancini A, Tonegatti L, Carli M, di Montezemolo LC, Di Cataldo A, Casale F, Mazzocco K, Cecchetto G, Rizzo A, Bernardi B. Localized unresectable neuroblastoma: Results of treatment based on clinical prognostic factors. *Annals of Oncology* 2002;13(6):956-964.
270. Matthay KK, Perez C, Seeger RC, Brodeur GM, Shimada H, Atkinson JB, Black CT, Gerbing R, Haase GM, Stram DO, Swift P, Lukens JN. Successful treatment of stage III neuroblastoma based on prospective biologic staging: A Children's Cancer Group study. *Journal of Clinical Oncology* 1998;16(4):1256-1264.
271. Rubie H, Michon J, Plantaz D, Peyroulet MC, Coze C, Frappaz D, Chastagner P, Baranzelli MC, Mechinaud F, Boutard P, Lutz P, Perel Y, Leverger G, de Lumley L, Millot F, Stephan JL, Marguerite G, Hartmann O. Unresectable localized neuroblastoma: Improved survival after primary chemotherapy including carboplatin-etoposide. Neuroblastoma Study Group of the Societe Francaise d'Oncologie Pediatrique (SFOP). *British Journal of Cancer* 1998;77(12):2310-2317.
272. Strother D, van Hoff J, Rao PV, Smith EI, Shamberger RC, Halperin EC, Murray KJ, Castleberry RP. Event-free survival of children with biologically favourable neuroblastoma based on the degree of initial tumour resection: Results from the Pediatric Oncology Group. *European Journal of Cancer* 1997;33(12):2121-2125.
273. Interiano RB, Davidoff AM. Current management of neonatal neuroblastoma. *Current Pediatric Reviews* 2015;11(3):179-187.
274. London WB, Frantz CN, Campbell LA, Seeger RC, Brumback BA, Cohn SL, Matthay KK, Castleberry RP, Diller L. Phase II randomized comparison of topotecan plus cyclophosphamide versus topotecan alone in children with recurrent or refractory neuroblastoma: A Children's Oncology Group study. *Journal of Clinical Oncology* 2010;28(24):3808-3815.
275. Sohara Y, Shimada H, DeClerck YA. Mechanisms of bone invasion and metastasis in human neuroblastoma. *Cancer Letters* 2005;228(1-2):203-209.
276. Kramer K, Kushner B, Heller G, Cheung NK. Neuroblastoma metastatic to the central nervous system. The Memorial Sloan-Kettering Cancer Center experience and a literature review. *Cancer* 2001;91(8):1510-1519.
277. Vandenhoute E, Stump-Guthier C, Lasierra Losada M, Tenenbaum T, Rudolph H, Ishikawa H, Schwerk C, Schrotten H, Durken M, Marz M, Karremann M. The choroid plexus may be an underestimated site of tumor invasion to the brain: An *in vitro* study using neuroblastoma cell lines. *Cancer Cell International* 2015;15:102.
278. Tsutsumimoto T, Williams P, Yoneda T. The SK-N-AS human neuroblastoma cell line develops osteolytic bone metastases with increased angiogenesis and COX-2 expression. *Journal of Bone Oncology* 2014;3(3-4):67-76.
279. Shankar V, Hori H, Kihira K, Lei Q, Toyoda H, Iwamoto S, Komada Y. Mesenchymal stromal cell secretome up-regulates 47 kDa CXCR4 expression, and induce invasiveness in neuroblastoma cell lines. *PLoS One* 2015;10(3):e0120069.
280. Matthay KK. Chemotherapy for neuroblastoma: Does it hit the target? *Lancet Oncology* 2008;9(3):195-196.
281. Granchi D, Corrias MV, Garaventa A, Baglio SR, Cangemi G, Carlini B, Paolucci P, Giunti A, Baldini N. Neuroblastoma and bone metastases: Clinical significance and prognostic value of Dickkopf 1 plasma levels. *Bone* 2011;48(1):152-159.



282. Komada Y, Zhang XL, Zhou YW, Inaba H, Deguchi T, Azuma E, Sakurai M. Flow cytometric analysis of peripheral blood and bone marrow for tumor cells in patients with neuroblastoma. *Cancer* 1998;82(3):591-599.
283. Hale GA, Arora M, Ahn KW, He W, Camitta B, Bishop MR, Bitan M, Cairo MS, Chan K, Childs RW, Copelan E, Davies SM, Perez MA, Doyle JJ, Gale RP, Vicent MG, Horn BN, Hussein AA, Jodele S, Kamani NR, Kasow KA, Kletzel M, Lazarus HM, Lewis VA, Myers KC, Olsson R, Pulsipher M, Qayed M, Sanders JE, Shaw PJ, Soni S, Stiff PJ, Stadtmauer EA, Ueno NT, Wall DA, Grupp SA. Allogeneic hematopoietic cell transplantation for neuroblastoma: The cibmtr experience. *Bone Marrow Transplantation* 2013;48(8):1056-1064.
284. Ootsuka S, Asami S, Sasaki T, Yoshida Y, Nemoto N, Shichino H, Chin M, Mugishima H, Suzuki T. Useful markers for detecting minimal residual disease in cases of neuroblastoma. *Biological & Pharmaceutical Bulletin* 2008;31(6):1071-1074.
285. Reynolds CP. Detection and treatment of minimal residual disease in high-risk neuroblastoma. *Pediatric Transplantation* 2004;8 Suppl 5(s5):56-66.
286. Fukuda M, Miyajima Y, Miyashita Y, Horibe K. Disease outcome may be predicted by molecular detection of minimal residual disease in bone marrow in advanced neuroblastoma: A pilot study. *Journal of Pediatric Hematology/Oncology* 2001;23(1):10-13.
287. Willems L, Waer M, Billiau AD. The graft-versus-neuroblastoma effect of allogeneic hematopoietic stem cell transplantation, a review of clinical and experimental evidence and a perspective on mechanisms. *Pediatric Blood & Cancer* 2014;61(12):2151-2157.
288. Burchill SA, Kinsey SE, Picton S, Roberts P, Pinkerton CR, Selby P, Lewis IJ. Minimal residual disease at the time of peripheral blood stem cell harvest in patients with advanced neuroblastoma. *Medical and Pediatric Oncology* 2001;36(1):213-219.
289. Jansen J, Hanks S, Thompson JM, Dugan MJ, Akard LP. Transplantation of hematopoietic stem cells from the peripheral blood. *Journal of Cellular and Molecular Medicine* 2005;9(1):37-50.
290. Ozyoruk D, Kibar AE, Surucu M, Azak E, Emir S, Cetin, II, Tunc B, Ozbek NY. Pulmonary arterial hypertension in a child with stage-IV neuroblastoma after autologous hematopoietic stem cell transplantation and review of the literature. *Pediatric Transplantation* 2015;19(7):E185-188.
291. Dandoy CE, Hirsch R, Chima R, Davies SM, Jodele S. Pulmonary hypertension after hematopoietic stem cell transplantation. *Biology of Blood and Marrow Transplantation* 2013;19(11):1546-1556.
292. Blatter S, Rottenberg S. Minimal residual disease in cancer therapy - small things make all the difference. *Drug Resistance Updates* 2015;21-22:1-10.
293. Borst P. Cancer drug pan-resistance: Pumps, cancer stem cells, quiescence, epithelial to mesenchymal transition, blocked cell death pathways, persists or what? *Open Biology* 2012;2(5):120066.
294. Alisi A, Cho WC, Locatelli F, Fruci D. Multidrug resistance and cancer stem cells in neuroblastoma and hepatoblastoma. *International Journal of Molecular Sciences* 2013;14(12):24706-24725.
295. Peinemann F, van Dalen EC, Tushabe DA, Berthold F. Retinoic acid post consolidation therapy for high-risk neuroblastoma patients treated with autologous hematopoietic stem cell transplantation. *Cochrane Database Systematic Reviews* 2015;1:CD010685.
296. Parsons K, Bernhardt B, Strickland B. Targeted immunotherapy for high-risk neuroblastoma--the role of monoclonal antibodies. *Annals of Pharmacotherapy* 2013;47(2):210-218.
297. Jackson JR, Kim Y, Seeger RC, Kim ES. A novel minimal residual disease model of neuroblastoma in mice. *Journal of Pediatric Surgery* 2016;51(6):991-994.
298. Reynolds CP, Matthay KK, Villablanca JG, Maurer BJ. Retinoid therapy of high-risk neuroblastoma. *Cancer Letters* 2003;197(1-2):185-192.
299. Matthay K, Reynolds C. Is there a role for retinoids to treat minimal residual disease in neuroblastoma? *British Journal of Cancer* 2000;83(9):1121.
300. DuBois SG, Allen S, Bent M, Hilton JF, Hollinger F, Hawkins R, Courtier J, Mosse YP, Matthay KK. Phase I/II study of (131)I-MIBG with vincristine and 5 days of irinotecan for advanced neuroblastoma. *British Journal of Cancer* 2015;112(4):644-649.
301. DuBois SG, Matthay KK. Radiolabeled metaiodobenzylguanidine for the treatment of neuroblastoma. *Nuclear Medicine and Biology* 2008;35(Suppl 1):S35-S48.
302. DuBois SG, Matthay KK. 131I-metaiodobenzylguanidine therapy in children with advanced neuroblastoma. *Quarterly Journal of Nuclear Medicine and Molecular Imaging* 2013;57(1):53-65.

303. Matthay KK, Yanik G, Messina J, Quach A, Huberty J, Cheng SC, Veatch J, Goldsby R, Brophy P, Kersun LS, Hawkins RA, Maris JM. Phase II study on the effect of disease sites, age, and prior therapy on response to iodine-131-metaiodobenzylguanidine therapy in refractory neuroblastoma. *Journal of Clinical Oncology* 2007;25(9):1054-1060.
304. DuBois SG, Chesler L, Groshen S, Hawkins R, Goodarzi F, Shimada H, Yanik G, Tagen M, Stewart C, Mosse YP, Maris JM, Tsao-Wei D, Marachelian A, Villablanca JG, Matthay KK. Phase I study of vincristine, irinotecan, and (1)(3)(1)I-metaiodobenzylguanidine for patients with relapsed or refractory neuroblastoma: A new approaches to neuroblastoma therapy trial. *Clinical Cancer Research* 2012;18(9):2679-2686.
305. George SL, Falzone N, Chittenden S, Kirk SJ, Lancaster D, Vaidya SJ, Mandeville H, Saran F, Pearson ADJ, Du Y, Meller ST, Denis-Bacelar AM, Flux GD. Individualized (131)I-MIBG therapy in the management of refractory and relapsed neuroblastoma. *Nuclear Medicine Communications* 2016;37(5):466-472.
306. Kushner BH, Ostrovnya I, Cheung IY, Kuk D, Modak S, Kramer K, Roberts SS, Basu EM, Yataghene K, Cheung N-KV. Lack of survival advantage with autologous stem-cell transplantation in high-risk neuroblastoma consolidated by anti-GD2 immunotherapy and isotretinoin. *Oncotarget* 2016;7(4):4155-4166.
307. Neil EC, Hanmantgad S, Khakoo Y. Neurological complications of pediatric cancer. *Journal of Child Neurology* 2015;[Epub ahead of print]:DOI: 10.1177/0883073815620673.
308. Sivakumar S, Poulik J, Sivaswamy L. Monocular blindness as presentation manifestation of neuroblastoma. *Neurohospitalist* 2016;6(1):41.
309. Noguchi K, Katayama K, Sugimoto Y. Human ABC transporter ABCG2/BCRP expression in chemoresistance: Basic and clinical perspectives for molecular cancer therapeutics. *Pharmacogenomics and Personalized Medicine* 2014;7:53-64.
310. Chen Z, Shi T, Zhang L, Zhu P, Deng M, Huang C, Hu T, Jiang L, Li J. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. *Cancer Letters* 2016;370(1):153-164.
311. Fletcher JI, Williams RT, Henderson MJ, Norris MD, Haber M. ABC transporters as mediators of drug resistance and contributors to cancer cell biology. *Drug Resistance Updates* 2016;26:1-9.
312. Gottesman MM, Lavi O, Hall MD, Gillet J-P. Toward a better understanding of the complexity of cancer drug resistance. *Annual Review of Pharmacology and Toxicology* 2016;56:85-102.
313. Li W, Zhang H, Assaraf YG, Zhao K, Xu X, Xie J, Yang D-H, Chen Z-S. Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies. *Drug Resistance Updates* 2016;27:14-29.
314. Wijdeven RH, Pang B, Assaraf YG, Neeffjes J. Old drugs, novel ways out: Drug resistance toward cytotoxic chemotherapeutics. *Drug Resistance Updates* 2016;28:65-81.
315. Wu Q, Yang Z, Nie Y, Shi Y, Fan D. Multi-drug resistance in cancer chemotherapeutics: Mechanisms and lab approaches. *Cancer Letters* 2014;347(2):159-166.
316. Buhagiar A, Ayers D. Chemoresistance, Cancer stem cells, and miRNA influences: The case for neuroblastoma. *Analytical Cellular Pathology (Amsterdam)* 2015;2015:150634.
317. Gillet JP, Gottesman MM. Mechanisms of multidrug resistance in cancer. In: Zhou J, ed. *Methods in Molecular Biology*. New York: Humana Press; 2010;596:47-76.
318. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Research* 2001;11(7):1156-1166.
319. Clevers H. The cancer stem cell: Premises, promises and challenges. *Nature Medicine* 2011;17(3):313-319.
320. Chen W, Dong J, Haiech J, Kilhoffer MC, Zeniou M. Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. *Stem Cells International* 2016;2016:1740936.
321. Boesch M, Wolf D, Sopper S. Optimized stem cell detection using the dyecycle-triggered side population phenotype. *Stem Cells International* 2015;2016.
322. Golebiewska A, Brons NH, Bjerkvig R, Niclou SP. Critical appraisal of the side population assay in stem cell and cancer stem cell research. *Cell Stem Cell* 2011;8(2):136-147.
323. Richard V, Nair MG, Santhosh Kumar TR, Pillai MR. Side population cells as prototype of chemoresistant, tumor-initiating cells. *BioMed Research International* 2013;2013:517237.

324. Koren E, Fuchs Y. The bad seed: Cancer stem cells in tumor development and resistance. *Drug Resistance Updates* 2016;28:1-12.
325. Szakacs G, Hall MD, Gottesman MM, Boumendjel A, Kachadourian R, Day BJ, Baubichon-Cortay H, Di Pietro A. Targeting the Achilles heel of multidrug-resistant cancer by exploiting the fitness cost of resistance. *Chemical Reviews* 2014;114(11):5753-5774.
326. Kathawala RJ, Gupta P, Ashby CR, Jr., Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: A review of the past decade. *Drug Resistance Updates* 2015;18:1-17.
327. Norris MD, Smith J, Tanabe K, Tobin P, Flemming C, Scheffer GL, Wielinga P, Cohn SL, London WB, Marshall GM. Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan *in vitro*. *Molecular Cancer Therapeutics* 2005;4(4):547-553.
328. Porro A, Haber M, Diolaiti D, Iraci N, Henderson M, Gherardi S, Valli E, Munoz MA, Xue C, Flemming C. Direct and coordinate regulation of ATP-binding cassette transporter genes by Myc factors generates specific transcription signatures that significantly affect the chemoresistance phenotype of cancer cells. *Journal of Biological Chemistry* 2010;285(25):19532-19543.
329. Haber M, Smith J, Bordow SB, Flemming C, Cohn SL, London WB, Marshall GM, Norris MD. Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma. *Journal of Clinical Oncology* 2006;24(10):1546-1553.
330. Henderson MJ, Haber M, Porro A, Munoz MA, Iraci N, Xue C, Murray J, Flemming CL, Smith J, Fletcher JI, Gherardi S, Kwek CK, Russell AJ, Valli E, London WB, Buxton AB, Ashton LJ, Sartorelli AC, Cohn SL, Schwab M, Marshall GM, Perini G, Norris MD. ABCC multidrug transporters in childhood neuroblastoma: Clinical and biological effects independent of cytotoxic drug efflux. *Journal of the National Cancer Institute* 2011;103(16):1236-1251.
331. Keshelava N, Zuo JJ, Chen P, Waidyaratne SN, Luna MC, Gomer CJ, Triche TJ, Reynolds CP. Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines. *Cancer Research* 2001;61(16):6185-6193.
332. Xue C, Haber M, Flemming C, Marshall GM, Lock RB, MacKenzie KL, Gurova KV, Norris MD, Gudkov AV. P53 determines multidrug sensitivity of childhood neuroblastoma. *Cancer Research* 2007;67(21):10351-10360.
333. Bourhis J, Benard J, Hartmann O, Boccon-Gibod L, Lemerle J, Riou G. Correlation of MDR1 gene expression with chemotherapy in neuroblastoma. *Journal of the National Cancer Institute* 1989;81(18):1401-1405.
334. Corrias MV, Cornaglia-Ferraris P, Di Martino D, Stenger AM, Lanino E, Boni L, Tonini GP. Expression of multiple drug resistance gene, MDR1, and N-myc oncogene in an Italian population of human neuroblastoma patients. *Anticancer Research* 1990;10(4):897-902.
335. Goldstein LJ, Fojo AT, Ueda K, Crist W, Green A, Brodeur G, Pastan I, Gottesman MM. Expression of the multidrug resistance, MDR1, gene in neuroblastomas. *Journal of Clinical Oncology* 1990;8(1):128-136.
336. Yoshida GJ, Saya H. Therapeutic strategies targeting cancer stem cells. *Cancer Science* 2016;107(1):5-11.
337. Dean M. ABC transporters, drug resistance, and cancer stem cells. *Journal of Mammary Gland Biology and Neoplasia* 2009;14(1):3-9.
338. Adkins ES, Doski JJ, Geiger JD, LaQuaglia MP, Mattei P, Nuchtern JG. Handbook for children with neuroblastoma. *American Pediatric Surgical Association* 2015;<http://www.eapsa.org/apsa/media/Documents/>.
339. Pearson AD, Pinkerton CR, Lewis IJ, Imeson J, Ellershaw C, Machin D, European Neuroblastoma Study G, Children's C, Leukaemia G. High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: A randomised trial. *Lancet Oncology* 2008;9(3):247-256.
340. Imamovic L, Sommer MO. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Science Translational Medicine* 2013;5(204):204ra132.
341. Pluchino KM, Hall MD, Goldsborough AS, Callaghan R, Gottesman MM. Collateral sensitivity as a strategy against cancer multidrug resistance. *Drug Resistance Updates* 2012;15(1-2):98-105.
342. Hall MD, Handley MD, Gottesman MM. Is resistance useless? Multidrug resistance and collateral sensitivity. *Trends in Pharmacological Sciences* 2009;30(10):546-556.

343. Laberge RM, Ambadipudi R, Georges E. P-glycoprotein mediates the collateral sensitivity of multidrug resistant cells to steroid hormones. *Biochemical and Biophysical Research Communications* 2014;447(4):574-579.
344. Kushner BH, Modak S, Kramer K, Basu EM, Roberts SS, Cheung NK. Ifosfamide, carboplatin, and etoposide for neuroblastoma: A high-dose salvage regimen and review of the literature. *Cancer* 2013;119(3):665-671.
345. Matthay KK, George RE, Yu AL. Promising therapeutic targets in neuroblastoma. *Clinical Cancer Research* 2012;18(10):2740-2753.
346. Bhatnagar SN, Sarin YK. Neuroblastoma: A review of management and outcome. *The Indian Journal of Pediatrics* 2012;79(6):787-792.
347. Meadows AT, D'Angio GJ, Evans AE, Harris CC, Miller RW, Mike V. Oncogenesis and other late effects of cancer treatment in children: Report of a single Hospital study 1. *Radiology* 1975;114(1):175-180.
348. Meadows AT, Baum E, Fossati-Bellani F, Green D, Jenkin R, Marsden B, Nesbit M, Newton W, Oberlin O, Sallan SG. Second malignant neoplasms in children: An update from the late effects Study Group. *Journal of Clinical Oncology* 1985;3(4):532-538.
349. Laverdière C, Cheung NKV, Kushner BH, Kramer K, Modak S, LaQuaglia MP, Wolden S, Ness KK, Gurney JG, Sklar CA. Long-term complications in survivors of advanced stage neuroblastoma. *Pediatric Blood & Cancer* 2005;45(3):324-332.
350. Laverdière C, Liu Q, Yasui Y, Nathan PC, Gurney JG, Stovall M, Diller LR, Cheung N-K, Wolden S, Robison LL. Long-term outcomes in survivors of neuroblastoma: A report from the childhood Cancer survivor Study. *Journal of the National Cancer Institute* 2009;101(16):1131-1140.
351. Moreno L, Vaidya SJ, Pinkerton CR, Lewis IJ, Imeson J, Machin D, Pearson AD. Long-term follow-up of children with high-risk neuroblastoma: The ens5 trial experience. *Pediatric Blood & Cancer* 2013;60(7):1135-1140.
352. Kushner BH, Kramer K, Modak S, Qin LX, Yataghena K, Jhanwar SC, Cheung NKV. Reduced risk of secondary leukemia with fewer cycles of dose-intensive induction chemotherapy in patients with neuroblastoma. *Pediatric Blood & Cancer* 2009;53(1):17-22.
353. Van Santen H, De Kraker J, Vulsma T. Endocrine late effects from multi-modality treatment of neuroblastoma. *European Journal of Cancer* 2005;41(12):1767-1774.
354. Uray IP, Dmitrovsky E, Brown PH. Retinoids and rexinoids in cancer prevention: From laboratory to clinic. *Seminars in Oncology* 2016;43(1):49-64.
355. Melino G, Thiele CJ, Knight RA, Piacentini M. Retinoids and the control of growth/death decisions in human neuroblastoma cell lines. *Journal of Neuro-Oncology* 1997;31(1-2):65-83.
356. Reynolds C, Schindler P, Jones D, Gentile J, Proffitt R, Einhorn P. Comparison of 13-cis-retinoic acid to trans-retinoic acid using human neuroblastoma cell lines. *Progress in Clinical and Biological Research* 1994;385:237.
357. di Masi A, Leboffe L, De Marinis E, Pagano F, Cicconi L, Rochette-Egly C, Lo-Coco F, Ascenzi P, Nervi C. Retinoic acid receptors: From molecular mechanisms to cancer therapy. *Molecular Aspects of Medicine* 2015;41:1-115.
358. Park JR, Villablanca JG, London WB, Gerbing RB, Haas-Kogan D, Adkins ES, Attiyeh EF, Maris JM, Seeger RC, Reynolds CP, Matthay KK, Children's Oncology G. Outcome of high-risk stage 3 neuroblastoma with myeloablative therapy and 13-cis-retinoic acid: A report from the Children's Oncology Group. *Pediatric Blood & Cancer* 2009;52(1):44-50.
359. Huang S, Laoukili J, Epping MT, Koster J, Hölzel M, Westerman BA, Nijkamp W, Hata A, Asgharzadeh S, Seeger RC. ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. *Cancer Cell* 2009;15(4):328-340.
360. Adamson PC, Matthay KK, O'Brien M, Reaman GH, Sato JK, Balis FM. A phase 2 trial of all-trans-retinoic acid in combination with interferon- $\alpha$ 2a in children with recurrent neuroblastoma or Wilms tumor: A Pediatric Oncology branch, NCI and Children's Oncology Group Study. *Pediatric Blood & Cancer* 2007;49(5):661-665.
361. Ponthan F, Johnsen JI, Klevenvall L, Castro J, Kogner P. The synthetic retinoid ro 13-6307 induces neuroblastoma differentiation *in vitro* and inhibits neuroblastoma tumour growth *in vivo*. *International Journal of Cancer* 2003;104(4):418-424.
362. Meseguer S, Laserna EJ, Escamilla JM, Masiá S, Baretino D. Regulation of neuroblastoma cell differentiation by retinoic acid: Role of alternative splicing and micro-rnas. In: Hayat MA, ed. *Pediatric Cancer*: Springer Netherlands; 2013;4:37-47.



363. Silvis AM, McCormick ML, Spitz DR, Kiningham KK. Redox balance influences differentiation status of neuroblastoma in the presence of all-trans retinoic acid. *Redox Biology* 2016;7:88-96.
364. Bochennek K, Esser R, Lehrnbecher T, Glienke W, Wehner S, Erben S, Soerensen J, Schwabe D, Bader P, Klingebiel T, Koehl U. Impact of minimal residual disease detection prior to autologous stem cell transplantation for post-transplant outcome in high risk neuroblastoma. *Klinische Padiatrie* 2012;224(3):139-142.
365. Cai JY, Pan C, Tang YJ, Chen J, Ye QD, Zhou M, Xue H, Tang JY. Minimal residual disease is a prognostic marker for neuroblastoma with bone marrow infiltration. *American Journal of Clinical Oncology: Cancer Clinical Trials* 2012;35(3):275-278.
366. Chambon F, Tchirkov A, Pereira B, Rochette E, Deméocq F, Kanold J. Molecular assessment of minimal residual disease in PBSC harvests provides prognostic information in neuroblastoma. *Pediatric Blood and Cancer* 2013;60(9):E109-E112.
367. van Wezel EM, Stutterheim J, Vree F, Zappeij-Kannegieter L, Decarolis B, Hero B, Berthold F, Schumacher-Kuckelkorn R, Simon T, Fiocco M, Voermans C, van Noesel MM, Caron HN, van der Schoot CE, Tytgat GAM, Koehl U, Schulte JH, Nigli F, Fruhwald MC, Niemeyer CM, Bode U, Schilling FH, Schultz C, Graf N, Nathrath M, Schmid I. Minimal residual disease detection in autologous stem cell grafts from patients with high risk neuroblastoma. *Pediatric Blood and Cancer* 2015;62(8):1368-1373.
368. Van Wezel EM, Decarolis B, Stutterheim J, Zappeij-Kannegieter L, Berthold F, Schumacher-Kuckelkorn R, Simon T, Fiocco M, Van Noesel MM, Caron HN, Van Der Schoot CE, Hero B, Tytgat GAM. Neuroblastoma messenger RNA is frequently detected in bone marrow at diagnosis of localised neuroblastoma patients. *European Journal of Cancer* 2016;54:149-158.
369. Reynolds C, Wang Y, Melton L, Einhorn P, Slamon D, Maurer B. Retinoic-acid-resistant neuroblastoma cell lines show altered MYC regulation and high sensitivity to fenretinide. *Medical and Pediatric Oncology* 2000;35(6):597-602.
370. Tee A, Marshall GM, Liu PY, Liu T. Neuroblastoma: A malignancy due to cell differentiation block. In: Shimada H, ed. *Neuroblastoma - present and future*. Available from: <http://www.intechopen.com/books/neuroblastoma-present-and-future/neuroblastoma-a-malignancy-due-to-cell-differentiation-block>: INTECH Open Access Publisher; 2012;DOI: 10.5772/27865:79-84.
371. Ribatti D, Alessandri G, Baronio M, Raffaghella L, Cosimo E, Marimpietri D, Montaldo PG, De Falco G, Caruso A, Vacca A, Ponzoni M. Inhibition of neuroblastoma-induced angiogenesis by fenretinide. *International Journal of Cancer* 2001;94(3):314-321.
372. Reynolds CP, Lemons RS. Retinoid therapy of childhood cancer. *Hematology/Oncology Clinics of North America* 2001;15(5):867-910.
373. Escamilla JM, López CM, Bäuerl C, Baretino D, Navarro S, Pekkala SP. Retinoic-acid-induced downregulation of the 67 kda laminin receptor correlates with reduced biological aggressiveness of human neuroblastoma cells. In: Shimada H, ed. *Neuroblastoma - present and future*. Available from: <http://www.intechopen.com/books/neuroblastoma-present-and-future/retinoic-acid-induced-downregulation-of-the-67-kda-laminin-receptor-correlates-with-reduced-biologic>: INTECH Open Access Publisher; 2012;DOI: 10.5772/27102:217-232.
374. Gudas LJ. Retinoids induce stem cell differentiation via epigenetic changes. *Seminars in Cell & Developmental Biology* 2013;24(10-12):701-705.
375. Gudas LJ, Wagner JA. Retinoids regulate stem cell differentiation. *Journal of Cellular Physiology* 2011;226(2):322-330.
376. Nicolai S, Pieraccioli M, Peschiaroli A, Melino G, Raschella G. Neuroblastoma: Oncogenic mechanisms and therapeutic exploitation of necroptosis. *Cell Death & Disease* 2015;6:e2010.
377. Villablanca JG, Khan AA, Avramis VI, Seeger RC, Matthay KK, Ramsay NK, Reynolds CP. Phase I trial of 13-cis-retinoic acid in children with neuroblastoma following bone marrow transplantation. *Journal of Clinical Oncology* 1995;13(4):894-901.
378. Adamson PC, Matthay KK, O'Brien M, Reaman GH, Sato JK, Balis FM. A phase 2 trial of all-trans-retinoic acid in combination with interferon- $\alpha$ 2a in children with recurrent neuroblastoma or Wilms tumor: A Pediatric Oncology branch, NCI and Children's Oncology Group Study. *Pediatric Blood & Cancer* 2007;49(5):661-665.
379. Matthay KK, Reynolds CP, Seeger RC, Shimada H, Adkins ES, Haas-Kogan D, Gerbing RB, London WB, Villablanca JG. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: A children's oncology group study. *Journal of Clinical Oncology* 2009;27(7):1007-1013.

380. Veal GJ, Errington J, Rowbotham SE, Illingworth NA, Malik G, Cole M, Daly AK, Pearson AD, Boddy AV. Adaptive dosing approaches to the individualization of 13-cis-retinoic acid (isotretinoin) treatment for children with high-risk neuroblastoma. *Clinical Cancer Research* 2013;19(2):469-479.
381. Efeyan A, Sabatini DM. mTOR and cancer: Many loops in one pathway. *Current Opinion in Cell Biology* 2010;22(2):169-176.
382. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes & Development* 2004;18(16):1926-1945.
383. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. *Nature Reviews: Drug Discovery* 2006;5(8):671-688.
384. Populo H, Lopes JM, Soares P. The mTOR signalling pathway in human cancer. *International Journal of Molecular Sciences* 2012;13(2):1886-1918.
385. Cheng H, Walls M, Baxi SM, Yin MJ. Targeting the mTOR pathway in tumor malignancy. *Current Cancer Drug Targets* 2013;13(3):267-277.
386. Moschetta M, Reale A, Marasco C, Vacca A, Carratu MR. Therapeutic targeting of the mTOR-signalling pathway in cancer: Benefits and limitations. *British Journal of Pharmacology* 2014;171(16):3801-3813.
387. Mei H, Wang Y, Lin Z, Tong Q. The mTOR signaling pathway in pediatric neuroblastoma. *Pediatric Hematology and Oncology* 2013;30(7):605-615.
388. Misawa A, Hosoi H, Tsuchiya K, Sugimoto T. Rapamycin inhibits proliferation of human neuroblastoma cells without suppression of MycN. *International Journal of Cancer* 2003;104(2):233-237.
389. Johnsen JJ, Segerstrom L, Orrego A, Elfman L, Henriksson M, Kagedal B, Eksborg S, Sveinbjornsson B, Kogner P. Inhibitors of mammalian target of rapamycin downregulate MYCN protein expression and inhibit neuroblastoma growth *in vitro* and *in vivo*. *Oncogene* 2008;27(20):2910-2922.
390. Opel D, Poremba C, Simon T, Debatin K-M, Fulda S. Activation of Akt predicts poor outcome in neuroblastoma. *Cancer Research* 2007;67(2):735-745.
391. Cella CA, Minucci S, Spada F, Galdy S, Elgendy M, Ravenda PS, Zampino MG, Murgioni S, Fazio N. Dual inhibition of mTOR pathway and VEGF signalling in neuroendocrine neoplasms: From bench to bedside. *Cancer Treatment Reviews* 2015;41(9):754-760.
392. Barone G, Anderson J, Pearson AD, Petrie K, Chesler L. New strategies in neuroblastoma: Therapeutic targeting of MYCN and ALK. *Clinical Cancer Research* 2013;19(21):5814-5821.
393. Corbacioglu S. Molecular-targeted therapy in refractory or relapsed neuroblastoma. In: Christiansen H, Christiansen NM, eds. *Progressive Neuroblastoma: Innovation and Novel Therapeutic Strategies*. Basel: Karger Publishers; 2015;20:121-137.
394. Gomez-Villafuertes R, Garcia-Huerta P, Diaz-Hernandez JI, Miras-Portugal MT. PI3K/Akt signaling pathway triggers p27 receptor expression as a pro-survival factor of neuroblastoma cells under limiting growth conditions. *Scientific Reports* 2015;5:18417.
395. King D, Yeomanson D, Bryant HE. Pi3king the lock: Targeting the PI3K/Akt/mTOR pathway as a novel therapeutic strategy in neuroblastoma. *Journal of Pediatric Hematology/Oncology* 2015;37(4):245-251.
396. Nam SO, Yotsumoto F, Miyata K, Souzaki R, Taguchi T, Kuroki M, Miyamoto S. Validity of hb-EGF as target for human neuroblastoma therapy. *Anticancer Research* 2015;35(8):4433-4440.
397. Zheng Y, Jiang Y. mTOR inhibitors at a glance. *Molecular and Cellular Pharmacology* 2015;7(2):15-20.
398. Chaturvedi NK, McGuire TR, Coulter DW, Shukla A, McIntyre EM, Sharp JG, Joshi SS. Improved therapy for neuroblastoma using a combination approach: Superior efficacy with vismodegib and topotecan. *Oncotarget* 2016;7(12):15215-15229.
399. Smith JR, Moreno L, Heaton SP, Chesler L, Pearson ADJ, Garrett MD. Novel pharmacodynamic biomarkers for MYCN protein and PI3K/AKT/mTOR pathway signaling in children with neuroblastoma. *Molecular Oncology* 2016;10(4):538-552.
400. Georger B, Kieran MW, Grupp S, Perek D, Clancy J, Krygowski M, Ananthakrishnan R, Boni JP, Berkenblit A, Spunt SL. Phase II trial of temsirolimus in children with high-grade glioma, neuroblastoma and rhabdomyosarcoma. *European Journal of Cancer* 2012;48(2):253-262.
401. Fouladi M, Laningham F, Wu J, O'Shaughnessy MA, Molina K, Broniscer A, Spunt SL, Lueckert I, Stewart CF, Houghton PJ, Gilbertson RJ, Furman WL. Phase I Study of everolimus in Pediatric patients with refractory solid tumors. *Journal of Clinical Oncology* 2007;25(30):4806-4812.
402. Gore L, Trippett TM, Katzenstein HM, Boklan J, Narendran A, Smith A, Macy ME, Rolla K, Narashimhan N, Squillace RM, Turner CD, Haluska FG, Nieder M. A multicenter, first-in-pediatrics,



- phase I, pharmacokinetic and pharmacodynamic study of ridaforolimus in patients with refractory solid tumors. *American Association for Cancer Research* 2013;19(13):3649-3658.
403. Zhang H, Dou J, Yu Y, Zhao Y, Fan Y, Cheng J, Xu X, Liu W, Guan S, Chen Z, Shi Y, Patel R, Vasudevan SA, Zage PE, Zhang H, Nuchtern JG, Kim ES, Fu S, Yang J. mTOR ATP-competitive inhibitor INK128 inhibits neuroblastoma growth via blocking mTOR signaling. *Apoptosis* 2015;20(1):50-62.
  404. Li Z, Yan S, Attayan N, Ramalingam S, Thiele CJ. Combination of an allosteric Akt inhibitor MK-2206 with etoposide or rapamycin enhances the antitumor growth effect in neuroblastoma. *American Association for Cancer Research* 2012;18(13):3603-3615.
  405. Otto T, Horn S, Brockmann M, Eilers U, Schuttrumpf L, Popov N, Kenney AM, Schulte JH, Beijersbergen R, Christiansen H, Berwanger B, Eilers M. Stabilization of N-Myc is a critical function of Aurora a in human neuroblastoma. *Cancer Cell* 2009;15(1):67-78.
  406. Bell E, Chen L, Liu T, Marshall GM, Lunec J, Tweddle DA. MYCN oncoprotein targets and their therapeutic potential. *Cancer Letters* 2010;293(2):144-157.
  407. Nikonova AS, Atsaturov I, Serebriiskii IG, Dunbrack RL, Golemis EA. Aurora a kinase (AURKA) in normal and pathological cell division. *Cellular and Molecular Life Sciences* 2013;70(4):661-687.
  408. Mosse YP, Lipsitz E, Fox E, Teachey DT, Maris JM, Weigel B, Adamson PC, Ingle MA, Ahern CH, Blaney SM. Pediatric phase I trial and pharmacokinetic study of MLN8237, an investigational oral selective small-molecule inhibitor of Aurora kinase a: A Children's Oncology Group Phase I consortium study. *Clinical Cancer Research* 2012;18(21):6058-6064.
  409. Shang X, Burlingame SM, Okcu MF, Ge N, Russell HV, Egler RA, David RD, Vasudevan SA, Yang J, Nuchtern JG. Aurora a is a negative prognostic factor and a new therapeutic target in human neuroblastoma. *American Association for Cancer Research* 2009;8(8):2461-2469.
  410. Maris JM, Morton CL, Gorlick R, Kolb EA, Lock R, Carol H, Keir ST, Reynolds CP, Kang MH, Wu J, Smith MA, Houghton PJ. Initial testing of the aurora kinase a inhibitor MLN8237 by the Pediatric Preclinical Testing Program (PPTP). *Pediatric Blood & Cancer* 2010;55(1):26-34.
  411. Brockmann M, Poon E, Berry T, Carstensen A, Deubzer HE, Rycak L, Jamin Y, Thway K, Robinson SP, Roels F, Witt O, Fischer M, Chesler L, Eilers M. Small molecule inhibitors of Aurora a induce proteasomal degradation of N-myc in childhood neuroblastoma. *Cancer Cell* 2013;24(1):75-89.
  412. Romain C, Paul P, Kim KW, Lee S, Qiao J, Chung DH. Targeting Aurora kinase a downregulates cell proliferation and angiogenesis in neuroblastoma. *Journal of Pediatric Surgery* 2014;49(1):159-165.
  413. Ramani P, Nash R, Rogers CA. Aurora kinase a is superior to Ki67 as a prognostic indicator of survival in neuroblastoma. *Histopathology* 2015;66(3):370-379.
  414. DuBois SG, Marachelian A, Fox E, Kudgus RA, Reid JM, Groshen S, Malvar J, Bagatell R, Wagner L, Maris JM, Hawkins R, Courtier J, Lai H, Goodarzi F, Shimada H, Czarnecki S, Tsao-Wei D, Matthay KK, Mosse YP. Phase I study of the Aurora a kinase inhibitor alisertib in combination with irinotecan and temozolomide for patients with relapsed or refractory neuroblastoma: A NANT (new approaches to Neuroblastoma therapy) trial. *Journal of Clinical Oncology* 2016;34(12):1368-1375.
  415. Cheung CH, Lin WH, Hsu JT, Hour TC, Yeh TK, Ko S, Lien TW, Coumar MS, Liu JF, Lai WY, Shiao HY, Lee TR, Hsieh HP, Chang JY. Bpr1k653, a novel Aurora kinase inhibitor, exhibits potent anti-proliferative activity in MDR1 (p-gp170)-mediated multidrug-resistant cancer cells. *PloS One* 2011;6(8):e23485.
  416. Michaelis M, Selt F, Rothweiler F, Loschmann N, Nusse B, Dirks WG, Zehner R, Cinatl J, Jr. Aurora kinases as targets in drug-resistant neuroblastoma cells. *PloS One* 2014;9(9):e108758.
  417. Michaelis M, Selt F, Rothweiler F, Wiese M, Cinatl J, Jr. ABCG2 impairs the activity of the aurora kinase inhibitor tozasertib but not of alisertib. *BMC Research Notes* 2015;8:484.
  418. Brodeur GM, Minturn JE, Ho R, Simpson AM, Iyer R, Varela CR, Light JE, Kolla V, Evans AE. Trk receptor expression and inhibition in neuroblastomas. *Clinical Cancer Research* 2009;15(10):3244-3250.
  419. Brodeur GM, Nakagawara A, Yamashiro DJ, Ikegaki N, Liu XG, Azar CG, Lee CP, Evans AE. Expression of TrkA, TrkB and TrkC in human neuroblastomas. *Journal of Neuro-Oncology* 1997;31(1-2):49-55.
  420. Thiele CJ, Li Z, McKee AE. On Trk—the TrkB signal transduction pathway is an increasingly important target in cancer biology. *Clinical Cancer Research* 2009;15(19):5962-5967.
  421. Nakagawara A. Trk receptor tyrosine kinases: A bridge between cancer and neural development. *Cancer Letters* 2001;169(2):107-114.

422. Kogner P, Barbany G, Dominici C, Castello MA, Raschella G, Persson H. Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. *Cancer Research* 1993;53(9):2044-2050.
423. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *New England Journal of Medicine* 1993;328(12):847-854.
424. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-b and BDNF in human neuroblastomas. *Molecular and Cellular Biology* 1994;14(1):759-767.
425. Nakagawara A, Brodeur G. Role of neurotrophins and their receptors in human neuroblastomas: A primary culture study. *European Journal of Cancer* 1997;33(12):2050-2053.
426. Ichim G, Tauszig-Delamasure S, Mehlen P. Neurotrophins and cell death. *Experimental Cell Research* 2012;318(11):1221-1228.
427. Huang EJ, Reichardt LF. Trk receptors: Roles in neuronal signal transduction. *Annual Review of Biochemistry* 2003;72(1):609-642.
428. Evans AE, Kisselbach KD, Yamashiro DJ, Ikegaki N, Camoratto AM, Dionne CA, Brodeur GM. Antitumor activity of CEP-751 (KT-6587) on human neuroblastoma and medulloblastoma xenografts. *Clinical Cancer Research* 1999;5(11):3594-3602.
429. Evans AE, Kisselbach KD, Liu X, Eggert A, Ikegaki N, Camoratto AM, Dionne C, Brodeur GM. Effect of CEP-751 (KT-6587) on neuroblastoma xenografts expressing TrkB. *Medical and Pediatric Oncology* 2001;36(1):181-184.
430. Ho R, Eggert A, Hishiki T, Minturn JE, Ikegaki N, Foster P, Camoratto AM, Evans AE, Brodeur GM. Resistance to chemotherapy mediated by TrkB in neuroblastomas. *Cancer Research* 2002;62(22):6462-6466.
431. Iyer R, Evans AE, Qi X, Ho R, Minturn JE, Zhao H, Balamuth N, Maris JM, Brodeur GM. Lestaurtinib enhances the antitumor efficacy of chemotherapy in murine xenograft models of neuroblastoma. *Clinical Cancer Research* 2010;16(5):1478-1485.
432. Minturn JE, Evans AE, Villablanca JG, Yanik GA, Park JR, Shusterman S, Groshen S, Hellriegel ET, Bensen-Kennedy D, Matthay KK, Brodeur GM, Maris JM. Phase I trial of lestaurtinib for children with refractory neuroblastoma: A new approaches to neuroblastoma therapy consortium study. *Cancer Chemotherapy and Pharmacology* 2011;68(4):1057-1065.
433. De Braud FG, Pilla L, Niger M, Damian S, Bardazza B, Martinetti A, Pelosi G, Marrapese G, Palmeri L, Cerea G. Phase 1 open label, dose escalation study of RXDX101, an oral PAN-TRK, ROS1, and ALK inhibitor, in patients with advanced solid tumors with relevant molecular alterations. *Journal of Clinical Oncology* 2014;32(5).
434. Vaishnavi A, Le AT, Doebele RC. Trking down an old oncogene in a new era of targeted therapy. *Cancer Discovery* 2015;5(1):25-34.
435. Lode HN. Approaches to passive and active vaccination against neuroblastoma. In: Christiansen H, Christiansen NM, eds. *Progressive Neuroblastoma: Innovation and Novel Therapeutic Strategies*. Basel: Karger Publishers; 2015;20:150-162.
436. Fest S, Starke S. Immune regulation in neuroblastoma. In: Christiansen H, Christiansen NM, eds. *Progressive Neuroblastoma: Innovation and Novel Therapeutic Strategies*. Basel: Karger Publishers; 2015;20:138-149.
437. Bremm M, Brehm C, Huenecke S, Rettinger E, Bader P. Role of cell therapy in neuroblastoma. In: Christiansen H, Christiansen NM, eds. *Progressive Neuroblastoma: Innovation and Novel Therapeutic Strategies*. Basel: Karger Publishers; 2015;20:163-177.
438. Bottino C, Dondero A, Bellora F, Moretta L, Locatelli F, Pistoia V, Moretta A, Castriconi R. Natural killer cells and neuroblastoma: Tumor recognition, escape mechanisms, and possible novel immunotherapeutic approaches. *Frontiers in Immunology* 2014;5:56.
439. Wu ZL, Schwartz E, Seeger R, Ladisch S. Expression of GD2 ganglioside by untreated primary human neuroblastomas. *Cancer Research* 1986;46(1):440-443.
440. Tarek N, Le Luavec JB, Gallagher MM, Zheng J, Venstrom JM, Chamberlain E, Modak S, Heller G, Dupont B, Cheung NK, Hsu KC. Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *Journal of Clinical Investigation* 2012;122(9):3260-3270.
441. Cheung NK, Cheung IY, Kushner BH, Ostrovnyaya I, Chamberlain E, Kramer K, Modak S. Murine anti-GD2 monoclonal antibody 3f8 combined with granulocyte-macrophage colony-stimulating factor and 13-

- cis-retinoic acid in high-risk patients with stage 4 neuroblastoma in first remission. *Journal of Clinical Oncology* 2012;30(26):3264-3270.
442. Pistoia V, Morandi F, Bianchi G, Pezzolo A, Prigione I, Raffaghello L. Immunosuppressive microenvironment in neuroblastoma. *Frontiers in Oncology* 2013;3:167.
443. Hillen F, Griffioen AW. Tumour vascularization: Sprouting angiogenesis and beyond. *Cancer and Metastasis Reviews* 2007;26(3-4):489-502.
444. Eggert A, Ikegaki N, Kwiatkowski J, Zhao H, Brodeur GM, Himelstein BP. High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. *Clinical Cancer Research* 2000;6(5):1900-1908.
445. Meitar D, Crawford SE, Rademaker AW, Cohn SL. Tumor angiogenesis correlates with metastatic disease, N-myc amplification, and poor outcome in human neuroblastoma. *Journal of Clinical Oncology* 1996;14(2):405-414.
446. Ribatti D. Anti-angiogenesis in neuroblastoma. *Critical Reviews in Oncology/Hematology* 2013;86(3):212-221.
447. Rössler J. Neuroblastoma and angiogenesis. In: Christiansen H, Christiansen NM, eds. *Progressive Neuroblastoma: Innovation and Novel Therapeutic Strategies*. Basel: Karger Publishers; 2015;20:89-106.
448. Roy Choudhury S, Karmakar S, Banik NL, Ray SK. Targeting angiogenesis for controlling neuroblastoma. *Journal of Oncology* 2012;2012:782020.
449. Becker J. Inhibition of Neuroblastoma progression by targeting lymphangiogenesis: Role of an endogenous soluble splice-variant of VEGFR-2. In: Hayat MA, ed. *Tumors of the Central Nervous System, Molecular mechanisms, Children's Cancer, Treatments, and Radiosurgery*. Dordrecht: Springer Netherlands; 2014;12:63-71.
450. Kim ES, Serur A, Huang J, Manley CA, McCrudden KW, Frischer JS, Soffer SZ, Ring L, New T, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Kandel JJ, Yamashiro DJ. Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99(17):11399-11404.
451. Treps L, Conradi LC, Harjes U, Carmeliet P. Manipulating angiogenesis by targeting endothelial metabolism: Hitting the engine rather than the drivers-a new perspective? *Pharmacological Reviews* 2016;68(3):872-887.
452. Xiang X, Zhao X, Qu H, Li D, Yang D, Pu J, Mei H, Zhao J, Huang K, Zheng L, Tong Q. Hepatocyte nuclear factor 4 alpha promotes the invasion, metastasis and angiogenesis of neuroblastoma cells via targeting matrix metalloproteinase 14. *Cancer Letters* 2015;359(2):187-197.
453. Amoroso F, Capece M, Rotondo A, Cangelosi D, Ferracin M, Franceschini A, Raffaghello L, Pistoia V, Varesio L, Adinolfi E. The p2x7 receptor is a key modulator of the PI3K/GSK3beta/VEGF signaling network: Evidence in experimental neuroblastoma. *Oncogene* 2015;34(41):5240-5251.
454. Zhang H, Pu J, Qi T, Qi M, Yang C, Li S, Huang K, Zheng L, Tong Q. MicroRNA-145 inhibits the growth, invasion, metastasis and angiogenesis of neuroblastoma cells through targeting hypoxia-inducible factor 2 alpha. *Oncogene* 2014;33(3):387-397.
455. Romain C, Paul P, Kim KW, Lee S, Qiao J, Chung DH. Targeting Aurora kinase-a downregulates cell proliferation and angiogenesis in neuroblastoma. *Journal of Pediatric Surgery* 2014;49(1):159-165.
456. Calero R, Morchon E, Johnsen JI, Serrano R. Sunitinib suppress neuroblastoma growth through degradation of MYCN and inhibition of angiogenesis. *PloS One* 2014;9(4):e95628.
457. Li D, Mei H, Qi M, Yang D, Zhao X, Xiang X, Pu J, Huang K, Zheng L, Tong Q. Foxd3 is a novel tumor suppressor that affects growth, invasion, metastasis and angiogenesis of neuroblastoma. *Oncotarget* 2013;4(11):2021-2044.
458. Zhang H, Qi M, Li S, Qi T, Mei H, Huang K, Zheng L, Tong Q. microRNA-9 targets matrix metalloproteinase 14 to inhibit invasion, metastasis, and angiogenesis of neuroblastoma cells. *Molecular Cancer Therapeutics* 2012;11(7):1454-1466.
459. Michaelis M, Hinsch N, Michaelis UR, Rothweiler F, Simon T, ilhelm Doerr HW, Cinatl J, Cinatl J, Jr. Chemotherapy-associated angiogenesis in neuroblastoma tumors. *American Journal of Pathology* 2012;180(4):1370-1377.
460. Kakodkar NC, Peddinti RR, Tian Y, Guerrero LJ, Chlenski A, Yang Q, Salwen HR, Maitland ML, Cohn SL. Sorafenib inhibits neuroblastoma cell proliferation and signaling, blocks angiogenesis, and impairs tumor growth. *Pediatric Blood & Cancer* 2012;59(4):642-647.

461. Di Paolo D, Ambrogio C, Pastorino F, Brignole C, Martinengo C, Carosio R, Loi M, Pagnan G, Emionite L, Cilli M, Ribatti D, Allen TM, Chiarle R, Ponzoni M, Perri P. Selective therapeutic targeting of the anaplastic lymphoma kinase with liposomal siRNA induces apoptosis and inhibits angiogenesis in neuroblastoma. *Molecular Therapy* 2011;19(12):2201-2212.
462. Yasuda C, Sakata S, Kakinoki S, Takeyama Y, Ohyanagi H, Shiozaki H. In vivo evaluation of microspheres containing the angiogenesis inhibitor, tnp-470, and the metastasis suppression with liver metastatic model implanted neuroblastoma. *Pathophysiology* 2010;17(2):149-155.
463. Peirce SK, Findley HW, Prince C, Dasgupta A, Cooper T, Durden DL. The PI-3 kinase-Akt-MDM2-survivin signaling axis in high-risk neuroblastoma: A target for PI-3 kinase inhibitor intervention. *Cancer Chemotherapy and Pharmacology* 2011;68(2):325-335.
464. Santo EE, Stroeken P, Sluis PV, Koster J, Versteeg R, Westerhout EM. Foxo3a is a major target of inactivation by PI3K/AKT signaling in aggressive neuroblastoma. *Cancer Research* 2013;73(7):2189-2198.
465. Fulda S. The PI3K/Akt/mTOR pathway as therapeutic target in neuroblastoma. *Current Cancer Drug Targets* 2009;9(6):729-737.
466. Opel D, Naumann I, Schneider M, Bertele D, Debatin KM, Fulda S. Targeting aberrant PI3K/Akt activation by PI103 restores sensitivity to TRAIL-induced apoptosis in neuroblastoma. *Clinical Cancer Research* 2011;17(10):3233-3247.
467. Smith JR, Moreno L, Heaton SP, Chesler L, Pearson AD, Garrett MD. Novel pharmacodynamic biomarkers for MYCN protein and PI3K/AKT/mTOR pathway signaling in children with neuroblastoma. *Molecular Oncology* 2016;10(4):538-552.
468. Westhoff MA, Faham N, Marx D, Nonnenmacher L, Jennewein C, Enzenmuller S, Gonzalez P, Fulda S, Debatin KM. Sequential dosing in chemosensitization: Targeting the PI3K/Akt/mTOR pathway in neuroblastoma. *PloS One* 2013;8(12):e83128.
469. Dal-Cim T, Molz S, Egea J, Parada E, Romero A, Budni J, Martin de Saavedra MD, del Barrio L, Tasca CI, Lopez MG. Guanosine protects human neuroblastoma SH-SY5Y cells against mitochondrial oxidative stress by inducing heme oxygenase-1 via PI3K/Akt/GSK-3beta pathway. *Neurochemistry International* 2012;61(3):397-404.
470. Qiao J, Paul P, Lee S, Qiao L, Josifi E, Tiao JR, Chung DH. PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation. *Biochemical and Biophysical Research Communications* 2012;424(3):421-426.
471. Sebesta JA, Young A, Bullock J, Moore KH, Azarow K, Sawin RS. Gastrin-releasing peptide: A potential growth factor expressed in human neuroblastoma tumors. *Current Surgery* 2001;58(1):86-89.
472. Kim S, Hu W, Kelly DR, Hellmich MR, Evers BM, Chung DH. Gastrin-releasing peptide is a growth factor for human neuroblastomas. *Annals of Surgery* 2002;235(5):621-630.
473. Qiao J, Kang J, Cree J, Evers BM, Chung DH. Gastrin-releasing peptide-induced down-regulation of tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome ten) in neuroblastomas. *Annals of Surgery* 2005;241(5):684-692.
474. Ishola TA, Kang J, Qiao J, Evers BM, Chung DH. Phosphatidylinositol 3-kinase regulation of gastrin-releasing peptide-induced cell cycle progression in neuroblastoma cells. *Biochimica et Biophysica Acta General Subjects* 2007;1770(6):927-932.
475. Paul P, Qiao J, Kim KW, Romain C, Lee S, Volny N, Mobley B, Correa H, Chung DH. Targeting gastrin-releasing peptide suppresses neuroblastoma progression via upregulation of PTEN signaling. *PloS One* 2013;8(9):e72570.
476. Patel O, Shulkes A, Baldwin GS. Gastrin-releasing peptide and cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2006;1766(1):23-41.
477. Baldwin GS, Patel O, Shulkes A. Phylogenetic analysis of the sequences of gastrin-releasing peptide and its receptors: Biological implications. *Regulatory Peptides* 2007;143(1-3):1-14.
478. Cornelio DB, Roesler R, Schwartzmann G. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. *Annals of Oncology* 2007;18(9):1457-1466.
479. Hohla F, Schally AV. Targeting gastrin releasing peptide receptors: New options for the therapy and diagnosis of cancer. *Cell Cycle* 2010;9(9):1738-1741.
480. Liu X, Carlisle DL, Swick MC, Gaither-Davis A, Grandis JR, Siegfried JM. Gastrin-releasing peptide activates Akt through the epidermal growth factor receptor pathway and abrogates the effect of gefitinib. *Experimental Cell Research* 2007;313(7):1361-1372.



481. Qiao J, Kang JH, Cree J, Evers BM, Chung DH. Ets1 transcription factor mediates gastrin-releasing peptide-induced IL-8 regulation in neuroblastoma cells. *Neoplasia* 2007;9(3):184-191.
482. Qiao J, Kang J, Ishola TA, Rychahou PG, Evers BM, Chung DH. Gastrin-releasing peptide receptor silencing suppresses the tumorigenesis and metastatic potential of neuroblastoma. *Proceedings of the National Academy of Sciences* 2008;105(35):12891-12896.
483. Paul P, Gillory LA, Kang J, Qiao J, Chung DH. Targeting gastrin-releasing peptide as a new approach to treat aggressive refractory neuroblastomas. *Surgery* 2011;149(3):425-432.
484. Qiao J, Hong T, Guo H, Xu Y-Q, Chung DH. Single-walled carbon nanotube-mediated small interfering RNA delivery for gastrin-releasing peptide receptor silencing in human neuroblastoma. *NanoBiotechnology Protocols* 2013:137-147.
485. Qiao J, Lee S, Paul P, Theiss L, Tiao J, Qiao L, Kong A, Chung DH. Mir-335 and mir-363 regulation of neuroblastoma tumorigenesis and metastasis. *Surgery* 2013;154(2):226-233.
486. Roesler R, Schwartzmann G. Gastrin-releasing peptide receptors in the central nervous system: Role in brain function and as a drug target. *Frontiers in Endocrinology* 2012;3:159.
487. Schulte JH, Schulte S, Heukamp LC, Astrahantseff K, Stephan H, Fischer M, Schramm A, Eggert A. Targeted therapy for Neuroblastoma: ALK inhibitors. *Klinische Padiatrie* 2013;225(6):303-308.
488. Wang Y, Wang L, Guan S, Cao W, Wang H, Chen Z, Zhao Y, Yu Y, Zhang H, Pang JC, Huang SL, Akiyama Y, Yang Y, Sun W, Xu X, Shi Y, Zhang H, Kim ES, Muscal JA, Lu F, Yang J. Novel ALK inhibitor AZD3463 inhibits neuroblastoma growth by overcoming crizotinib resistance and inducing apoptosis. *Scientific Reports* 2016;6.
489. Infarinato NR, Park JH, Krytska K, Ryles HT, Sano R, Szigety KM, Li Y, Zou HY, Lee NV, Smeal T, Lemmon MA, Mossé YP. The ALK/ROS1 inhibitor PF-06463922 overcomes primary resistance to crizotinib in ALK-driven neuroblastoma. *Cancer Discovery* 2016;6(1):96-107.
490. Versteeg R, George RE. Targeting ALK: The ten lives of a tumor. *Cancer Discovery* 2016;6(1):20-21.
491. Varki A. Biological roles of glycans. *Glycobiology* 2016:1-47.
492. Schnaar RL. Glycobiology simplified: Diverse roles of glycan recognition in inflammation. *Journal of Leukocyte Biology* 2016;99(6):825-838.
493. Holst S, Wuhler M, Rombouts Y. Chapter 6: Glycosylation characteristics of colorectal Cancer. In: Richard RD, Lauren EB, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;126:203-256.
494. Ho WL, Hsu WM, Huang MC, Kadomatsu K, Nakagawara A. Protein glycosylation in cancers and its potential therapeutic applications in neuroblastoma. *Journal of Hematology & Oncology* 2016;9(1):100.
495. Springer SA, Gagneux P. Glycan evolution in response to collaboration, conflict, and constraint. *Journal of Biological Chemistry* 2013;288(10):6904-6911.
496. Cummings RD, Pierce JM. The challenge and promise of glycomics. *Chemistry & Biology* 2014;21(1):1-15.
497. Bieberich E. Synthesis, processing, and function of N-glycans in N-glycoproteins. *Advances in Neurobiology* 2014;9:47-70.
498. Scott RA, Panitch A. Glycosaminoglycans in biomedicine. *Wiley Interdisciplinary Reviews Nanomedicine and Nanobiotechnology* 2013;5(4):388-398.
499. Afratis N, Gialeli C, Nikitovic D, Tseganidis T, Karousou E, Theocharis AD, Pavao MS, Tzanakakis GN, Karamanos NK. Glycosaminoglycans: Key players in cancer cell biology and treatment. *FEBS Journal* 2012;279(7):1177-1197.
500. Gulati K, Poluri KM. Mechanistic and therapeutic overview of glycosaminoglycans: The unsung heroes of biomolecular signaling. *Glycoconjugate Journal* 2016;33(1):1-17.
501. Zhang R, Loers G, Schachner M, Boelens R, Wienk H, Siebert S, Eckert T, Kraan S, Rojas-Macias MA, Lütke T, Galuska SP, Scheidig A, Petridis AK, Liang S, Billeter M, Schauer R, Steinmeyer J, Schröder J-M, Siebert H-C. Molecular basis of the receptor interactions of polysialic acid (polySia), polySia mimetics, and sulfated polysaccharides. *ChemMedChem* 2016;11(9):990-1002.
502. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, Stanley JD, Darvill A, Kinoshita T, Prestegard JJ, Schnaar RL, Freeze HH, Marth JD, Bertozzi CR, Etzler ME, Frank M, Vliegthart JF, Lutteke T, Perez S, Bolton E, Rudd P, Paulson J, Kanehisa M, Toukach P, Aoki-Kinoshita KF, Dell A, Narimatsu H, York W, Taniguchi N, Kornfeld S. Symbol nomenclature for graphical representations of glycans. *Glycobiology* 2015;25(12):1323-1324.

503. Agard NJ, Bertozzi CR. Chemical approaches to perturb, profile, and perceive glycans. *Accounts of Chemical Research* 2009;42(6):788-797.
504. Davis TP, Sanchez-Covarubias L, Tome ME. P-Glycoprotein trafficking as a therapeutic target to optimize CNS drug delivery. *Advances in Pharmacology* 2014;71:25-44.
505. Hartz AM, Zhong Y, Wolf A, LeVine H, 3rd, Miller DS, Bauer B. Abeta40 reduces P-Glycoprotein at the blood-brain barrier through the ubiquitin-proteasome pathway. *Journal of Neuroscience* 2016;36(6):1930-1941.
506. Poulain FE, Yost HJ. Heparan sulfate proteoglycans: A sugar code for vertebrate development? *Development* 2015;142(20):3456-3467.
507. Nigam SK, Bush KT. Growth factor-heparan sulfate "switches" regulating stages of branching morphogenesis. *Pediatric Nephrology* 2014;29(4):727-735.
508. Coulson-Thomas VJ. The role of heparan sulphate in development: The ectodermal story. *International Journal of Experimental Pathology* 2016;97(3):213-229.
509. Itakura Y, Sasaki N, Kami D, Gojo S, Umezawa A, Toyoda M. N- and O-glycan cell surface protein modifications associated with cellular senescence and human aging. *Cell & Bioscience* 2016;6:14.
510. Haines N, Irvine KD. Glycosylation regulates notch signalling. *Nature Reviews: Molecular Cell Biology* 2003;4(10):786-797.
511. Romero-Brey I, Bartenschlager R. Endoplasmic reticulum: The favorite intracellular niche for viral replication and assembly. *Viruses* 2016;8(6).
512. Zhang S, Shang S, Li W, Qin X, Liu Y. Insights on N-glycosylation of human haptoglobin and its association with cancers. *Glycobiology* 2016;26(7):684-692.
513. Lee HS, Qi Y, Im W. Effects of N-glycosylation on protein conformation and dynamics: Protein data bank analysis and molecular dynamics simulation study. *Scientific Reports* 2015;5:8926.
514. Dube DH, Bertozzi CR. Glycans in cancer and inflammation - potential for therapeutics and diagnostics. *Nature Reviews: Drug Discovery* 2005;4(6):477-488.
515. Takahashi M, Kizuka Y, Ohtsubo K, Gu J, Taniguchi N. Disease-associated glycans on cell surface proteins. *Molecular Aspects of Medicine* 2016;51:56-70.
516. Takahashi M, Hasegawa Y, Gao C, Kuroki Y, Taniguchi N. N-glycans of growth factor receptors: Their role in receptor function and disease implications. *Clinical Science (London, England: 1979)* 2016;130(20):1781-1792.
517. Kudelka MR, Ju T, Heimburg-Molinaro J, Cummings RD. Simple sugars to complex disease-mucin-type O-glycans in cancer. *Advances in Cancer Research* 2015;126:53-135.
518. Sonawane A, Mohanty S, Jagannathan L, Bekolay A, Banerjee S. Role of glycans and glycoproteins in disease development by Mycobacterium tuberculosis. *Critical Reviews in Microbiology* 2012;38(3):250-266.
519. Xu C, Ng DT. Glycosylation-directed quality control of protein folding. *Nature Reviews: Molecular Cell Biology* 2015;16(12):742-752.
520. Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: Diversity, synthesis and function. *Nature Reviews: Molecular Cell Biology* 2012;13(7):448-462.
521. Caramelo JJ, Parodi AJ. A sweet code for glycoprotein folding. *FEBS Letters* 2015;589(22):3379-3387.
522. Mikami T, Kitagawa H. Sulfated glycosaminoglycans: Their distinct roles in stem cell biology. *Glycoconjugate Journal* 2016.
523. Song Y. Function of membrane-associated proteoglycans in the regulation of satellite cell growth. *Advances in Experimental Medicine and Biology* 2016;900:61-95.
524. Couchman JR, Mulhaupt H, Sanderson RD. Recent insights into cell surface heparan sulphate proteoglycans and Cancer. *faculty of F1000Res* 2016;5.
525. Cadete A, Alonso MJ. Targeting cancer with hyaluronic acid-based nanocarriers: Recent advances and translational perspectives. *Nanomedicine (Lond)* 2016;11(17):2341-2357.
526. Wang A, de la Motte C, Lauer M, Hascall V. Hyaluronan matrices in pathobiological processes. *FEBS Journal* 2011;278(9):1412-1418.



527. Berois N, Osinaga E. Glycobiology of neuroblastoma: Impact on tumor behavior, prognosis, and therapeutic strategies. *Frontiers in Oncology* 2014;4:114.
528. Hakomori S-i. Tumor-associated carbohydrate antigens defining tumor malignancy: Basis for development of anti-Cancer vaccines. In: Wu AM, ed. *The Molecular immunology of complex carbohydrates* —2. Boston, MA: Springer US; 2001:369-402.
529. Saito M, Wu G, Hui M, Masiello K, Dobrenis K, Ledeen RW, Saito M. Ganglioside accumulation in activated glia in the developing brain: Comparison between WT and GalNAcT KO mice. *Journal of Lipid Research* 2015;56(8):1434-1448.
530. Saito M, Chakraborty G, Hui M, Masiello K, Saito M. Ethanol-induced neurodegeneration and glial activation in the developing brain. *Brain Sci* 2016;6(3).
531. Ledeen RW, Yu RK. Gangliosides: Structure, isolation, and analysis. *Methods in Enzymology* 1982;83:139-191.
532. Kolter T. Ganglioside biochemistry. *ISRN Biochem* 2012;2012:506160.
533. Dong L, Liu Y, Colberg-Poley AM, Kaucic K, Ladisch S. Induction of gm1a/GD1b synthase triggers complex ganglioside expression and alters neuroblastoma cell behavior; a new tumor cell model of ganglioside function. *Glycoconjugate Journal* 2011;28(3-4):137-147.
534. Pochehueva T, Chinarev A, Schoetzau A, Fedier A, Bovin NV, Hacker NF, Jacob F, Heinzelmann-Schwarz V. Blood plasma-derived anti-glycan antibodies to sialylated and sulfated glycans identify ovarian cancer patients. *PLoS One* 2016;11(10):e0164230.
535. Valentino L, Moss T, Olson E, Wang HJ, Elashoff R, Ladisch S. Shed tumor gangliosides and progression of human neuroblastoma. *Blood* 1990;75(7):1564-1567.
536. Li RX, Ladisch S. Shedding of human neuroblastoma gangliosides. *Biochimica et Biophysica Acta* 1991;1083(1):57-64.
537. Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Research* 1996;56(23):5309-5318.
538. Kaucic K, Etue N, LaFleur B, Woods W, Ladisch S. Neuroblastomas of infancy exhibit a characteristic ganglioside pattern. *Cancer* 2001;91(4):785-793.
539. Hettmer S, Malott C, Woods W, Ladisch S, Kaucic K. Biological stratification of human neuroblastoma by complex "b" pathway ganglioside expression. *Cancer Research* 2003;63(21):7270-7276.
540. Schengrund CL, Repman MA, Shochat SJ. Ganglioside composition of human neuroblastomas correlation with prognosis a pediatric oncology group study. *Cancer* 1985;56(11):2640-2646.
541. Mirkin BL, Clark SH, Zhang C. Inhibition of human neuroblastoma cell proliferation and EGF receptor phosphorylation by gangliosides GM1, GM3, GD1A and GT1B. *Cell Proliferation* 2002;35(2):105-115.
542. Kregel U, Bousquet PA. Molecular recognition of gangliosides and their potential for cancer immunotherapies. *Frontiers in Immunology* 2014;5:325.
543. Hettmer S, McCarter R, Ladisch S, Kaucic K. Alterations in neuroblastoma ganglioside synthesis by induction of GD1b synthase by retinoic acid. *British Journal of Cancer* 2004;91(2):389-397.
544. Lauc G, Pezer M, Rudan I, Campbell H. Mechanisms of disease: The human N-glycome. *Biochimica et Biophysica Acta* 2016;1860(8):1574-1582.
545. Taniguchi N, Kizuka Y. Chapter 2: Glycans and Cancer: Role of n-glycans in Cancer biomarker, progression and metastasis, and therapeutics. In: Richard RD, Lauren EB, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;126:11-51.
546. Tang H, Hsueh P, Kletter D, Bern M, Haab B. Chapter 5: The detection and discovery of glycan motifs in biological samples using lectins and antibodies: New methods and opportunities. In: Richard RD, Lauren EB, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;126:167-202.
547. Hockl PF, Wolosiuk A, Perez-Saez JM, Bordoni AV, Croci DO, Toum-Terrones Y, Soler-Illia GJ, Rabinovich GA. Glyco-nano-oncology: Novel therapeutic opportunities by combining small and sweet. *Pharmacological Research* 2016;109:45-54.
548. Sandhoff K, van Echten G. Ganglioside metabolism: Enzymology, topology and regulation. *Progress in Brain Research* 1994;101:17-29.
549. Zhong X, Drgonova J, Li CY, Uhl GR. Human cell adhesion molecules: Annotated functional subtypes and overrepresentation of addiction-associated genes. *Annals of the New York Academy of Sciences* 2015;1349:83-95.

550. Xin M, Dong XW, Guo XL. Role of the interaction between galectin-3 and cell adhesion molecules in cancer metastasis. *Biomedicine and Pharmacotherapy* 2015;69:179-185.
551. Missaire M, Hindges R. The role of cell adhesion molecules in visual circuit formation: From neurite outgrowth to maps and synaptic specificity. *Developmental Neurobiology* 2015;75(6):569-583.
552. Schwankhaus N, Gathmann C, Wicklein D, Riecken K, Schumacher U, Valentiner U. Cell adhesion molecules in metastatic neuroblastoma models. *Clinical & Experimental Metastasis* 2014;31(4):483-496.
553. Yoon KJ, Phelps DA, Bush RA, Remack JS, Billups CA, Khoury JD. ICAM-2 expression mediates a membrane-actin link, confers a nonmetastatic phenotype and reflects favorable tumor stage or histology in neuroblastoma. *PloS One* 2008;3(11):e3629.
554. Feduska JM, Garcia PL, Brennan SB, Bu S, Council LN, Yoon KJ. N-glycosylation of ICAM-2 is required for ICAM-2-mediated complete suppression of metastatic potential of SK-N-AS neuroblastoma cells. *BMC Cancer* 2013;13(1):1.
555. Feduska JM, Aller SG, Garcia PL, Cramer SL, Council LN, van Waardenburg RC, Yoon KJ. ICAM-2 confers a non-metastatic phenotype in neuroblastoma cells by interaction with alpha-actinin. *Oncogene* 2015;34(12):1553-1562.
556. Del Grosso F, De Mariano M, Passoni L, Luksch R, Tonini GP, Longo L. Inhibition of N-linked glycosylation impairs ALK phosphorylation and disrupts pro-survival signaling in neuroblastoma cell lines. *BMC Cancer* 2011;11(1):525.
557. Bennett EP, Mandel U, Clausen H, Gerken TA, Fritz TA, Tabak LA. Control of mucin-type O-glycosylation: A classification of the polypeptide GalNAc-transferase gene family. *Glycobiology* 2011;22(6):736-756.
558. Munkley J. The role of sialyl-Tn in Cancer. *International Journal of Molecular Sciences* 2016;17(3):275.
559. Julien S, Videira PA, Delannoy P. Sialyl-Tn in cancer: (how) did we miss the target? *Biomolecules* 2012;2(4):435-466.
560. Pearce OMT, Läubli H. Sialic acids in cancer biology and immunity. *Glycobiology* 2016;26(2):111-128.
561. Berois N, Gattoliat C-H, Barrios E, Capandeguy L, Douc-Rasy S, Valteau-Couanet D, Bénard J, Osinaga E. GALNT9 expression is a prognostic marker in neuroblastoma patients. *Clinical Chemistry* 2013;59(1):225-233.
562. Ho WL, Che MI, Chou CH, Chang HH, Jeng YM, Hsu WM, Lin KH, Huang MC. B3GNT3 expression suppresses cell migration and invasion and predicts favorable outcomes in neuroblastoma. *Cancer Science* 2013;104(12):1600-1608.
563. Glick MC, Livingston BD, Shaw GW, Jacobs JL, Troy FA. Expression of polysialic acid on human neuroblastoma. *Progress in Clinical and Biological Research* 1991;366:267-274.
564. Livingston BD, Jacobs JL, Glick MC, Troy FA. Extended polysialic acid chains (n greater than 55) in glycoproteins from human neuroblastoma cells. *Journal of Biological Chemistry* 1988;263(19):9443-9448.
565. Hildebrandt H, Becker C, Gluer S, Rosner H, Gerardy-Schahn R, Rahmann H. Polysialic acid on the neural cell adhesion molecule correlates with expression of polysialyltransferases and promotes neuroblastoma cell growth. *Cancer Research* 1998;58(4):779-784.
566. Valentiner U, Muhlenhoff M, Lehmann U, Hildebrandt H, Schumacher U. Expression of the neural cell adhesion molecule and polysialic acid in human neuroblastoma cell lines. *International Journal of Oncology* 2011;39(2):417-424.
567. Cheung IY, Vickers A, Cheung NK. Sialyltransferase STX (ST8SiaII): A novel molecular marker of metastatic neuroblastoma. *International Journal of Cancer* 2006;119(1):152-156.
568. Seifert A, Glanz D, Glaubitz N, Horstkorte R, Bork K. Polysialylation of the neural cell adhesion molecule: Interfering with polysialylation and migration in neuroblastoma cells. *Archives of Biochemistry and Biophysics* 2012;524(1):56-63.
569. Falconer RA, Errington RJ, Shnyder SD, Smith PJ, Patterson LH. Polysialyltransferase: A new target in metastatic Cancer. *Current Cancer Drug Targets* 2012;12(8):925-939.
570. Al-Saraireh YM, Sutherland M, Springett BR, Freiburger F, Ribeiro Morais G, Loadman PM, Errington RJ, Smith PJ, Fukuda M, Gerardy-Schahn R, Patterson LH, Shnyder SD, Falconer RA. Pharmacological inhibition of polysialyltransferase ST8SiaII modulates tumour cell migration. *PloS One* 2013;8(8):e73366.
571. Fuster MM, Esko JD. The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nature Reviews: Cancer* 2005;5(7):526-542.

572. Miyazaki K, Sakuma K, Kawamura YI, Izawa M, Ohmori K, Mitsuki M, Yamaji T, Hashimoto Y, Suzuki A, Saito Y, Dohi T, Kannagi R. Colonic epithelial cells express specific ligands for mucosal macrophage immunosuppressive receptors siglec-7 and -9. *Journal of Immunology* 2012;188(9):4690-4700.
573. Attrill H, Takazawa H, Witt S, Kelm S, Isecke R, Brossmer R, Ando T, Ishida H, Kiso M, Crocker PR, van Aalten DM. The structure of siglec-7 in complex with sialosides: Leads for rational structure-based inhibitor design. *Biochemical Journal* 2006;397(2):271-278.
574. Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker PR. Ganglioside gd3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and-independent mechanisms. *European Journal of Immunology* 2003;33(6):1642-1648.
575. Ito K, Stannard K, Gabutero E, Clark AM, Neo S-Y, Onturk S, Blanchard H, Ralph SJ. Galectin-1 as a potent target for cancer therapy: Role in the tumor microenvironment. *Cancer and Metastasis Reviews* 2012;31(3):763-778.
576. Croci DO, Cerliani JP, Dalotto-Moreno T, Mendez-Huergo SP, Mascanfroni ID, Dergan-Dylon S, Toscano MA, Caramelo JJ, Garcia-Vallejo JJ, Ouyang J, Mesri EA, Junttila MR, Bais C, Shipp MA, Salatino M, Rabinovich GA. Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. *Cell* 2014;156(4):744-758.
577. Banh A, Zhang J, Cao H, Bouley DM, Kwok S, Kong C, Giaccia AJ, Koong AC, Le Q-T. Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-Cell apoptosis. *Cancer Research* 2011;71(13):4423-4431.
578. Soldati R, Berger E, Zenclussen AC, Jorch G, Lode HN, Salatino M, Rabinovich GA, Fest S. Neuroblastoma triggers an immunoevasive program involving galectin-1-dependent modulation of T cell and dendritic cell compartments. *International Journal of Cancer* 2012;131(5):1131-1141.
579. Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Iarregui JM, Bravo A, Mordoh J, Fainboim L, Podhajcer OL, Rabinovich GA. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; a potential mechanism of tumor-immune privilege. *Cancer Cell* 2004;5(3):241-251.
580. Cimmino F, Schulte JH, Zollo M, Koster J, Versteeg R, Iolascon A, Eggert A, Schramm A. Galectin-1 is a major effector of TrkB-mediated neuroblastoma aggressiveness. *Oncogene* 2009;28(19):2015-2023.
581. Dalotto-Moreno T, Croci DO, Cerliani JP, Martinez-Allo VC, Dergan-Dylon S, Méndez-Huergo SP, Stupirski JC, Mazal D, Osinaga E, Toscano MA, Sundblad V, Rabinovich GA, Salatino M. Targeting galectin-1 overcomes breast cancer-associated immunosuppression and prevents metastatic disease. *Cancer Research* 2013;73(3):1107-1117.
582. Hakomori S. Glycosylation defining cancer malignancy: New wine in an old bottle. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99(16):10231-10233.
583. Schultz MJ, Swindall AF, Bellis SL. Regulation of the metastatic cell phenotype by sialylated glycans. *Cancer and Metastasis Reviews* 2012;31(3-4):501-518.
584. Berois N, Blanc E, Ripoche H, Mergui X, Trajtenberg F, Cantais S, Barrois M, Dessen P, Kågedal B, Bénard J. ppGalNAc-T13: A new molecular marker of bone marrow involvement in neuroblastoma. *Clinical Chemistry* 2006;52(9):1701-1712.
585. Inamori K, Gu J, Ohira M, Kawasaki A, Nakamura Y, Nakagawa T, Kondo A, Miyoshi E, Nakagawara A, Taniguchi N. High expression of N-acetylglucosaminyltransferase V in favorable neuroblastomas: Involvement of its effect on apoptosis. *FEBS Letters* 2006;580(2):627-632.
586. Cheung IY, Feng Y, Gerald W, Cheung N-KV. Exploiting gene expression profiling to identify novel minimal residual disease markers of neuroblastoma. *Clinical Cancer Research* 2008;14(21):7020-7027.
587. Meany DL, Chan DW. Aberrant glycosylation associated with enzymes as cancer biomarkers. *Clinical Proteomics* 2011;8(1):7.
588. Hsu WM, Che MI, Liao YF, Chang HH, Chen CH, Huang YM, Jeng YM, Huang J, Quon MJ, Lee H, Huang HC, Huang MC. B4GALNT3 expression predicts a favorable prognosis and suppresses cell migration and invasion via beta(1) integrin signaling in neuroblastoma. *American Journal of Pathology* 2011;179(3):1394-1404.
589. Yu DMT, Huynh T, Truong AM, Haber M, Norris MD. Chapter 5: ABC transporters and Neuroblastoma. In: John DS, Toshihisa I, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;125:139-170.
590. Ferrandis E, Da Silva J, Riou G, Benard I. Coactivation of the MDR1 and MYCN genes in human neuroblastoma cells during the metastatic process in the nude mouse. *Cancer Research* 1994;54(8):2256-2261.

591. Blanc E, Goldschneider D, Ferrandis E, Barrois M, Le Roux G, Leonce S, Douc-Rasy S, Benard J, Raguenez G. MYCN enhances P-gp/MDR1 gene expression in the human metastatic neuroblastoma IGR-N-91 model. *American Journal of Pathology* 2003;163(1):321-331.
592. Zhang Y, Iwasaki H, Wang H, Kudo T, Kalka TB, Hennet T, Kubota T, Cheng L, Inaba N, Gotoh M, Togayachi A, Guo J, Hisatomi H, Nakajima K, Nishihara S, Nakamura M, Marth JD, Narimatsu H. Cloning and characterization of a new human UDP-N-acetyl- $\alpha$ -D-galactosamine:Polypeptidyl-N-acetylgalactosaminyltransferase, designated pp-GalNAc-T13, that is specifically expressed in neurons and synthesizes GalNAc  $\alpha$ -serine/threonine antigen. *Journal of Biological Chemistry* 2003;278(1):573-584.
593. Wojtowicz K, Januchowski R, Nowicki M, Zabel M. Inhibition of protein glycosylation reverses the MDR phenotype of cancer cell lines. *Biomedicine and Pharmacotherapy* 2015;74:49-56.
594. Wojtowicz K, Szaflarski W, Januchowski R, Zawierucha P, Nowicki M, Zabel M. Inhibitors of N-glycosylation as a potential tool for analysis of the mechanism of action and cellular localisation of glycoprotein P. *Acta Biochimica Polonica* 2012;59(4):445-450.
595. Draheim V, Reichel A, Weitschies W, Moenning U. N-glycosylation of ABC transporters is associated with functional activity in sandwich-cultured rat hepatocytes. *European Journal of Pharmaceutical Sciences* 2010;41(2):201-209.
596. Kramer R, Weber TK, Arceci R, Ramchurren N, Kastrinakis WV, Steele G, Jr., Summerhayes IC. Inhibition of N-linked glycosylation of P-glycoprotein by tunicamycin results in a reduced multidrug resistance phenotype. *British Journal of Cancer* 1995;71(4):670-675.
597. Hiss DC, Gabriels GA, Folb PI. Combination of tunicamycin with anticancer drugs synergistically enhances their toxicity in multidrug-resistant human ovarian cystadenocarcinoma cells. *Cancer Cell International* 2007;7:5.
598. Schinkel A, Kemp S, Dolle M, Rudenko G, Wagenaar E. N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein. *Journal of Biological Chemistry* 1993;268(10):7474-7481.
599. Sereš M, Cholujova D, Bubencikova T, Breier A, Sulova Z. Tunicamycin depresses P-glycoprotein glycosylation without an effect on its membrane localization and drug efflux activity in L1210 cells. *International Journal of Molecular Sciences* 2011;12(11):7772-7784.
600. Breier A, Gibalova L, Seres M, Barancik M, Sulova Z. New insight into P-glycoprotein as a drug target. *Anti-Cancer Agents in Medicinal Chemistry* 2013;13(1):159-170.
601. Elbein AD. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annual Review of Biochemistry* 1987;56(1):497-534.
602. Elbein AD. Glycosidase inhibitors: Inhibitors of N-linked oligosaccharide processing. *FASEB Journal* 1991;5(15):3055-3063.
603. Youakim A, Shur BD. Alteration of oligosaccharide biosynthesis by genetic manipulation of glycosyltransferases. *Annals of the New York Academy of Sciences* 1994;745:331-335.
604. Elbein AD, Molyneux RJ. Alkaloid glycosidase inhibitors. In: Mander L, Liu H-W, eds. *Comprehensive Natural Products II: Chemistry and Biology*. Amsterdam: Elsevier Science; 2010;1:225-259.
605. Dennis JW, Granovsky M, Warren CE. Protein glycosylation in development and disease. *Bioessays* 1999;21(5):412-421.
606. Varki A. Evolutionary forces shaping the Golgi glycosylation machinery: Why cell surface glycans are universal to living cells. *Cold Spring Harbor Perspectives in Biology* 2011;3(6).
607. Varki A, Etzler ME, Cummings RD, Esko JD. Discovery and classification of glycan-binding proteins. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, eds. *Essentials of Glycobiology*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.
608. Wu F, Lukinius A, Bergstrom M, Eriksson B, Watanabe Y, Langstrom B. A mechanism behind the antitumour effect of 6-diazo-5-oxo-l-norleucine (don): Disruption of mitochondria. *European Journal of Cancer* 1999;35(7):1155-1161.
609. Cervantes-Madrid D, Romero Y, Duenas-Gonzalez A. Reviving lonidamine and 6-diazo-5-oxo-l-norleucine to be used in combination for metabolic cancer therapy. *BioMed Research International* 2015;2015:690492.
610. Klausner RD, Donaldson JG, Lippincott-Schwartz J, Brefeldin A: Insights into the control of membrane traffic and organelle structure. *Journal of Cell Biology* 1992;116(5):1071-1080.
611. Nebenführ A, Ritzenthaler C, Robinson DG, Brefeldin A: Deciphering an enigmatic inhibitor of secretion. *Plant Physiology* 2002;130(3):1102-1108.



612. Dwarakanath BS. Cytotoxicity, radiosensitization, and chemosensitization of tumor cells by 2-deoxy-D-glucose *in vitro*. *Journal of Cancer Research and Therapeutics* 2009;5 Suppl 1:S27-31.
613. Gaddameedhi S, Chatterjee S. Association between the unfolded protein response, induced by 2-deoxyglucose, and hypersensitivity to cisplatin: A mechanistic study employing molecular genomics. *Journal of Cancer Research and Therapeutics* 2009;5(9):61.
614. Andresen L, Skovbakke SL, Persson G, Hagemann-Jensen M, Hansen KA, Jensen H, Skov S. 2-Deoxy-D-glucose prevents cell surface expression of NKG2D ligands through inhibition of N-linked glycosylation. *The Journal of Immunology* 2012;188(4):1847-1855.
615. Lugemwa FN, Sarkar AK, Esko JD. Unusual  $\beta$ -D-xylosides that prime glycosaminoglycans in animal cells. *Journal of Biological Chemistry* 1996;271(32):19159-19165.
616. Thorsheim K, Siegbahn A, Johnsson RE, Stalbrand H, Manner S, Widmalm G, Ellervik U. Chemistry of xylopyranosides. *Carbohydrate Research* 2015;418:65-88.
617. Saliba M, Ramalanjaona N, Gulberti S, Bertin-Jung I, Thomas A, Dahbi S, Lopin-Bon C, Jacquinet JC, Breton C, Ouzzine M, Fournel-Gigleux S. Probing the acceptor active site organization of the human recombinant beta1,4-galactosyltransferase 7 and design of xyloside-based inhibitors. *Journal of Biological Chemistry* 2015;290(12):7658-7670.
618. Stegelmeier BL, Molyneux RJ, Asano N, Watson AA, Nash RJ. The comparative pathology of the glycosidase inhibitors swainsonine, castanospermine, and calystegines A3, B2, and C1 in mice. *Toxicologic Pathology* 2008;36(5):651-659.
619. Kato A, Hirokami Y, Kinami K, Tsuji Y, Miyawaki S, Adachi I, Hollinshead J, Nash RJ, Kiappes J, Zitzmann N. Isolation and SAR studies of bicyclic iminosugars from *Castanospermum australe* as glycosidase inhibitors. *Phytochemistry* 2015;111:124-131.
620. Bras NF, Cerqueira NM, Ramos MJ, Fernandes PA. Glycosidase inhibitors: A patent review (2008-2013). *Expert Opinion on Therapeutic Patents* 2014;24(8):857-874.
621. Zhao Y, Zhou Y, KM OB, Murphy PV. Biological study of the angiogenesis inhibitor N-(8-(3-ethynylphenoxy)octyl)-1-deoxynojirimycin. *Chemical Biology & Drug Design* 2010;75(6):570-577.
622. Gross V, Tran-Thi TA, Schwarz RT, Elbein AD, Decker K, Heinrich PC. Different effects of the glucosidase inhibitors 1-deoxynojirimycin, n-methyl-1-deoxynojirimycin and castanospermine on the glycosylation of rat alpha 1-proteinase inhibitor and alpha 1-acid glycoprotein. *Biochemical Journal* 1986;236(3):853-860.
623. Wang R-J, Yang C-H, Hu M-L. 1-Deoxynojirimycin inhibits metastasis of B16F10 melanoma cells by attenuating the activity and expression of matrix metalloproteinases-2 and-9 and altering cell surface glycosylation. *Journal of Agricultural and Food Chemistry* 2010;58(16):8988-8993.
624. Goss PE, Baker MA, Carver JP, Dennis JW. Inhibitors of carbohydrate processing: A new class of anticancer agents. *Clinical Cancer Research* 1995;1(9):935-944.
625. de Freitas Junior JCM, Bárbara Du Rocher D, de Souza WF, de Araújo WM, Abdelhay ESFW, Morgado-Díaz JA. Inhibition of N-linked glycosylation by tunicamycin induces E-cadherin-mediated cell-cell adhesion and inhibits cell proliferation in undifferentiated human colon cancer cells. *Cancer Chemotherapy and Pharmacology* 2011;68(1):227-238.
626. Noda I, Fujieda S, Seki M, Tanaka N, Sunaga H, Ohtsubo T, Tsuzuki H, Fan GK, Saito H. Inhibition of N-linked glycosylation by tunicamycin enhances sensitivity to cisplatin in human head-and-neck carcinoma cells. *International Journal of Cancer* 1999;80(2):279-284.
627. Molinari A, Calcabrini A, Meschini S, Stringaro A, Crateri P, Toccaceli L, Marra M, Colone M, Cianfriglia M, Arancia G. Subcellular detection and localization of the drug transporter P-glycoprotein in cultured tumor cells. *Current Protein and Peptide Science* 2002;3(6):653-670.
628. Li S, Li C, Ryu HH, Lim SH, Jang WY, Jung S. Bacitracin inhibits the migration of U87-MG glioma cells via interferences of the integrin outside-in signaling pathway. *Journal of the Korean Neurosurgical Society* 2016;59(2):106-116.
629. Dickerhof N, Kleffmann T, Jack R, McCormick S. Bacitracin inhibits the reductive activity of protein disulfide isomerase by disulfide bond formation with free cysteines in the substrate-binding domain. *FEBS Journal* 2011;278(12):2034-2043.
630. Karala AR, Ruddock LW. Bacitracin is not a specific inhibitor of protein disulfide isomerase. *FEBS Journal* 2010;277(11):2454-2462.
631. Lovat PE, Corazzari M, Armstrong JL, Martin S, Pagliarini V, Hill D, Brown AM, Piacentini M, Birch-Machin MA, Redfern CP. Increasing melanoma cell death using inhibitors of protein disulphide

- isomerases to abrogate survival responses to endoplasmic reticulum stress. *Cancer Research* 2008;68(13):5363.
632. Gerlach JQ, Sharma S, Leister KJ, Joshi L. A tight-knit Group: Protein glycosylation, endoplasmic reticulum stress and the unfolded protein response. *Endoplasmic Reticulum Stress in Health and Disease*. Dordrecht: Springer Science; 2012:23-39.
633. Bah A, Forman-Kay JD. Modulation of intrinsically disordered protein function by post-translational modifications. *Journal of Biological Chemistry* 2016;291(13):6696-6705.
634. Basak S, Lu C, Basak A. Post-translational protein modifications of rare and unconventional types: Implications in functions and diseases. *Current Medicinal Chemistry* 2016;23(7):714-745.
635. Duan G, Walther D. The roles of post-translational modifications in the context of protein interaction networks. *PLoS Computational Biology* 2015;11(2):e1004049.
636. Walsh CT, Garneau-Tsodikova S, Gatto GJ, Jr. Protein posttranslational modifications: The chemistry of proteome diversifications. *Angewandte Chemie, International Edition in English* 2005;44(45):7342-7372.
637. Hulsmeier AJ, Welte M, Hennet T. Glycoprotein maturation and the UPR. *Methods in Enzymology* 2011;491:163-182.
638. McCaffrey K, Braakman I. Protein quality control at the endoplasmic reticulum. *Essays in Biochemistry* 2016;60(2):227-235.
639. Boscher C, Dennis JW, Nabi IR. Glycosylation, galectins and cellular signaling. *Current Opinion in Cell Biology* 2011;23(4):383-392.
640. Benham AM. Protein secretion and the endoplasmic reticulum. *Cold Spring Harbor Perspectives in Biology* 2012;4(8):a012872.
641. Varki A, Freeze HH. Glycans in acquired human diseases. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, eds. *Essentials of Glycobiology*. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press. The Consortium of Glycobiology Editors, La Jolla, California.; 2009.
642. Ruddock LW, Molinari M. N-glycan processing in ER quality control. *Journal of Cell Science* 2006;119(Pt 21):4373-4380.
643. Aebi M, Bernasconi R, Clerc S, Molinari M. N-glycan structures: Recognition and processing in the ER. *Trends in Biochemical Sciences* 2010;35(2):74-82.
644. Penaranda Fajardo NM, Meijer C, Kruyt FA. The endoplasmic reticulum stress/unfolded protein response in gliomagenesis, tumor progression and as a therapeutic target in glioblastoma. *Biochemical Pharmacology* 2016;118:1-8.
645. Lobo GP, Ebke LA, Au A, Hagstrom SA. Tulp1 missense mutations induces the endoplasmic reticulum unfolded protein response stress complex (ER-UPR). *Advances in Experimental Medicine and Biology* 2016;854:223-230.
646. Stowell SR, Ju T, Cummings RD. Protein glycosylation in cancer. *Annual Review of Pathology* 2015;10:473-510.
647. Lai E, Teodoro T, Volchuk A. Endoplasmic reticulum stress: Signaling the unfolded protein response. *Physiology* 2007;22:193-201.
648. Zhang K, Kaufman RJ. The unfolded protein response: A stress signaling pathway critical for health and disease. *Neurology* 2006;66(2 Suppl 1):S102-109.
649. Kirstein-Miles J, Scior A, Deuerling E, Morimoto RI. The nascent polypeptide-associated complex is a key regulator of proteostasis. *EMBO Journal* 2013;32(10):1451-1468.
650. Schröder M, Kaufman RJ. The mammalian unfolded protein response. *Annual Review of Biochemistry* 2005;74:739-789.
651. Walter P, Ron D. The unfolded protein response: From stress pathway to homeostatic regulation. *Science* 2011;334(6059):1081-1086.
652. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews: Molecular Cell Biology* 2007;8(7):519-529.
653. Smith MH, Ploegh HL, Weissman JS. Road to ruin: Targeting proteins for degradation in the endoplasmic reticulum. *Science* 2011;334(6059):1086-1090.
654. Ruggiano A, Foresti O, Carvalho P. Quality control: ER-associated degradation: Protein quality control and beyond. *Journal of Cell Biology* 2014;204(6):869-879.



655. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 2000;101(3):249-258.
656. Hetz C. The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nature Reviews: Molecular Cell Biology* 2012;13(2):89-102.
657. Radhakrishnan P, Dabelsteen S, Madsen FB, Francavilla C, Kopp KL, Steentoft C, Vakhrushev SY, Olsen JV, Hansen L, Bennett EP, Woetmann A, Yin G, Chen L, Song H, Bak M, Hlady RA, Peters SL, Opavsky R, Thode C, Qvortrup K, Schjoldager KT, Clausen H, Hollingsworth MA, Wandall HH. Immature truncated o-glycophenotype of cancer directly induces oncogenic features. *Proceedings of the National Academy of Sciences of the United States of America* 2014;111(39):E4066-4075.
658. Mottis A, Jovaisaite V, Auwerx J. The mitochondrial unfolded protein response in mammalian physiology. *Mammalian Genome* 2014;25(9-10):424-433.
659. Snapp EL. Unfolded protein responses with or without unfolded proteins? *Cells* 2012;1(4):926-950.
660. Jorgensen E, Stinson A, Shan L, Yang J, Gietl D, Albino AP. Cigarette smoke induces endoplasmic reticulum stress and the unfolded protein response in normal and malignant human lung cells. *BMC Cancer* 2008;8:229.
661. Calamini B, Morimoto RI. Protein homeostasis as a therapeutic target for diseases of protein conformation. *Current Topics in Medicinal Chemistry* 2012;12(22):2623-2640.
662. Park SW, Ozcan U. Potential for therapeutic manipulation of the UPR in disease. *Seminars in Immunopathology* 2013;35(3):351-373.
663. DuRose JB, Tam AB, Niwa M. Intrinsic capacities of molecular sensors of the unfolded protein response to sense alternate forms of endoplasmic reticulum stress. *Molecular Biology of the Cell* 2006;17(7):3095-3107.
664. Smith HL, Mallucci GR. The unfolded protein response: Mechanisms and therapy of neurodegeneration. *Brain* 2016;139(8):2113-2121.
665. Wang P, Li J, Sha B. The ER stress sensor perk luminal domain functions as a molecular chaperone to interact with misfolded proteins. *Acta Crystallographica Section D, Structural Biology* 2016;72(Pt 12):1290-1297.
666. Hiss DC, Gabriels GA. Implications of endoplasmic reticulum stress, the unfolded protein response and apoptosis for molecular cancer therapy. Part I: Targeting p53, Mdm2, GADD153/CHOP, GRP78/BiP and heat shock proteins. *Expert Opinion on Drug Discovery* 2009;4(8):799-821.
667. Hirano M, Adachi Y, Ito Y, Totani K. Calreticulin discriminates the proximal region at the N-glycosylation site of Glc1Man9GlcNAc2 ligand. *Biochemical and Biophysical Research Communications* 2015;466(3):350-355.
668. Genereux JC, Wiseman RL. Regulating extracellular proteostasis capacity through the unfolded protein response. *Prion* 2015;9(1):10-21.
669. Genereux JC, Qu S, Zhou M, Ryno LM, Wang S, Shoulders MD, Kaufman RJ, Lasmezas CI, Kelly JW, Wiseman RL. Unfolded protein response-induced erdj3 secretion links ER stress to extracellular proteostasis. *EMBO Journal* 2015;34(1):4-19.
670. Thomas CG, Spyrou G. Erdj5 sensitizes neuroblastoma cells to endoplasmic reticulum stress-induced apoptosis. *Journal of Biological Chemistry* 2009;284(10):6282-6290.
671. Maattanen P, Gehring K, Bergeron JJ, Thomas DY. Protein quality control in the ER: The recognition of misfolded proteins. *Seminars in Cell & Developmental Biology* 2010;21(5):500-511.
672. Hiramatsu N, Chiang WC, Kurt TD, Sigurdson CJ, Lin JH. Multiple mechanisms of unfolded protein response-induced cell death. *American Journal of Pathology* 2015;185(7):1800-1808.
673. Diaz-Villanueva JF, Diaz-Molina R, Garcia-Gonzalez V. Protein folding and mechanisms of proteostasis. *International Journal of Molecular Sciences* 2015;16(8):17193-17230.
674. Pincus D, Walter P. A first line of defense against ER stress. *Journal of Cell Biology* 2012;198(3):277-279.
675. Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death and Differentiation* 2008;15(9):1460-1471.
676. Schönthal AH. Pharmacological targeting of endoplasmic reticulum stress signaling in cancer. *Biochemical Pharmacology* 2013;85(5):653-666.

677. Fu XL, Gao DS. Endoplasmic reticulum proteins quality control and the unfolded protein response: The regulative mechanism of organisms against stress injuries. *BioFactors* 2014;40(6):569-585.
678. Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene* 2003;22(53):8608-8618.
679. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. *Mutation Research* 2005;569(1-2):29-63.
680. Rutkowski DT, Kaufman RJ. That which does not kill me makes me stronger: Adapting to chronic ER stress. *Trends in Biochemical Sciences* 2007;32(10):469-476.
681. Lee WS, Yoo WH, Chae HJ. ER stress and autophagy. *Current Molecular Medicine* 2015;15(8):735-745.
682. Benbrook DM, Long A. Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis. *Experimental Oncology* 2012;34(3):286-297.
683. Sano R, Reed JC. ER stress-induced cell death mechanisms. *Biochimica et Biophysica Acta* 2013;1833(12):3460-3470.
684. Giampietri C, Petrungaro S, Conti S, Facchiano A, Filippini A, Ziparo E. Cancer microenvironment and endoplasmic reticulum stress response. *Mediators of Inflammation* 2015;2015:417281.
685. Manie SN, Lebeau J, Chevet E. Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 3. Orchestrating the unfolded protein response in oncogenesis: An update. *American Journal of Physiology Cell Physiology* 2014;307(10):C901-907.
686. Yadav RK, Chae SW, Kim HR, Chae HJ. Endoplasmic reticulum stress and cancer. *Journal of Cancer Prevention* 2014;19(2):75-88.
687. Garg AD, Maes H, van Vliet AR, Agostinis P. Targeting the hallmarks of cancer with therapy-induced endoplasmic reticulum (ER) stress. *Molecular and Cellular Oncology* 2015;2(1):e975089.
688. Hammadi M, Oulidi A, Gackiere F, Katsogiannou M, Slomianny C, Roudbaraki M, Dewailly E, Delcourt P, Lepage G, Lotteau S, Ducreux S, Prevarskaya N, Van Coppenolle F. Modulation of ER stress and apoptosis by endoplasmic reticulum calcium leak via translocon during unfolded protein response: Involvement of GRP78. *FASEB Journal* 2013;27(4):1600-1609.
689. Buontempo F, Orsini E, Lonetti A, Cappellini A, Chiarini F, Evangelisti C, Evangelisti C, Melchionda F, Pession A, Bertaina A, Locatelli F, Bertacchini J, Neri LM, McCubrey JA, Martelli AM. Synergistic cytotoxic effects of bortezomib and CK2 inhibitor CX-4945 in acute lymphoblastic leukemia: Turning off the prosurvival ER chaperone BIP/Grp78 and turning on the pro-apoptotic NF-kappaB. *Oncotarget* 2016;7(2):1323-1340.
690. Carvalho HH, Silva PA, Mendes GC, Brustolini OJ, Pimenta MR, Gouveia BC, Valente MA, Ramos HJ, Soares-Ramos JR, Fontes EP. The endoplasmic reticulum binding protein BiP displays dual function in modulating cell death events. *Plant Physiology* 2014;164(2):654-670.
691. Hiss DC, Gabriels GA. Implications of endoplasmic reticulum stress, the unfolded protein response and apoptosis for molecular cancer therapy. Part II: Targeting cell cycle events, caspases, NF-kappaB and the proteasome. *Expert Opinion on Drug Discovery* 2009;4(9):907-921.
692. Maly DJ, Papa FR. Druggable sensors of the unfolded protein response. *Nature Chemical Biology* 2014;10(11):892-901.
693. Wang WA, Groenendyk J, Michalak M. Endoplasmic reticulum stress associated responses in cancer. *Biochimica et Biophysica Acta* 2014;1843(10):2143-2149.
694. Devisscher L, Vieri M, Logue SE, Panse J, Geerts A, van Vlierberghe H, Chevet E, Gorman AM, Samali A, Kharabi Masouleh B. Targeting the angio-proteostasis network: Combining the forces against cancer. *Pharmacology & Therapeutics* 2016;167:1-12.
695. Taouji S, Chevet E. Modulation pharmacologique de la réponse au stress du réticulum endoplasmique. Potentiel thérapeutique en cancérologie [modulating endoplasmic reticulum stress in the treatment of cancer]. *Médecine Sciences* 2015;31(6-7):667-673.
696. Xipell E, Aragon T, Martinez-Velez N, Vera B, Idoate MA, Martinez-Irujo JJ, Garzon AG, Gonzalez-Huarriz M, Acanda AM, Jones C, Lang FF, Fueyo J, Gomez-Manzano C, Alonso MM. Endoplasmic reticulum stress-inducing drugs sensitize glioma cells to temozolomide through downregulation of MGMT, MPG, and Rad51. *Neuro-Oncology* 2016;18(8):1109-1119.
697. Nagelkerke A, Bussink J, Sweep FCGJ, Span PN. The unfolded protein response as a target for cancer therapy. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2014;1846(2):277-284.
698. Liu H, Yang J, Li L, Shi W, Yuan X, Wu L. The natural occurring compounds targeting endoplasmic reticulum stress. *Evidence-Based Complementary and Alternative Medicine* 2016;2016:7831282.

699. Ma Z, Fan C, Yang Y, Di S, Hu W, Li T, Zhu Y, Han J, Xin Z, Wu G, Zhao J, Li X, Yan X. Thapsigargin sensitizes human esophageal cancer to TRAIL-induced apoptosis via AMPK activation. *Scientific Reports* 2016;6:35196.
700. Inesi G, Sagara Y. Thapsigargin, a high affinity and global inhibitor of intracellular Ca<sup>2+</sup> transport ATPases. *Archives of Biochemistry and Biophysics* 1992;298(2):313-317.
701. Kardosh A, Golden EB, Pyrko P, Uddin J, Hofman FM, Chen TC, Louie SG, Petasis NA, Schönthal AH. Aggravated endoplasmic reticulum stress as a basis for enhanced glioblastoma cell killing by bortezomib in combination with celecoxib or its non-coxib analogue, 2, 5-dimethyl-celecoxib. *Cancer Research* 2008;68(3):843-851.
702. Lee AS. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* 2005;35(4):373-381.
703. Sasaya H, Utsumi T, Shimoke K, Nakayama H, Matsumura Y, Fukunaga K, Ikeuchi T. Nicotine suppresses tunicamycin-induced, but not thapsigargin-induced, expression of GRP78 during ER stress-mediated apoptosis in PC12 cells. *Journal of Biochemistry* 2008;144(2):251-257.
704. Elbein AD. The tunicamycins—useful tools for studies on glycoproteins. *Trends in Biochemical Sciences* 1981;6:219-221.
705. Contessa JN, Bhojani MS, Freeze HH, Rehemtulla A, Lawrence TS. Inhibition of N-linked glycosylation disrupts receptor tyrosine kinase signaling in tumor cells. *Cancer Research* 2008;68(10):3803-3809.
706. Banerjee A, Johnson KT, Banerjee IA, Banerjee DK. Nanof ormulation enhances anti-angiogenic efficacy of tunicamycin. *Translational Cancer Research* 2013;2(4):240-255.
707. Banerjee A, Lang J-Y, Hung M-C, Sengupta K, Banerjee SK, Baksi K, Banerjee DK. Unfolded protein response is required in nu/nu mice microvasculature for treating breast tumor with tunicamycin. *Journal of Biological Chemistry* 2011;286(33):29127-29138.
708. Han X, Zhang X, Li H, Huang S, Zhang S, Wang F, Shi Y. Tunicamycin enhances the antitumor activity of trastuzumab on breast cancer *in vitro* and *in vivo*. *Oncotarget* 2015;6(36):38912.
709. Peñaranda Fajardo NM, Meijer C, Kruyt FAE. The endoplasmic reticulum stress/unfolded protein response in gliomagenesis, tumor progression and as a therapeutic target in glioblastoma. *Biochemical Pharmacology* 2016;118:1-8.
710. Carman KMI, Yin J, K.S. Ng P, Lin S-Y, B. Mills G. Genomic-glycosylation aberrations in tumor initiation, progression and management. *AIMS Medical Science* 2016;3(4):386-416.
711. Silva R, Vilas-Boas V, Carmo H, Dinis-Oliveira RJ, Carvalho F, de Lourdes Bastos M, Remiao F. Modulation of P-glycoprotein efflux pump: Induction and activation as a therapeutic strategy. *Pharmacology & Therapeutics* 2015;149:1-123.
712. Liu ES, Ou JH, Lee AS. Brefeldin A as a regulator of grp78 gene expression in mammalian cells. *Journal of Biological Chemistry* 1992;267(10):7128-7133.
713. Lee AS. GRP78 induction in cancer: Therapeutic and prognostic implications. *Cancer Research* 2007;67(8):3496-3499.
714. Graham RM, Hernandez F, Puerta N, De Angulo G, Webster KA, Vanni S. Resveratrol augments ER stress and the cytotoxic effects of glycolytic inhibition in neuroblastoma by downregulating Akt in a mechanism independent of SIRT1. *Experimental and Molecular Medicine* 2016;48:e210.
715. Wang M, Wey S, Zhang Y, Ye R, Lee AS. Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. *Antioxidants & Redox Signaling* 2009;11(9):2307-2316.
716. Booth L, Roberts JL, Cash DR, Tavallai S, Jean S, Fidanza A, Cruz-Luna T, Siembiba P, Cycon KA, Cornelissen CN, Dent P. GRP78/BiP/HSPA5/Dna K is a universal therapeutic target for human disease. *Journal of Cellular Physiology* 2015;230(7):1661-1676.
717. Han KS, Li N, Raven PA, Fazli L, Frees S, Ettinger S, Park KC, Hong SJ, Gleave ME, So AI. Inhibition of endoplasmic reticulum chaperone protein glucose-regulated protein 78 potentiates anti-angiogenic therapy in renal cell carcinoma through inactivation of the PERK/eIF2alpha pathway. *Oncotarget* 2015;6(33):34818-34830.
718. Cerezo M, Lehraiki A, Millet A, Rouaud F, Plaisant M, Jaune E, Botton T, Ronco C, Abbe P, Amdouni H, Passeron T, Hofman V, Mograbi B, Dabert-Gay AS, Debayle D, Alcor D, Rabhi N, Annicotte JS, Heliot L, Gonzalez-Pisfil M, Robert C, Morera S, Virougoux A, Gual P, Ali MM, Bertolotto C, Hofman P, Ballotti R, Benhida R, Rocchi S. Compounds triggering ER stress exert anti-melanoma effects and overcome BRAF inhibitor resistance. *Cancer Cell* 2016;29(6):805-819.

719. Ponthan F, Wickstrom M, Gleissman H, Fuskevag OM, Segerstrom L, Sveinbjornsson B, Redfern CP, Eksborg S, Kogner P, Johnsen JI. Celecoxib prevents neuroblastoma tumor development and potentiates the effect of chemotherapeutic drugs *in vitro* and *in vivo*. *Clinical Cancer Research* 2007;13(3):1036-1044.
720. Bahar E, Kim H, Yoon H. ER stress-mediated signaling: Action potential and ca(2+) as key players. *International Journal of Molecular Sciences* 2016;17(9).
721. Jendrossek V. Targeting apoptosis pathways by celecoxib in cancer. *Cancer Letters* 2013;332(2):313-324.
722. van Roosmalen IAM, Reis CR, Sestroikromo R, Yuvaraj S, Joseph JV, Tepper PG, Krut FAE, Quax WJ. The ER stress inducer dmc enhances TRAIL-induced apoptosis in glioblastoma. *SpringerPlus* 2014;3(1):495.
723. Sobolewski C, Rhim J, Legrand N, Muller F, Cerella C, Mack F, Chateauvieux S, Kim J-G, Yoon A-Y, Kim K-W, Dicato M, Diederich M. 2,5-dimethyl-celecoxib inhibits cell cycle progression and induces apoptosis in human leukemia cells. *Journal of Pharmacology and Experimental Therapeutics* 2015;355(2):322-342.
724. Silva AM, Wang D, Komar AA, Castilho BA, Williams BR. Salicylates trigger protein synthesis inhibition in a protein kinase R-like endoplasmic reticulum kinase-dependent manner. *Journal of Biological Chemistry* 2007;282(14):10164-10171.
725. Yamazaki T, Muramoto M, Oe T, Morikawa N, Okitsu O, Nagashima T, Nishimura S, Katayama Y, Kita Y. Diclofenac, a non-steroidal anti-inflammatory drug, suppresses apoptosis induced by endoplasmic reticulum stresses by inhibiting caspase signaling. *Neuropharmacology* 2006;50(5):558-567.
726. Mügge FLB, Silva AM. Endoplasmic reticulum stress response in the roadway for the effects of non-steroidal anti-inflammatory drugs. *Endoplasmic Reticulum Stress in Diseases* 2015;2(1):1-17.
727. Thun MJ, Jacobs EJ, Patrono C. The role of aspirin in cancer prevention. *Nature Reviews: Clinical Oncology* 2012;9(5):259-267.
728. Henderson B, Kaiser F. Do reciprocal interactions between cell stress proteins and cytokines create a new intra-/extra-cellular signalling nexus? *Cell Stress & Chaperones* 2013;18(6):685-701.
729. Fukuyo Y, Hunt CR, Horikoshi N. Geldanamycin and its anti-cancer activities. *Cancer Letters* 2010;290(1):24-35.
730. Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E, Quest AF, Lavandero S. Endoplasmic reticulum and the unfolded protein response: Dynamics and metabolic integration. *International Review of Cell and Molecular Biology* 2013;301:215-290.
731. Chen D, Frezza M, Schmitt S, Kanwar J, Dou QP. Bortezomib as the first proteasome inhibitor anticancer drug: Current status and future perspectives. *Current Cancer Drug Targets* 2011;11(3):239-253.
732. Buac D, Shen M, Schmitt S, Kona FR, Deshmukh R, Zhang Z, Neslund-Dudas C, Mitra B, Dou QP. From bortezomib to other inhibitors of the proteasome and beyond. *Current Pharmaceutical Design* 2013;19(22):4025-4038.
733. Rashid HO, Yadav RK, Kim HR, Chae HJ. ER stress: Autophagy induction, inhibition and selection. *Autophagy* 2015;11(11):1956-1977.
734. Zha W, Wang G, Xu W, Liu X, Wang Y, Zha BS, Shi J, Zhao Q, Gerk PM, Studer E, Hylemon PB, Pandak WM, Jr., Zhou H. Inhibition of P-glycoprotein by HIV protease inhibitors increases intracellular accumulation of berberine in murine and human macrophages. *PLoS One* 2013;8(1):e54349.
735. König J, Müller F, Fromm MF. Transporters and drug-drug interactions: Important determinants of drug disposition and effects. *Pharmacological Reviews* 2013;65(3):944-966.
736. Srivalli KMR, Lakshmi P. Overview of P-glycoprotein inhibitors: A rational outlook. *Brazilian Journal of Pharmaceutical Sciences* 2012;48(3):353-367.
737. Pereira ER, Preston AM, Hendershot LM. UPR activation in Cancer cells: A double-edged sword. In: Agostinis P, Afshin S, eds. *Endoplasmic Reticulum Stress in Health and Disease*: Springer; 2012:383-412.
738. Wang L, Wang C, Jia Y, Liu Z, Shu X, Liu K. Resveratrol increases anti-proliferative activity of bestatin through downregulating P-Glycoprotein expression via inhibiting PI3K/Akt/mTOR pathway in K562/ADR cells. *Journal of Cellular Biochemistry* 2016;117(5):1233-1239.
739. Bedada SK, Yellu NR, Neerati P. Effect of resveratrol on the pharmacokinetics of fexofenadine in rats: Involvement of P-glycoprotein inhibition. *Pharmacological Reports* 2016;68(2):338-343.



740. Zeeshan HM, Lee GH, Kim HR, Chae HJ. Endoplasmic reticulum stress and associated ROS. *International Journal of Molecular Sciences* 2016;17(3):327.
741. Pluquet O, Pourtier A, Abbadie C. The unfolded protein response and cellular senescence. A review in the theme: Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. *American Journal of Physiology Cell Physiology* 2015;308(6):C415-425.
742. Nakato R, Ohkubo Y, Konishi A, Shibata M, Kaneko Y, Iwawaki T, Nakamura T, Lipton SA, Uehara T. Regulation of the unfolded protein response via S-nitrosylation of sensors of endoplasmic reticulum stress. *Scientific Reports* 2015;5:14812.
743. Shen M, Chan TH, Dou QP. Targeting tumor ubiquitin-proteasome pathway with polyphenols for chemosensitization. *Anti-Cancer Agents in Medicinal Chemistry* 2012;12(8):891-901.
744. Xu Y, Wang C, Li Z. A new strategy of promoting cisplatin chemotherapeutic efficiency by targeting endoplasmic reticulum stress. *Molecular and Clinical Oncology* 2014;2(1):3-7.
745. Wang C, Li T, Tang S, Zhao D, Zhang C, Zhang S, Deng S, Zhou Y, Xiao X. Thapsigargin induces apoptosis when autophagy is inhibited in HepG2 cells and both processes are regulated by ROS-dependent pathway. *Environmental Toxicology and Pharmacology* 2016;41:167-179.
746. Demirsoy S, Martin S, Maes H, Agostinis P. Adapt, recycle, and move on: Proteostasis and trafficking mechanisms in melanoma. *Frontiers in Oncology* 2016;6:240.
747. Johnson CE, Hunt DK, Wiltshire M, Herbert TP, Sampson JR, Errington RJ, Davies DM, Tee AR. Endoplasmic reticulum stress and cell death in mTORC1-overactive cells is induced by nelfinavir and enhanced by chloroquine. *Molecular Oncology* 2015;9(3):675-688.
748. Roller DG, Capaldo B, Bekiranov S, Mackey AJ, Conaway MR, Petricoin EF, Gioeli D, Weber MJ. Combinatorial drug screening and molecular profiling reveal diverse mechanisms of intrinsic and adaptive resistance to BRAF inhibition in v600e BRAF mutant melanomas. *Oncotarget* 2016;7(3):2734-2753.
749. Garg B, Pathria G, Wagner C, Maurer M, Wagner SN. Signal sequence receptor 2 is required for survival of human melanoma cells as part of an unfolded protein response to endoplasmic reticulum stress. *Mutagenesis* 2016;31(5):573-582.
750. Ma X-H, Piao S-F, Dey S, McAfee Q, Karakousis G, Villanueva J, Hart LS, Levi S, Hu J, Zhang G, Lazova R, Klump V, Pawelek JM, Xu X, Xu W, Schuchter LM, Davies MA, Herlyn M, Winkler J, Koumenis C, Amaravadi RK. Targeting ER stress-induced autophagy overcomes BRAF inhibitor resistance in melanoma. *The Journal of Clinical Investigation* 2014;124(3):1406-1417.
751. Holland ML, Panetta JA, Hoskins JM, Bebawy M, Roufogalis BD, Allen JD, Arnold JC. The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochemical Pharmacology* 2006;71(8):1146-1154.
752. Godwin P, Baird AM, Heavey S, Barr MP, O'Byrne KJ, Gately K. Targeting nuclear factor-kappa b to overcome resistance to chemotherapy. *Frontiers in Oncology* 2013;3:120.
753. Gallagher CM, Garri C, Cain EL, Ang KK, Wilson CG, Chen S, Hearn BR, Jaishankar P, Aranda-Diaz A, Arkin MR, Renslo AR, Walter P. Ceapins are a new class of unfolded protein response inhibitors, selectively targeting the ATF6alpha branch. *Elife* 2016;5:DOI: 10.7554/eLife.11878.
754. !!! INVALID CITATION !!! {Keshelava, 2001 #4384}{Xue, 2007 #4385}
755. Aebi M. N-linked protein glycosylation in the ER. *Biochimica et Biophysica Acta* 2013;1833(11):2430-2437.
756. Wu J, Qin H, Li T, Cheng K, Dong J, Tian M, Chai N, Guo H, Li J, You X, Dong M, Ye M, Nie Y, Zou H, Fan D. Characterization of site-specific glycosylation of secreted proteins associated with multi-drug resistance of gastric cancer. *Oncotarget* 2016;7(18):25315-25327.
757. Pavlikova L, Seres M, Imrichova D, Hano M, Rusnak A, Zamorova M, Katrlík J, Breier A, Sulova Z. The expression of P-gp in leukemia cells is associated with cross-resistance to protein N-glycosylation inhibitor tunicamycin. *General Physiology and Biophysics* 2016;35(4):497-510.
758. Hagenbuchner J, Kiechl-Kohlendorfer U, Obexer P, Ausserlechner MJ. Birc5/survivin as a target for glycolysis inhibition in high-stage neuroblastoma. *Oncogene* 2016;35(16):2052-2061.
759. Ferreira JA, Peixoto A, Neves M, Gaitheiro C, Reis CA, Assaraf YG, Santos LL. Mechanisms of cisplatin resistance and targeting of cancer stem cells: Adding glycosylation to the equation. *Drug Resistance Updates* 2016;24:34-54.
760. Lannoo N, Van Damme EJ. Review/N-glycans: The making of a varied toolbox. *Plant Science* 2015;239:67-83.

761. Guo H, Abbott KL. Chapter 8: Functional impact of tumor-specific N-linked glycan changes in breast and ovarian cancers. In: Richard RD, Lauren EB, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;126:281-303.
762. Drake RR, Jones EE, Powers TW, Nyalwidhe JO. Chapter 10: Altered glycosylation in prostate Cancer. In: Richard RD, Lauren EB, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;126:345-382.
763. Drake RR. Chapter 1: Glycosylation and Cancer: Moving glycomics to the forefront. In: Richard RD, Lauren EB, eds. *Advances in Cancer Research*. San Diego, CA: Academic Press; 2015;126:1-10.
764. Lantz TB, Jie C, Clark S, Naiditch JA, Jafari N, Qiu YY, Zheng X, Chu F, Madonna MB. The effect of vorinostat on the development of resistance to doxorubicin in neuroblastoma. *PLoS One* 2012;7(7):e40816.
765. Biedler JL, Spengler BA. Collateral sensitivity in multidrug resistance. In: Kellen JA, ed. *Reversal of multidrug resistance in cancer*. Ann Arbor: CRC Press; 1994:21-46.
766. Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Research* 1978;38(11 Pt 1):3751-3757.
767. Barnes EN, Biedler JL, Spengler BA, Lyser KM. The fine structure of continuous human neuroblastoma lines SK-N-SH, SK-N-BE(2), and SK-N-MC. *In vitro* 1981;17(7):619-631.
768. Biedler JL, Spengler BA. A novel chromosome abnormality in human neuroblastoma and antifolate-resistant Chinese hamster cell lines in culture. *Journal of the National Cancer Institute* 1976;57(3):683-695.
769. Biedler J, Spengler B. Metaphase chromosome anomaly: Association with drug resistance and cell-specific products. *Science* 1976;191(4223):185-187.
770. Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y, Ueno K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta* 1997;44(7):1299-1305.
771. Tsukatani T, Suenaga H, Higuchi T, Akao T, Ishiyama M, Ezoe K, Matsumoto K. Colorimetric cell proliferation assay for microorganisms in microtiter plate using water-soluble tetrazolium salts. *Journal of Microbiological Methods* 2008;75(1):109-116.
772. Tominaga H, Ishiyama M, Ohseto F, Sasamoto K, Hamamoto T, Suzuki K, Watanabe M. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Analytical Communications* 1999;36(2):47-50.
773. Niles AL, Moravec RA, Riss TL. *In vitro* viability and cytotoxicity testing and same-well multi-parametric combinations for high throughput screening. *Current Chemical Genomics* 2009;3:33-41.
774. Steinberg P, editor. High-throughput screening methods in toxicity Testing. New Jersey: Wiley & Sons; 2013.
775. Kleinjans J. Toxicogenomics-based cellular models: Alternatives to animal testing for safety assessment. Oxford: Academic Press; 2014.
776. Niles AL, Moravec RA, Riss TL. Update on *in vitro* cytotoxicity assays for drug development. *Expert Opinion on Drug Discovery* 2008;3(6):655-669.
777. Saunders DN, Falkenberg KJ, Simpson KJ. High-throughput approaches to measuring cell death. *Cold Spring Harbor Protoc* 2014;2014(6):591-601.
778. Liminga G, Nygren P, Larsson R. Microfluorometric evaluation of calcein acetoxyethyl ester as a probe for P-Glycoprotein-mediated resistance: Effects of cyclosporin a and its nonimmunosuppressive analogue SDZ PSC 833. *Experimental Cell Research* 1994;212(2):291-296.
779. Holló Z, Homolya L, Davis CW, Sarkadi B. Calcein accumulation as a fluorometric functional assay of the multidrug transporter. *Biochimica et Biophysica Acta - Biomembranes* 1994;1191(2):384-388.
780. Feller N, Broxterman H, Währer D, Pinedo H. ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): No inhibition by intracellular glutathione depletion. *FEBS Letters* 1995;368(2):385-388.
781. Tiberghien F, Loo F. Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay. *Anti-Cancer Drugs* 1996;7(5):568-578.
782. Shukla S, Kouanda A, Silverton L, Talele TT, Ambudkar SV. Pharmacophore modeling of nilotinib as an inhibitor of ATP-binding cassette drug transporters and bcr-abl kinase using a three-dimensional quantitative structure-activity relationship approach. *Molecular Pharmaceutics* 2014;11(7):2313-2322.
783. Eckford PD, Sharom FJ. ABC efflux pump-based resistance to chemotherapy drugs. *Chemical Reviews* 2009;109(7):2989-3011.



784. Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, Serabjit-Singh CS. Rational use of *in vitro* P-glycoprotein assays in drug discovery. *Journal of Pharmacology and Experimental Therapeutics* 2001;299(2):620-628.
785. Bates SE, Shieh CY, Tsokos M. Expression of mdr-1/P-glycoprotein in human neuroblastoma. *American Journal of Pathology* 1991;139(2):305-315.
786. Bates SE, Mickley LA, Chen YN, Richert N, Rudick J, Biedler JL, Fojo AT. Expression of a drug resistance gene in human neuroblastoma cell lines: Modulation by retinoic acid-induced differentiation. *Molecular and Cellular Biology* 1989;9(10):4337-4344.
787. Holló Z, Homolya L, Hegedüs T, Sarkadi B. Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. *FEBS Letters* 1996;383(1-2):99-104.
788. Ward E, DeSantis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics, 2014. *CA: A Cancer Journal for Clinicians* 2014;64(2):83-103.
789. Schwab M, Westermann F, Hero B, Berthold F. Neuroblastoma: Biology and molecular and chromosomal pathology. *Lancet Oncology* 2003;4(8):472-480.
790. Wang LL, Teshiba R, Ikegaki N, Tang XX, Naranjo A, London WB, Hogarty MD, Gastier-Foster JM, Look AT, Park JR, Maris JM, Cohn SL, Seeger RC, Asgharzadeh S, Shimada H. Augmented expression of MYC and/or MYCN protein defines highly aggressive MYC-driven neuroblastoma: A Children's Oncology Group study. *British Journal of Cancer* 2015;113(1):57-63.
791. Walton JD, Kattan DR, Thomas SK, Spengler BA, Guo HF, Biedler JL, Cheung NK, Ross RA. Characteristics of stem cells from human neuroblastoma cell lines and in tumors. *Neoplasia* 2004;6(6):838-845.
792. Xing LL, Sha YL, Wu YM, Hu JM, Zhang M, Lv F. Preliminary analysis of stem cell-like cells in human neuroblastoma. *World Journal of Pediatrics* 2015;11(1):54-60.
793. Ross RA, Spengler BA. Human neuroblastoma stem cells. *Seminars in Cancer Biology* 2007;17(3):241-247.
794. Kamijo T. Role of stemness-related molecules in neuroblastoma. *Pediatric Research* 2012;71(4-2):511-515.
795. Chang JC. Cancer stem cells: Role in tumor growth, recurrence, metastasis, and treatment resistance. *Medicine* 2016;95(1S):S20-S25.
796. Rich JN. Cancer stem cells: Understanding tumor hierarchy and heterogeneity. *Medicine* 2016;95(1S):S2-S7.
797. Ross RA, Walton JD, Han D, Guo HF, Cheung NK. A distinct gene expression signature characterizes human neuroblastoma cancer stem cells. *Stem Cell Research* 2015;15(2):419-426.
798. Qi S, Zheng J, Zhu H, Yang L, Xiao X. Identification of neuroblastoma stem cells by characterization of side population cells in the human neuroblastoma SK-N-SH cell line. *Journal of Pediatric Surgery* 2010;45(12):2305-2311.
799. Mei H, Lin ZY, Tong QS. The roles of micrnas in neuroblastoma. *World Journal of Pediatrics* 2014;10(1):10-16.
800. Kim YS, Kim E, Park YJ, Kim Y. Retinoic acid receptor beta enhanced the anti-cancer stem cells effect of beta-carotene by down-regulating expression of delta-like 1 homologue in human neuroblastoma cells. *Biochemical and Biophysical Research Communications* 2016;480(2):254-260.
801. Scott H, Panin VM. N-glycosylation in regulation of the nervous system. *Advances in Neurobiology* 2014;9:367-394.
802. Morgenstern DA, London WB, Stephens D, Volchenboum SL, Hero B, Di Cataldo A, Nakagawara A, Shimada H, Ambros PF, Matthay KK, Cohn SL, Pearson AD, Irwin MS. Metastatic neuroblastoma confined to distant lymph nodes (stage 4N) predicts outcome in patients with stage 4 disease: A study from the International Neuroblastoma Risk Group database. *Journal of Clinical Oncology* 2014;32(12):1228-1235.
803. Hansford LM, McKee AE, Zhang L, George RE, Gerstle JT, Thorner PS, Smith KM, Look AT, Yeger H, Miller FD, Irwin MS, Thiele CJ, Kaplan DR. Neuroblastoma cells isolated from bone marrow metastases contain a naturally enriched tumor-initiating cell. *Cancer Research* 2007;67(23):11234-11243.
804. Norris M, Bordow S, Haber P, Marshall G, Kavallaris M, Madafiglio J, Cohn S, Salwen H, Schmidt M, Hipfner D. Evidence that the MYCN oncogene regulates MRP gene expression in neuroblastoma. *European Journal of Cancer* 1997;33(12):1911-1916.

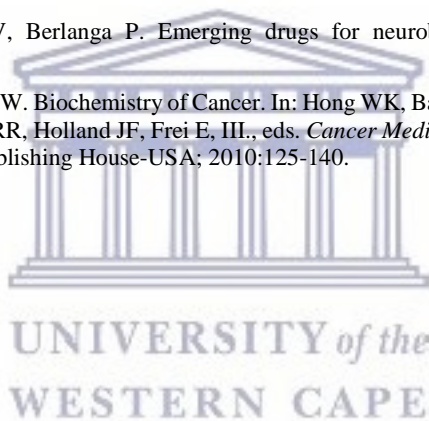
805. Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. *Cancer Research* 1994;54(19):5036-5040.
806. Dhooge CR, De Moerloose BM, Benoit YC, Van Roy N, Philippe, Laureys GG. Expression of the MDR1 gene product P-glycoprotein in childhood neuroblastoma. *Cancer* 1997;80(7):1250-1257.
807. Sieczkowski E, Lehner C, Ambros PF, Hohenegger M. Double impact on p-glycoprotein by statins enhances doxorubicin cytotoxicity in human neuroblastoma cells. *International Journal of Cancer* 2010;126(9):2025-2035.
808. Bader P, Schilling F, Schlaud M, Girgert R, Handgretinger R, Klingebiel T, Treuner J, Liu C, Niethammer D, Beck JF. Expression analysis of multidrug resistance associated genes in neuroblastomas. *Oncology Reports* 1999;6(5):1143-1146.
809. Vinogradova TV, Chernov IP, Monastyrskaya GS, Kondratyeva LG, Sverdlov ED. Cancer stem cells: Plasticity works against therapy. *Acta Naturae* 2015;7(4):46-55.
810. Matsui WH. Cancer stem cell signaling pathways. *Medicine* 2016;95(1S):S8-S19.
811. Liu G, Neelamegham S. Integration of systems glycobiology with bioinformatics toolboxes, glycoinformatics resources, and glycoproteomics data. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* 2015;7(4):163-181.
812. Lauc G, Huffman JE, Pucic M, Zgaga L, Adamczyk B, Muzinic A, Novokmet M, Polasek O, Gornik O, Kristic J, Keser T, Vitart V, Scheijen B, Uh HW, Molokhia M, Patrick AL, McKeigue P, Kolcic I, Lukic IK, Swann O, van Leeuwen FN, Ruhaak LR, Houwing-Duistermaat JJ, Slagboom PE, Beekman M, de Craen AJ, Deelder AM, Zeng Q, Wang W, Hastie ND, Gyllensten U, Wilson JF, Wuhler M, Wright AF, Rudd PM, Hayward C, Aulchenko Y, Campbell H, Rudan I. Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genetics* 2013;9(1):e1003225.
813. Kyselova Z, Mechref Y, Al Bataineh MM, Dobrolecki LE, Hickey RJ, Vinson J, Sweeney CJ, Novotny MV. Alterations in the serum glycome due to metastatic prostate cancer. *Journal of Proteome Research* 2007;6(5):1822-1832.
814. Lee LY, Thaysen-Andersen M, Baker MS, Packer NH, Hancock WS, Fanayan S. Comprehensive N-glycome profiling of cultured human epithelial breast cells identifies unique secretome N-glycosylation signatures enabling tumorigenic subtype classification. *Journal of Proteome Research* 2014;13(11):4783-4795.
815. Sethi MK, Kim H, Park CK, Baker MS, Paik YK, Packer NH, Hancock WS, Fanayan S, Thaysen-Andersen M. In-depth N-glycome profiling of paired colorectal cancer and non-tumorigenic tissues reveals cancer-, stage- and EGFR-specific protein N-glycosylation. *Glycobiology* 2015;25(10):1064-1078.
816. Rombouts Y, Jonasdottir HS, Hipgrave Ederveen AL, Reiding KR, Jansen BC, Freysdottir J, Hardardottir I, Ioan-Facsinay A, Giera M, Wuhler M. Acute phase inflammation is characterized by rapid changes in plasma/peritoneal fluid N-glycosylation in mice. *Glycoconjugate Journal* 2016.
817. Priglinger CS, Obermann J, Szober CM, Merl-Pham J, Ohmayer U, Behler J, Gruhn F, Kreutzer TC, Wertheimer C, Geerlof A, Priglinger SG, Hauck SM. Epithelial-to-mesenchymal transition of RPE cells *in vitro* confers increased beta1,6-N-glycosylation and increased susceptibility to galectin-3 binding. *PLoS One* 2016;11(1):e0146887.
818. Bubka M, Link-Lenczowski P, Janik M, Pohec E, Litynska A. Overexpression of n-acetylglucosaminyltransferases III and V in human melanoma cells. Implications for MCAM N-glycosylation. *Biochimie* 2014;103:37-49.
819. Scott H, Panin VM. The role of protein N-glycosylation in neural transmission. *Glycobiology* 2014;24(5):407-417.
820. Ruhaak LR, Taylor SL, Stroble C, Nguyen UT, Parker EA, Song T, Lebrilla CB, Rom WN, Pass H, Kim K, Kelly K, Miyamoto S. Differential N-glycosylation patterns in lung adenocarcinoma tissue. *Journal of Proteome Research* 2015;14(11):4538-4549.
821. Hamouda H, Ullah M, Berger M, Sittinger M, Tauber R, Ringe J, Blanchard V. N-glycosylation profile of undifferentiated and adipogenically differentiated human bone marrow mesenchymal stem cells: Towards a next generation of stem cell markers. *Stem Cells Dev* 2013;22(23):3100-3113.
822. Leight JL, Drain AP, Weaver VM. Extracellular matrix remodeling and stiffening modulate tumor phenotype and treatment response. *Annual Review of Cancer Biology* 2017;1(1):313-334.

823. Peanne R, Vanbeselaere J, Vicogne D, Mir AM, Biot C, Matthijs G, Guerardel Y, Foulquier F. Assessing ER and Golgi N-glycosylation process using metabolic labeling in mammalian cultured cells. *Methods in Cell Biology* 2013;118:157-176.
824. Miyahara K, Nouse K, Morimoto Y, Kinugasa H, Kato H, Yamamoto N, Tsutsumi K, Kuwaki K, Onishi H, Ikeda F, Nakamura S, Shiraha H, Takaki A, Nakahara T, Miura Y, Asada H, Amano M, Nishimura S, Yamamoto K. Prognostic value of altered N-glycosylation of circulating glycoproteins in patients with unresectable pancreatic cancer treated with gemcitabine. *Pancreas* 2015;44(4):551-556.
825. Saldova R, Asadi Shehni A, Haakensen VD, Steinfeld I, Hilliard M, Kifer I, Helland A, Yakhini Z, Borresen-Dale AL, Rudd PM. Association of N-glycosylation with breast carcinoma and systemic features using high-resolution quantitative UPLC. *Journal of Proteome Research* 2014;13(5):2314-2327.
826. Huang Y, Wu H, Xue R, Liu T, Dong L, Yao J, Zhang Y, Shen X. Identification of N-glycosylation in hepatocellular carcinoma patients' serum with a comparative proteomic approach. *PLoS One* 2013;8(10):e77161.
827. Roth J, Zuber C, Park S, Jang I, Lee Y, Kysela KG, Le Fourn V, Santimaria R, Guhl B, Cho JW. Protein N-glycosylation, protein folding, and protein quality control. *Molecules and Cells* 2010;30(6):497-506.
828. Parmar VM, Schroder M. Sensing endoplasmic reticulum stress. *Advances in Experimental Medicine and Biology* 2012;738:153-168.
829. Koumenis C. ER stress, hypoxia tolerance and tumor progression. *Current Molecular Medicine* 2006;6(1):55-69.
830. Shah PP, Dupre TV, Siskind LJ, Beverly LJ. Common cytotoxic chemotherapeutics induce epithelial-mesenchymal transition (EMT) downstream of ER stress. *Oncotarget* 2017;doi:10.18632/oncotarget.15150.
831. Martinon F. Targeting endoplasmic reticulum signaling pathways in cancer. *Acta Oncologica* 2012;51(7):822-830.
832. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proceedings of the National Academy of Sciences* 1998;95(12):7024-7029.
833. Zhang Y, Lozano G. P53: Multiple facets of a rubik's cube. *Annual Review of Cancer Biology* 2017;1(1):185-201.
834. Shrimal S, Cherepanova NA, Gilmore R. Cotranslational and posttranslational N-glycosylation of proteins in the endoplasmic reticulum. *Seminars in Cell & Developmental Biology* 2015;41:71-78.
835. Anania VG, Yu K, Gnad F, Pferdehirt RR, Li H, Ma TP, Jeon D, Fortelny N, Forrest W, Ashkenazi A, Overall CM, Lill JR. Uncovering a dual regulatory role for caspases during endoplasmic reticulum stress-induced cell death. *Molecular and Cellular Proteomics* 2016;15(7):2293-2307.
836. Deshaies RJ. Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. *BMC Biology* 2014;12:94.
837. Waechter CJ, Schmidt JW, Catterall WA. Glycosylation is required for maintenance of functional sodium channels in neuroblastoma cells. *Journal of Biological Chemistry* 1983;258(8):5117-5123.
838. Milenkovic AG, Johnson TC. The relationship between glycosylation and glycoprotein metabolism of mouse neuroblastoma N18 cells. *Biochemical Journal* 1980;191(1):21-28.
839. Hiss D, Gabriels G, Jacobs P, Folb P. Tunicamycin potentiates drug cytotoxicity and vincristine retention in multidrug resistant cell lines. *European Journal of Cancer* 1996;32A(12):2164-2172.
840. Loo TW, Clarke DM. The human multidrug resistance P-glycoprotein is inactive when its maturation is inhibited: Potential for a role in cancer chemotherapy. *The FASEB Journal* 1999;13(13):1724-1732.
841. Cojoc M, Mabert K, Muders MH, Dubrovskaya A. A role for cancer stem cells in therapy resistance: Cellular and molecular mechanisms. *Seminars in Cancer Biology* 2015;31:16-27.
842. Sui H, Zhu L, Deng W, Li Q. Epithelial-mesenchymal transition and drug resistance: Role, molecular mechanisms, and therapeutic strategies. *Oncol Res Treat* 2014;37(10):584-589.
843. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: An awakening field. *Nature Reviews: Cancer* 2014;14(9):611-622.
844. Varelas X, Bouchie MP, Kukuruzinska MA. Protein N-glycosylation in oral cancer: Dysregulated cellular networks among dpagt1, E-cadherin adhesion and canonical wnt signaling. *Glycobiology* 2014;24(7):579-591.

845. Li X, Wang X, Tan Z, Chen S, Guan F. Role of glycans in cancer cells undergoing epithelial-mesenchymal transition. *Frontiers in Oncology* 2016;6:33.
846. Peiris D, Spector AF, Lomax-Browne H, Azimi T, Ramesh B, Loizidou M, Welch H, Dwek MV. Cellular glycosylation affects herceptin binding and sensitivity of breast cancer cells to doxorubicin and growth factors. *Scientific Reports* 2017;7:43006.
847. Hebert DN, Lamriben L, Powers ET, Kelly JW. The intrinsic and extrinsic effects of N-linked glycans on glycoproteostasis. *Nature Chemical Biology* 2014;10(11):902-910.
848. Bischoff J, Kornfeld R. The effect of 1-deoxymannojirimycin on rat liver alpha-mannosidases. *Biochemical and Biophysical Research Communications* 1984;125(1):324-331.
849. Muta K, Fukami T, Nakajima M, Yokoi T. N-glycosylation during translation is essential for human arylacetamide deacetylase enzyme activity. *Biochemical Pharmacology* 2014;87(2):352-359.
850. Hammond C, Braakman I, Helenius A. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91(3):913-917.
851. Johnson AJ, Hsu A-L, Lin H-P, Song X, Chen C-S. The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca<sup>2+</sup>-ATPases: A plausible link with its anti-tumour effect and cardiovascular risks. *Biochemical Journal* 2002;366(3):831-837.
852. Wang Z, Fuentes CF, Shapshay SM. Antiangiogenic and chemopreventive activities of celecoxib in oral carcinoma cell. *Laryngoscope* 2002;112(5):839-843.
853. Roy KR, Reddy GV, Maitreyi L, Agarwal S, Achari C, Vali S, Reddanna P. Celecoxib inhibits MDR1 expression through COX-2-dependent mechanism in human hepatocellular carcinoma (HepG2) cell line. *Cancer Chemotherapy and Pharmacology* 2010;65(5):903-911.
854. Tsai YT, Lozanski G, Lehman A, Sass EJ, Hertlein E, Salunke SB, Chen CS, Grever MR, Byrd JC, Lucas DM. BRAF induces ABCB1/P-glycoprotein expression and drug resistance in b-cells via ap-1 activation. *Leukemia Research* 2015.
855. Chen C, Shen HL, Yang J, Chen QY, Xu WL. Preventing chemoresistance of human breast cancer cell line, MCF-7 with celecoxib. *Journal of Cancer Research and Clinical Oncology* 2011;137(1):9-17.
856. Avemary J, Salvamoser JD, Peraud A, Remi J, Noachter S, Fricker G, Potschka H. Dynamic regulation of P-glycoprotein in human brain capillaries. *Molecular Pharmaceutics* 2013;10(9):3333-3341.
857. Robison NJ, Campigotto F, Chi SN, Manley PE, Turner CD, Zimmerman MA, Chordas CA, Werger AM, Allen JC, Goldman S, Rubin JB, Isakoff MS, Pan WJ, Khatib ZA, Comito MA, Bendel AE, Pietrantonio JB, Kondrat L, Hubbs SM, Neuberg DS, Kieran MW. A phase II trial of a multi-agent oral antiangiogenic (metronomic) regimen in children with recurrent or progressive cancer. *Pediatric Blood & Cancer* 2014;61(4):636-642.
858. Kaneko M, Kaneko S, Suzuki K. Prolonged low-dose administration of the cyclooxygenase-2 inhibitor celecoxib enhances the antitumor activity of irinotecan against neuroblastoma xenografts. *Cancer Science* 2009;100(11):2193-2201.
859. Park SH, Hong H, Han YM, Kangwan N, Kim SJ, Kim EH, Hahm KB. Nonsteroidal anti-inflammatory drugs (NSAID) sparing effects of glucosamine hydrochloride through N-glycosylation inhibition; strategy to rescue stomach from NSAID damage. *Journal of Physiology and Pharmacology* 2013;64(2):157-165.
860. Unzueta A, Vargas HE. Nonsteroidal anti-inflammatory drug-induced hepatotoxicity. *Clinics in Liver Disease* 2013;17(4):643-656, ix.
861. Elbein AD, Solf R, Dorling PR, Vosbeck K. Swainsonine: An inhibitor of glycoprotein processing. *Proceedings of the National Academy of Sciences of the United States of America* 1981;78(12):7393-7397.
862. Elbein AD, Pan YT, Solf R, Vosbeck K. Effect of swainsonine, an inhibitor of glycoprotein processing, on cultured mammalian cells. *Journal of Cellular Physiology* 1983;115(3):265-275.
863. Henics T, Wheatley DN. Cytoplasmic vacuolation, adaptation and cell death: A view on new perspectives and features. *Biology of the Cell* 1999;91(7):485-498.
864. Morissette G, Moreau E, C.-Gaudreault R, Marceau F. Massive cell vacuolization induced by organic amines such as procainamide. *Journal of Pharmacology and Experimental Therapeutics* 2004;310(1):395-406.
865. Dennis JW, Granovsky M, Warren CE. Glycoprotein glycosylation and cancer progression. *Biochimica et Biophysica Acta* 1999;1473(1):21-34.



866. Ranjan A, Kalraiya RD. Invasive potential of melanoma cells correlates with the expression of mt1-mmp and regulated by modulating its association with motility receptors via N-glycosylation on the receptors. *BioMed Research International* 2014;2014:804680.
867. Hamaguchi J, Nakagawa H, Takahashi M, Kudo T, Kamiyama N, Sun B, Oshima T, Sato Y, Deguchi K, Todo S, Nishimura S. Swainsonine reduces 5-fluorouracil tolerance in the multistage resistance of colorectal cancer cell lines. *Molecular Cancer* 2007;6:58.
868. Goss PE, Reid CL, Bailey D, Dennis JW. Phase I clinical trial of the oligosaccharide processing inhibitor swainsonine in patients with advanced malignancies. *Clinical Cancer Research* 1997;3(7):1077-1086.
869. Huang W, Luo WJ, Zhu P, Tang J, Yu XL, Cui HY, Wang B, Zhang Y, Jiang JL, Chen ZN. Modulation of cd147-induced matrix metalloproteinase activity: Role of cd147 N-glycosylation. *Biochemical Journal* 2013;449(2):437-448.
870. Potter-Perigo S, Braun KR, Schönherr E, Wight TN. Altered proteoglycan synthesis via the false acceptor pathway can be dissociated from  $\beta$ -D-xyloside inhibition of proliferation. *Archives of Biochemistry and Biophysics* 1992;297(1):101-109.
871. Feng YX, Sokol ES, Gupta PB. The endoplasmic reticulum may be an Achilles' heel of cancer cells that have undergone an epithelial-to-mesenchymal transition. *Molecular & Cellular Oncology* 2014;1(2):e961822.
872. Saggar JK, Yu M, Tan Q, Tannock IF. The tumor microenvironment and strategies to improve drug distribution. *Frontiers in Oncology* 2013;3:154.
873. Masetti R, Biagi C, Zama D, Vendemini F, Martoni A, Morello W, Gasperini P, Pession A. Retinoids in pediatric onco-hematology: The model of acute promyelocytic leukemia and neuroblastoma. *Advances in Therapy* 2012;29(9):747-762.
874. Castel V, Segura V, Berlanga P. Emerging drugs for neuroblastoma. *Expert Opin Emerg Drugs* 2013;18(2):155-171.
875. Ruddon RW, Kufe DW. Biochemistry of Cancer. In: Hong WK, Bast RC, Jr., Hait WN, Kufe DW, Pollock RE, Weichselbaum RR, Holland JF, Frei E, III., eds. *Cancer Medicine*. Shelton, Connecticut: BC Decker, People's Medical Publishing House-USA; 2010:125-140.





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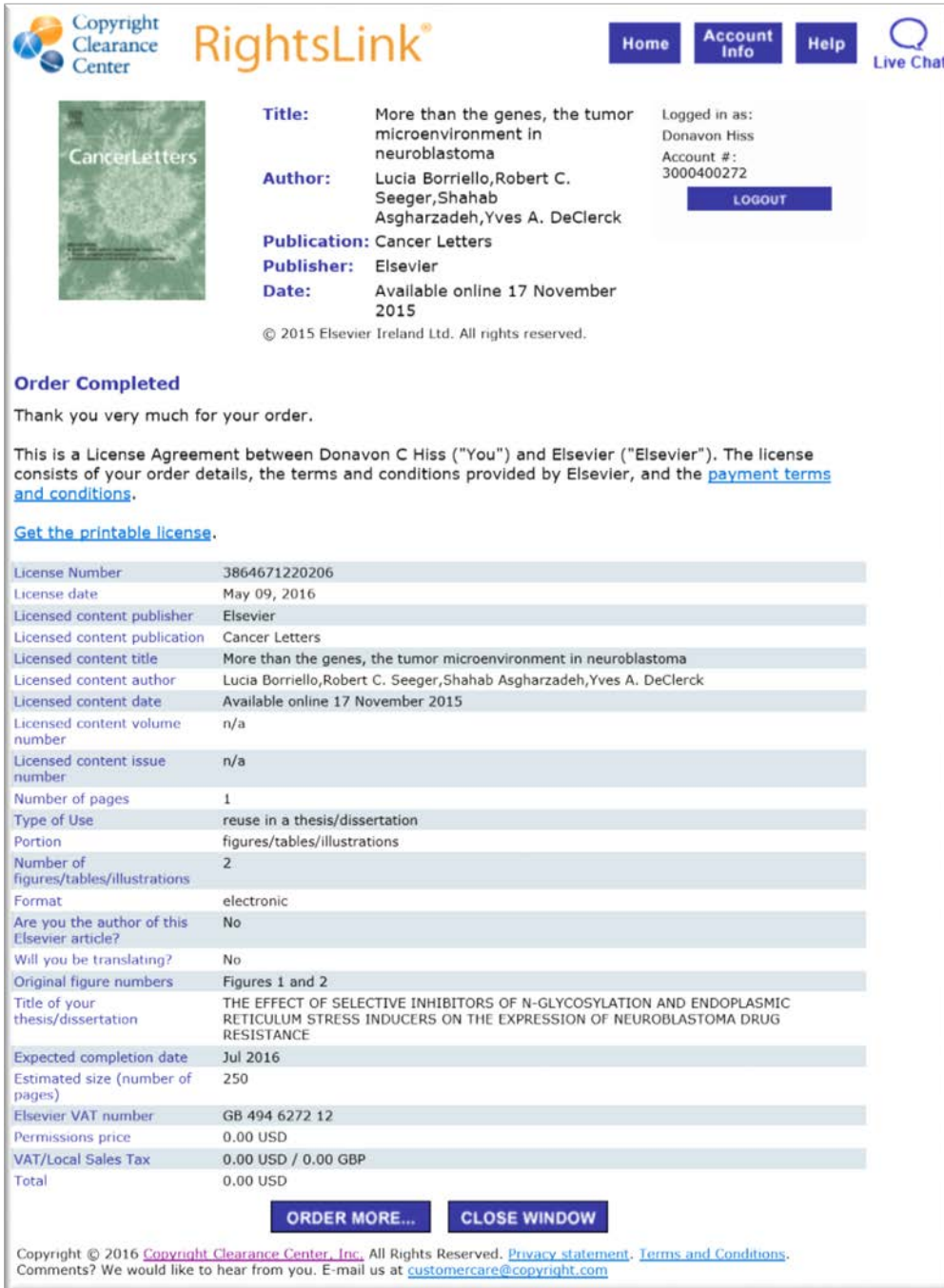
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- Author:** Lucia Borriello, Robert C. Seeger, Shahab Asgharzadeh, Yves A. DeClerck
- Publication:** Cancer Letters
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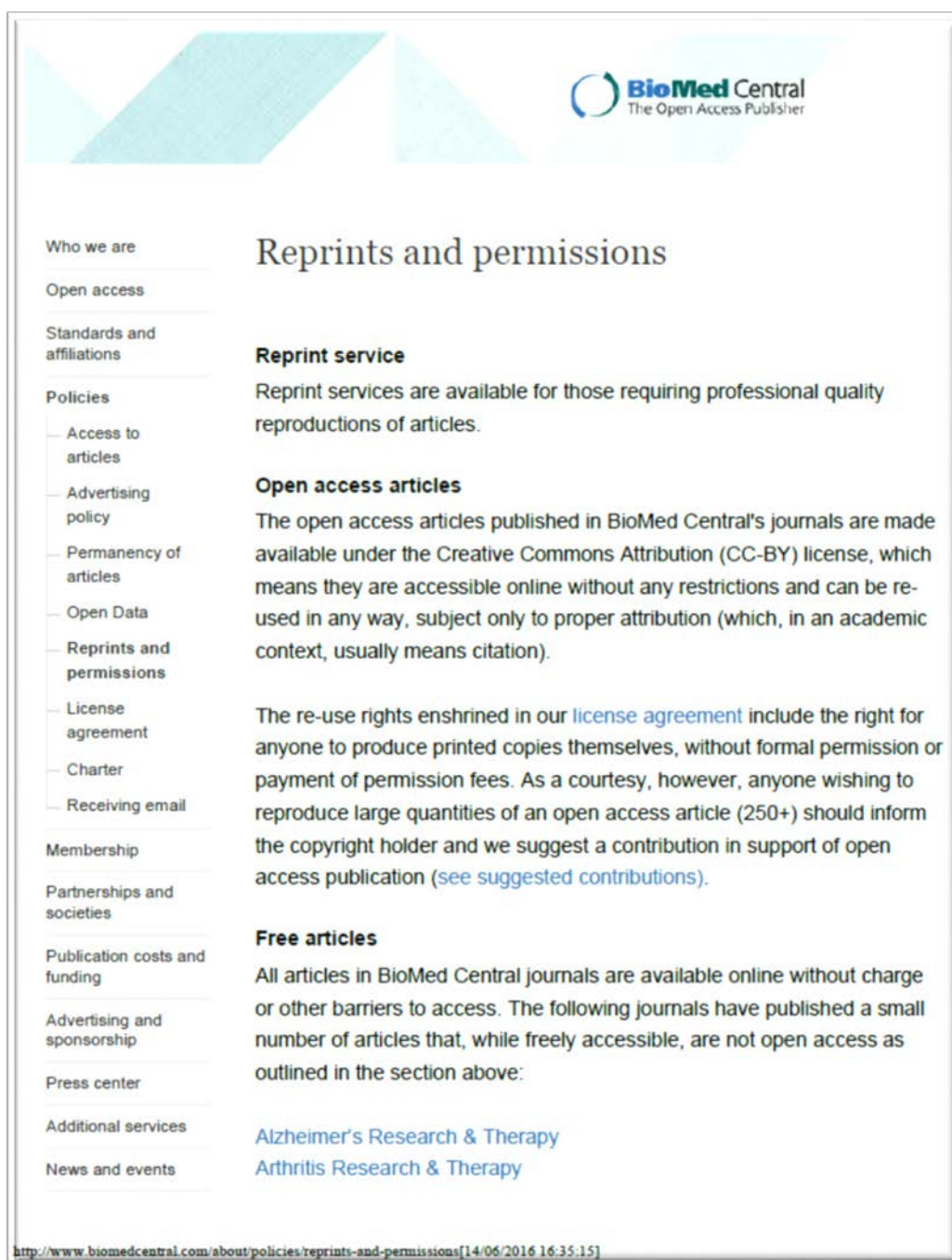
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**Author:** Qiong Wu,Zhiping Yang,Yongzhan Nie,Yongquan Shi,Daiming Fan

**Publication:** Cancer Letters

**Publisher:** Elsevier

**Date:** 1 June 2014

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**Author:** C.Patrick Reynolds,Katherine K. Matthay,Judith G. Villablanca,Barry J. Maurer

**Publication:** Cancer Letters

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**Title:** mTOR and cancer: many loops in one pathway

**Author:** Alejo Efeyan, David M Sabatini

**Publication:** [Current Opinion in Cell Biology](#)

**Publisher:** Elsevier

**Date:** April 2010

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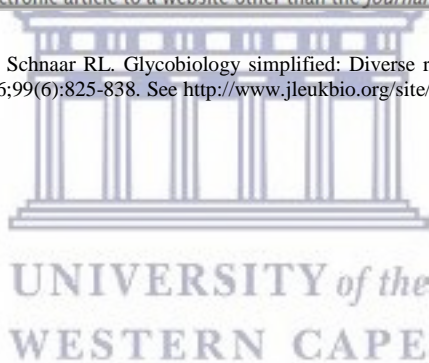
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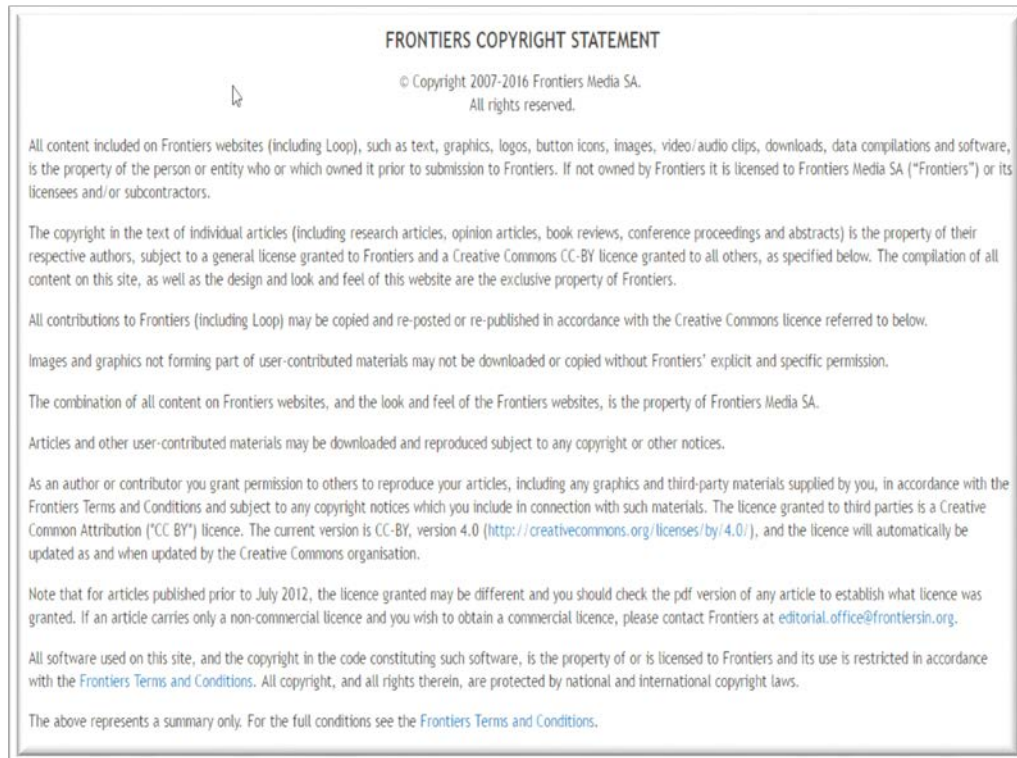
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