The Integrated Effects of Selected Inducers of Endoplasmic Reticulum Stress, the Unfolded Protein Response and Apoptosis on P-Glycoprotein-Mediated Drug Resistance in MCF-7 Breast Carcinoma Cells

by

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DECLARATION

I, **Leeshan Pillay**, hereby declare that the dissertation "**The Integrated Effects of Selected Inducers of Endoplasmic Reticulum Stress, the Unfolded Protein Response and Apoptosis on P-Glycoprotein-Mediated Drug Resistance in MCF-7 Breast Carcinoma Cells**" for the Masters degree in Medical BioSciences at the University of the Western Cape hereby submitted by me has not been submitted previously at this or any other university, and that it is my own work in design and in execution, and that all reference materials contained herein have been duly acknowledged.

Leeshan Pillay : .....

Date Signed : .....

DEDICATION

This dissertation is dedicated to first and foremost to my parents, Ameta and Rajin Pillay, who through their sacrifices granted me the opportunity to further my education by allowing me to undertake this study, and providing me with the necessary emotional, mental and physical support required to complete this dissertation. To my siblings, Dinesan and Shantini Pillay, who encouraged me to persevere, and providing me the reassurance that I was never alone in this exciting and eventful, yet stressful adventure. Your continuous support, abundance of love, patience and understanding during my studies is highly appreciated.

This dissertation is also dedicated to all individuals currently affected by breast cancer, in material of stage or state, you will always be in our memories and prayers. It is a long and scary road to walk through, but as long as cancer research continues, there will always be hope that one day the road will not need to be pursued at all, and that the ongoing battle faced by breast cancer patients will soon be won.

LITERARY QUOTATIONS

With over 3 million women battling breast cancer today, everywhere you turn there is a mother, daughter, sister, or friend who has been affected by breast cancer.

BETSEY JOHNSON (1942)

American designer.

(http://www.brainyquote.com/quotes/quotes/b/betseyjon441042.html, accessed November 3, 2014).

Growth for the sake of growth is the ideology of the cancer cell.

EDWARD ABBEY (1927-1989)

American author.

(http://www.brainyquote.com/quotes/quotes/e/edwardabbe104709.ht ml, accessed November 3, 2014).

In order to pursue chemotherapy successfully we must look for substances which possess a high affinity and high lethal potency in relation to the parasites, but have a low toxicity in relation to the body, so that it becomes possible to kill the parasites without damaging the body to any great extent. We want to hit the parasites as selectively as possible. In other words, we must learn to aim and to aim in a chemical sense. The way to do this is to synthesize by chemical means as many derivatives as possible of relevant substances.

PAUL EHRLICH (1854-1915)

German bacteriologist, haematologist and immunologist who discovered Salvarsan, the first effective treatment for syphilis. He shared the 1908 Nobel Prize for Physiology or Medicine with Élie Metchnikoff. 'Partial Cell Functions', Nobel Lecture, 11 Dec 1908. In: *Nobel Lectures: Physiology or Medicine 1901-1921* (1967), 304. (http://todayinsci.com/E/Ehrlich_Paul/EhrlichPaul-Quotations.htm accessed November 3, 2014), 'Ueber den jetzigen Stand der Chemotherapie'. Berichte der Deutschen Chemischen Gesellschagt, 1909, 42, 17-47. Translated in B. Holmstedt and G. Liljestrand (eds), *Readings in Pharmacology* (1963), (http://todayinsci.com/QuotationsCategories/C_Cat/Chemotherapy-Quotations.htm).

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My supervisor, Prof Donavon Charles Hiss, Your assistance, encouragement and support has had a major impact on the outcome of my dissertation. Your guidance in my research project was highly appreciated and it has been a pleasure and honour to have been under your supervision.

The office of the Deputy Vice-Chancellor (in particular, Prof Ramesh Bharuthram) for the financial support towards the Molecular Oncology Laboratory.

The NRF for providing the necessary funding to undertake and complete this study.

ABSTRACT

Purpose: One of the leading causes of death reported in women worldwide is breast cancer. Many tumours, including breast cancer, associated with poor prognosis, have received a renewed focus and increased perspective with regard to drug discovery and innovation towards developing rational combination regimens of first-line anticancer drugs with novel compounds that target diverse hallmarks of the cancer phenotype. Multidrug resistance (MDR), which has been found to significantly decrease the efficacy of anticancer drugs and causes tumor recurrence, has been a major challenge in clinical cancer treatment with chemotherapeutic drugs for decades. Several mechanisms of overcoming drug resistance have been postulated and the well known P-glycoprotein (P-gp) including other drug efflux transporters are considered to be critical in pumping anticancer drugs out of cells which in turn results in unsuccessful chemotherapy treatments. The endoplasmic reticulum (ER) is an interconnecting organelle which synthesizes proteins and its quality control processes ensures the proper protein folding, post-translational modifications and conformation of secretory and trans-membrane proteins. Previous studies demonstrated that geldanamycin (GA), a benzoquinone ansamycin antibiotic, the antibiotic, tunicamycin (TM) and the sesquiterpene lactone, thapsigargin (TG) have been found to cause ER stress and consequently, cellular arrest. GA is known to manifest anti-cancer activity through the inhibition of Hsp90-chaperone, TM interferes with N-glycosylation of newly synthesized proteins triggering the unfolded protein response, while TG inhibits intracellular Ca^{2+} ATPases resulting in increased cytosolic Ca^{2+}. Cellular stress conditions, lead to accumulation of unfolded or misfolded proteins in the endoplasmic reticulum lumen which results in a unfolded protein response (UPR) to maintain cell survival in cancer cells. ERS has been previously reported to enhance MDR1 transcriptional induction and P-gp transport function in cancer cells, however, prolonged endoplasmic reticulum stress conditions and inadequate unfolded protein response force cells undergo apoptosis. In this study, we examined the effects of GA, TG and TM alone and in combination to determine the cellular response of the MCF-7 breast carcinoma cell line with regard to proliferation and P-gp-mediated drug efflux activity and apoptosis.

Methods: Analyses of MCF-7 breast carcinoma cells exposed to Endoplasmic Reticulum Stress

(ERS) inducers geldanamycin, thapsigargin and tunicamycin, alone and in combination, included growth curves alone and in the presence of 24 hour IC_{50} inhibitory concentrations of the 3 ERS inducers alone, dose-response curves (MTT cytotoxicity assays) of the ERS alone and in combination, analysis of P-glycoprotein-mediated efflux pump activity in the presence of the ERS inducers alone and in combination (Calcein-AM efflux assays), analysis of viability, cytotoxicity and early apoptosis via caspase-3/7 expression (Triplex assay) and morphological staining of apoptotic and/or necrotic cells in the presence of IC_{50} inhibitory concentrations of the ERS inducers alone with Annexin V-FITC.

Results: This study investigated the effects of Endoplasmic Reticulum Stress (ERS) inducers on growth and proliferation of MCF-7 breast carcinoma cells in culture. The MCF-7 cell line was exposed to different concentrations of ERS inducers alone and in combination with each other. All responses occurred in a dose- and time- dependent manner. When combined at equimolar log dose concentrations, integrated effects yielded enhanced cytotoxic properties as IC_{50} values were drastically decreased in combination as opposed to single ERS inducer responses. Combined effect on P-glycoprotein-mediated drug efflux activity yielded minor but insignificant decreases in efflux pump activity at different time intervals as opposed to the increase in cellular efflux in the presence of the ERS inducers alone at different time intervals. Caspase-3/7 apoptotic protein expression was increased as log doses of ERS inducers alone were increased, leading to cell necrosis at higher cytotoxic concentrations. The determined IC_{50} growth inhibitory concentrations after 24 hours were confirmed by the Annexin V-FITC demonstrating early apoptotic, necrotic and viable cells in the presence of the ERS inducers alone.

Conclusion: This study demonstrated a significant growth inhibition of MCF-7 breast carcinoma cells upon exposure to ERS inducers alone. Results suggested that when ERS inducers are used in combination, their efficacy is enhanced as 50 percent inhibitory concentrations were considerably lower in combination as opposed to when used alone. The present study is consistent with previous studies with geldanamycin, and was the 1st to investigate the effects of geldanamycin, thapsigargin and tunicamycin in combination and with reference to P-gp efflux activity. Results suggested that in combination, efflux activity may be reduced, and efficacy may be enhanced. To enhance efficacy would be a major breakthrough in cancer drug discovery and development-targeting specific populations of cancer cells and reducing ERS-induced toxicity to normal cells and vital organs.

KEYWORDS

- Breast Cancer
- Multidrug Resistance
- P-Glycoprotein
- Endoplasmic Reticulum
- Endoplasmic Reticulum Stress
- Geldanamycin
- Thapsigargin
- Tunicamycin
- Unfolded Protein Response
- Human MCF-7 Breast Carcinoma Cells
- Dose-response Curves
- Calcein-AM
- IC_{50}
- Annexin V-Cy3 Fluorescent Staining
- Apoptosis

ABBREVIATIONS, NOTATIONS AND SYMBOLS

°C	Degrees Celsius/Centigrade
ABC	ATP Binding Cassette
ATF6	Activating Transcription Factor 6
BCRP	Breast Cancer Resistance Protein
BCS	Breast-Conserving Surgery
BiP	Immunoglobulin heavy chain Binding Protein
B-TNBC	Basaloid Triple-Negative Breast Cancer
6-CFDA	6-Carboxyfluorescein Diacetate
95% CI	95% Confidence Interval
DCIS	Ductal Carcinoma In Situ
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EGCG	Epigallocatechin Gallate
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation Response

ERSE	Endoplasmic Reticulum Stress-Response Elements
GA	Geldanamycin
GPT	GlcNAc-1-P Transferase
GRP 78	Glucose Regulated Protein 78
h	Hour(s)
hBSCs	Human Breast milk Stem Cells
HDI	Human Development Index
HER2	Human Epidermal Growth Factor Receptor 2
HIFBS	Heat-Inactivated Foetal Bovine Serum
Hsp/Hsps	Heat Shock Protein/Proteins
H/Mdm2	Human/Mouse double minute 2
IAPS	Inhibitor of Apoptosis Proteins
IARC	International Agency for Research on Cancer
IBC	Invasive Breast Carcinoma
IC_{50}	50% Inhibitory Concentration
IDC	Invasive Ductal Carcinoma
IGF-1R	Insulin-Like Growth Factor 1 Receptor
IHC	Immunohistochemical
IRE1	Inositol-Requiring Enzyme 1
ILC	Invasive Lobular Carcinoma

kDa	Kilo Dalton
LCIS	Lobular Carcinoma In Situ
MBC	Metastatic Breast Cancer
MCF-7	Michigan Cancer Foundation—human cell line 7 from a pleural effusion derived from a breast carcinoma
MDR	Multidrug Resistance
MECs / MEPs	Mammary Epithelial Cells / Myoepithelial Cells
MMG	Mammography
MPR1	Multidrug Resistance Protein 1
MRI	Magnetic Resonance Imaging
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MXR	Multi-Xenobiotic Resistance
NCI	National Cancer Institute
NF-κB	Nuclear Factor kappa B
PBS	Phosphate-Buffered Saline
PCD	Programmed Cell Death
PERK	Kinase-like ER Kinase
P-gp	P-glycoprotein
PLC	Pregnancy and Lactation Cycle
R2	Regression or Correlation Coefficient
SERCA	Sarcoplasmic Endoplasmic Reticulum ATPase

TG	Thapsigargin
TM	Tunicamycin
TNBC	Triple-Negative Breast Cancers
TRAIL	Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand
UPP	Ubiquitin Proteasome Pathway
UPR	Unfolded Protein Response
UPRE	Unfolded Protein Response Elements
UPS	Ubiquitin Proteasome System
WHO	World Health Organization
Xbp1	X-Box-Binding Protein

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction and Background to the Study

The objective of this chapter is to outline and briefly elaborate on our understanding of the functional anatomy and physiology of the female breast. It also covers breast cancer with regard to epidemiology, incidence and mortality statistics and the research efforts directed towards its prevention and the development of targeted molecular and personalized therapies. In addition, it includes a review of pertinent literature to introduce and contextualize the specialized areas of breast cancer research, the hypothesis to be tested, and a discussion of the rationale for the choice of methods to investigate the research problem and analyze the results to achieve the set objectives.

1.2 Functional Anatomy of the Breast

Breasts, also referred to as mammae, are composed of breast tissue, fat, nerves, veins, arteries, lymphatic vessels, connective tissue and Cooper's ligaments which serve for breast support and provide its shape[1]. Figure 1.1 illustrates the different parts of the lactating female breast. Anatomically, the breast overlies the 2nd to 6th ribs, with the main chest muscle anatomically termed the pectoralis major muscle located between the chest and ribs.

The basic parts of a mature mammary gland are the alveoli lined with milk-secreting cuboidal cells which are surrounded by myoepithelial cells (MEPs or MECs). The alveoli (glandular tissue) are contained in lobules. Each lobule contains a duct that drains into the nipple known as the lactiferous duct. Contractions of the MECs are synchronized by oxytocin and results in milk secretion by alveolar units into the lobule lumen toward the nipple.

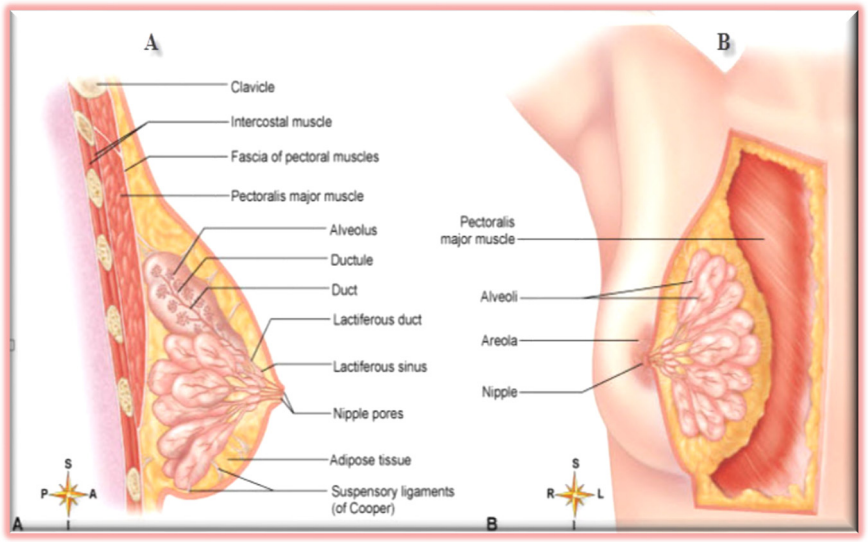
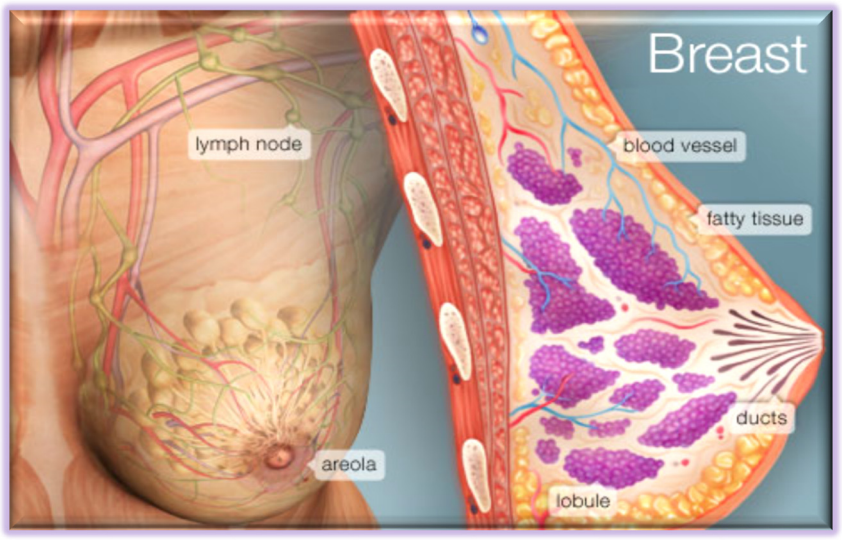


Figure 1.1: Micro-anatomical structure of the lactating female breast

A.: Saggital section showing how glandular structures are anchored to the overlying skin and to the pectoral muscles by the suspensory ligaments of Cooper. Each lobule of glandular tissue is drained by a lactiferous duct that opens through the nipple. **B:** Anterior view showing overlying skin and connective tissue removed from the medial side to reveal the internal structure of the breast and underlying skeletal muscle. In the non-lactating breast, the glandular tissue is much less prominent with adipose tissue constituting most of the breast. Source: Patton & Thibodeau: Anatomy & Physiology, 7th edition.[2]

The complex interconnections of lobules (minute oval sacs that produce milk) and ducts (that transport milk from the lobules to the nipple openings during lactation) form a structure that resembles bunches of grapes, referred to as lobes (Figure 1.2). The dark area of skin surrounding the nipple is termed the areola. Nerves, in turn, impart sensation to the breast.

Breast development occurs during foetal growth, infancy (prepuberty), puberty, pregnancy as well as lactation-associated remodelling, and post-lactational and post-menopausal involution (shrinking). Mammary differentiation and specialization surge during the pregnancy and the lactation cycle (PLC) once the mammary gland transforms into a mature and functional milk-secretory organ.



Demonstrated above includes the areola, nipple arteries and veins, lymph nodes and lymphatic vessels, ducts and fatty tissue. Source: http://women.webmd.com/picture-of-the-breasts.

Figure1.2: Structure of the lactating female breast

The PLC can recur in multiparous females and therefore, childbearing and breastfeeding offer some protection against the development of breast cancer in the long term.[3-6] However, in humans, crosstalk occurs between the signalling pathways that drive normal mammary morphogenesis during PLC and those oncogenic (aberrantly activated or suppressed) signals associated with breast cancer initiation, progression and metastasis. In particular, self-renewal transcription factors are shared between normal human breast milk stem cells (hBSCs) and various types of aggressive breast tumours, implying that generalizations about the protective effects of PLC should be made with prudence.[7,8]

The onset of post-menopausal involution is paralleled by a decreased ovarian function and blood levels of oestrogen and progesterone which cause regression and atrophy of the glandular tissue of the breast and a concomitant buildup of adipose tissue and correlated sequalae such as dysregulated mammary cell transformation and the development of breast cancer.[8]

1.3 Breast Cancer

Breast cancer has been found to originate within the breast tissue, commonly the milk ducts or lobules, and has the propensity to spread to the lymph nodes in the armpits as well. The type of cancer can be classified by TNM staging of malignant tumors (T designates the size of the tumor and whether it has been colonized by the adjacent tissue; N refers to the regional lymph nodes that are implicated; M represents the distant metastasis or spread of the cancer from one body part to another), pathology, grade, receptor status and the presence or absence of genes as determined by DNA (deoxyribonucleic acid) analysis.[9,10]

Many risk factors pertaining to breast cancer include unchangeable circumstances, such as gender, aging, genetic risk factors, family history of breast cancer, personal history of breast cancer, race and ethnicity, however other cancer causing factors are related to personal lifestyle.[11]

Based on distinctive histopathological types, dissemination patterns to distant sites, therapeutic responses and patient outcomes, breast cancer is referred to as a heterogeneous group of diseases.[12,13] Some breast cancers are referred to as *in situ*, as they are confined between the ducts (ductal carcinoma *in situ* or DCIS) or lobules (lobular carcinoma *in situ* or LCIS) from which they originated. Of the two, it was been found that approximately 83% of *in situ* cases diagnosed were DCIS, and LCIS being less common only accounting for 11% of *in situ* breast cancers diagnosed during 2004-2008.[14]

1.4 Breast Cancer Epidemiology: Incidence and Mortality Statistics

According to the World Health Organization (WHO) and its cancer research agency, the International Agency for Research on Cancer (IARC), an estimated 12.7 million new cancer cases were diagnosed worldwide in 2008. It was also found that cancer caused more than 7.6 million deaths in that year (http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900; accessed 14 October 2013). The most frequently diagnosed cancers which exceeded 40% of all cases were lung, female breast, colorectal and stomach cancers.

Globally, breast cancer is found to be the most common type known to affect women and approximately 1.38 million new cases of this neoplasm was diagnosed in 2008, this found to represent approximately 11% of new cases and roughly 23% of all female cancers. (http://www.wcrf.org/cancer_statistics/data_specific_cancers/breast_cancer_statistics.php).

Figure 1.3 depicts the world incidence and mortality rates for breast cancer by region. Recent estimates have corroborated that breast cancer persists to be the most prevalent cancer in the vast majority of countries globally, i.e., by world region, country and human development index (HDI).[15] Incidence of breast cancer is higher in developed countries than in developing countries, however, this difference may be due, in part, to factors such as lifestyle, screening and incidence reporting practices.[16,17] A recent systematic analysis of the Global Burden of Disease reported 438,000 breast cancer deaths globally in 2010.[18]

Estimates for Europe in 2012 were 3.45 million new cases of cancer and 1.75 million deaths from cancer—most notably female breast cancer (464,000 cases), colorectal (447,000), prostate (417,000) and lung (410,000). Together, these four cancers represented almost half of the overall burden of cancer in Europe. The most common causes of death from cancer were cancers of the lung (353,000 deaths), colorectal (215,000), breast (131,000) and stomach (107,000).[19] In the United States alone, it was estimated that 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths would occur among women in 2013.[20]

Breast cancer persists to be the most prevalent female cancer in the vast majority of countries worldwide (Figure 1.4).[15] An appraisal of current trends approximate that in 2030 more than 747,802 women will die from breast cancer worldwide if surveillance, i.e., detection of life-threatening familial or sporadic disease at an earlier or more curable stage using diagnostic mammography (MMG) and clinical breast examination, prevention and treatment programmes are not implemented or improved.[21,22]

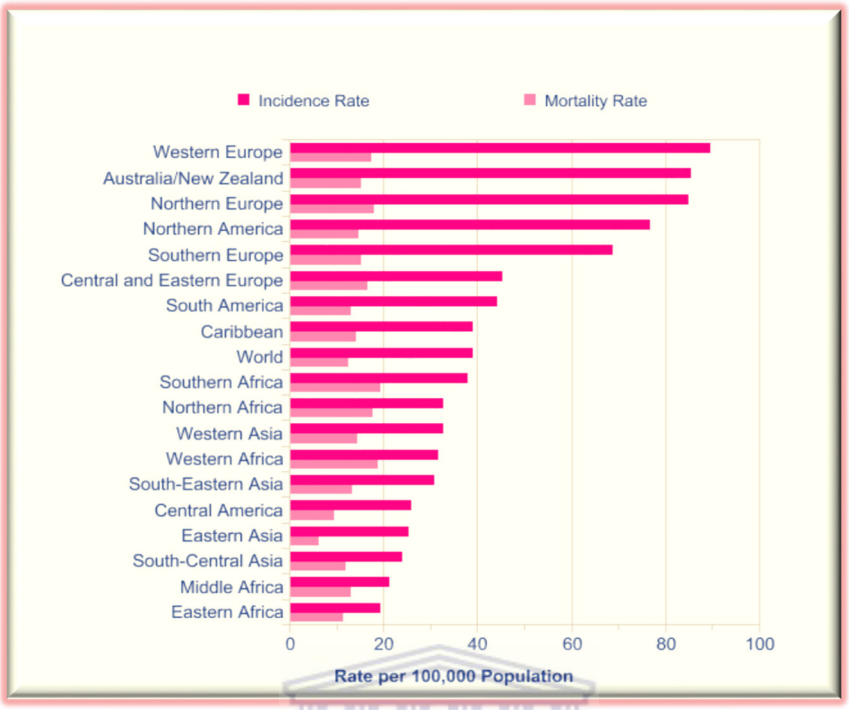


Figure1.3: World incidence and mortality rates for breast cancer by region

The estimates for 2008 presented here were taken from the International Agency for Research on Cancer GLOBOCAN database which presents cancer statistics for all cancers combined and for specific types of cancer for most countries or territories of the world.

(Source: Cancer Research UK: http://www.cancerresearchuk.org/cancer-info/cancerstats/world/cancer-worldwide-the-global-picture, Accessed 13 October 2013; See also Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://globocan.iarc.fr, Accessed 13 October 2013).

The intensification of breast cancer MMG (an X-ray of the breast) and frequency reporting in the United States and the rest of the world since 1980 has seen an increase in incidence registration of ductal carcinoma in situ (DCIS).[22] The abnormal DCIS cells are mostly confined to the milk ducts and do not transform into invasive cancer cells. Presently, it is difficult to discriminate DCIS that will develop into aggressive malignant cancer from the benign type, and thus many women are exposed to redundant therapeutic regimens that may lead to short- and long-term morbidities. However, a recent study has shown, using univariate and multivariate analyses, that basal-like DCIS

correlates with a higher risk of invasive- or general recurrence compared with non-basal-like DCIS.[23]

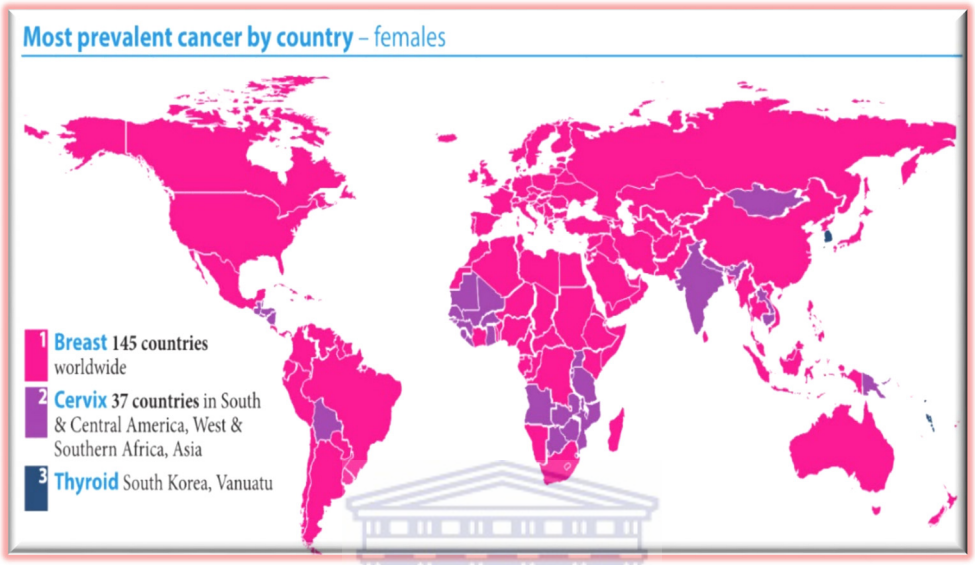


Figure: 1.4: Most prevalent female cancers by country

Data for 2008 presented here were taken from the International Agency for Research on Cancer GLOBOCAN database which presents cancer statistics for all cancers combined and for specific types of cancer for most countries or territories of the world. (Source: Cancer Research UK: http://www.cancerresearchuk.org/cancer-info/cancerstats/world/cancer-worldwide-the-global-picture, Accessed 13 October 2013; See also: http://globocan.iarc.fr, Accessed 13 October 2013).

Therefore, DCIS does necessitate therapy with breast-conserving surgery (BCS) and radiation to block development of invasive breast cancer. It should be noted though that women do not only die of tumours limited to the breast or draining lymph nodes, but that the majority of breast cancer deaths may be caused by metastasis to vital organs such as bone, lung, liver, and brain, and consequential failure of these organ systems.

It is currently estimated that triple-negative breast cancers (TNBC), i.e., tumours that tested negative for oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), contribute to 10-17% of all breast carcinomas, with higher incidence rates and poor

survival outcomes observed for certain ethnic patient populations (such as African Americans) and young age groups,[24-27] and women who are obese, premenopausal or of low socioeconomic background.[28] A recent study has demonstrated that guideline-adherent adjuvant treatment significantly improves survival of TNBC patients <50 and ≥65 years old.[29]

Several histochemical distinct subtypes of TNBC have been identified that express varying responses to chemo- and radiation therapy,[30,31] but basaloid triple-negative breast cancer (B-TNBC) probably represents one of the most invasive and therapy-resistant metastatic tumours of this type.[32,33] TNBC has defective DNA repair pathways, including BRCA1 mutations, which correlate with increased genomic instability, aggressive tumour behaviour and worst clinical history, even in the face of initial, yet short-term, extreme chemosensitivity.[34] Current classifications based on histologic and immunohistochemical profiles may contribute to a substantial advance in predicting the outcome in TNBC patients.[35]

1.5 Breast Cancer Therapies

According to the Global Cancer Report issued by the World Health Organization (WHO), there are over 10 million new cases of cancer each year and over 7.9 million annual deaths from the disease.[36] The various types of treatments currently used in the management of breast cancer include surgery, chemotherapy, hormone therapy, radiation therapy and targeted therapy.[37-40]

Due to dissemination of the disease to other organs and sites, surgery has not been found to be a very effective method of treatment for metastatic breast cancer. Major factors influencing the decision making of whether or not to resort to surgery as a form of treatment are most likely to be influenced by prognostic factors such as clinically undetectable or small tumour size, few metastatic sites (1 vs many), fewer liver metastases, metastases in bones and soft tissues rather than visceral negligible lymph node invasion, tumour grade, presence of ERs, HER2 overexpression, and the optimal choice of chemotherapy as first-line treatment. However, the primary objective of the tumor removal in

breast conserving surgery is to maintain the contour and shape of the breast while removing the tumour bulk and a perimeter of adjacent normal tissues.

Despite variability amongst patients, resistance to conventional chemo-and radiation- therapies have been linked with breast cancer relapse and metastasis, as well as complications in clinical responses.[37,41,42] In recent studies, it was concluded that low doses of doxorubicin produced minimal toxicity in T47D and SKBR3 breast cancer cells, particularly when combined with ionizing radiation. This combined therapeutic modality may prove advantageous if extended to patients with localized breast cancer, and principally a method to overcome the adverse effects of doxorubicin, such as cardiomyopathy, acute arrhythmia, irreversible congestive heart failure, radiation recall dermatitis (an acute inflammatory reaction or focal lesion at previously irradiated areas triggered by the administration of precipitating systemic anticancer agents after radiation treatment),[43] decreased full blood counts and associated risks of infection and haemorrhage, loss of appetite, stomatitis, alopecia, nausea and vomiting, mouth sores, birth defects and hepatotoxicity.[44]

Neoadjuvent chemotherapy is known as chemotherapy administered before surgery and after surgery administration referred to as adjuvant chemotherapy. There are several classes of conventional cytotoxic agents known to have distinct and overlapping mechanisms of action with moderate toxic effects which have proved to be efficacious as mono- or combination therapies in patients with metastatic breast cancer. The most active antineoplastics agents against metastatic breast cancer include the anthracyclines (doxorubicin, epirubicin and liposomal formulations of doxorubicin)[45] alkylating agents (cyclophosphamide, melphalan, thiotepa and cisplatin), the anthraquinones (mitoxantrone), antimetabolites (methotrexate, 5-fluorouracil, capecitabine and gemcitabine), the vinca alkaloids (vinorelbine, vinblastine and vincristine), epothilone-derivatives (ixabepilone) and the taxanes (paclitaxel, nab-paclitaxel and docetaxel).[46] Regrettably, continuous use of the above mentioned antineoplastic agents may result in multidrug resistance (MDR), this posing a major problem in the successive treatment and eradication of cancers.

1.6 Multidrug Resistance

Multidrug resistance (MDR), which has been found to significantly decrease the efficacy of anticancer drugs and causes tumor recurrence, has been a major challenge in clinical cancer treatment with chemotherapeutic drugs for decades.[36] Several mechanisms of overcoming drug resistance have been postulated and the well known P-glycoprotein (P-gp) and other drug efflux transporters are considered to be critical in pumping anticancer drugs out of cells which in turn results in unsuccessful chemotherapy treatments.[36]

Tumors generally develop significant resistance to repeated anticancer treatment with one kind of agent and often become resistant to similar or completely different drugs.[36] This mechanism for tumor survival under chemotherapeutic treatment is known as multidrug resistance (MDR).

MDR can be intrinsic or acquired through chemotherapeutic drug exposure, and multiple mechanisms are likely to contribute to clinical MDR. Historically, the most significant discovery about MDR was the identification of P-glycoprotein (P-gp),[36] which was found to be overexpressed on the plasma membrane of cancer cells with MDR.[36] Following P-gp, other transporters, such as multidrug resistance-associated protein 1 (MRP1)[36] and multixenobiotic resistance (MXR),[47] are also recognized to relate with drug efflux.

The changes found to drive antitumor drug resistance include increased activity of the drug eflux pump, such as the ATP-binding cassette (ABC) superfamily, decreased drug influx, activation of DNA repair, metabolic modification or detoxification and altered expression of apoptosis associated protein Bcl-2[36]and tumor supressor protein p53.[47,48] Of the above mentioned mechanisms, overexpression of ABC transporters is the most frequent.[36]

ABC transport molecules are generally expressed on plasma membranes and on the membranes of cellular vesicles. They are transmembrane proteins that use the energy of ATP hydrolysis to shuttle various substrates across the cell membrane as well as affect the pharmacokinetic properties of

chemotherapeutics in humans and thus play vital physiologic functions within the cell.[36] To date, there are 48 known transporters in the ABC family,[49]of which thirteen ABC transporters have been found to contribute to tumor MDR, including P-gp (MDR1/ABCB1), multidrug resistance proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/ABCG2), all of which are the most characterized ABC transporters.[50]

1.7 P-Glycoprotein

The normal function of ABC transporters as pumps is to extrude toxins and foreign substances out of the cell. P-glycoprotein (P-gp) has been found to be the best-known membrane pump molecule of the ABC transporters involved in MDR.[36] The Human P-gp, a 170 kDa membrane-associated protein which contains 1280 aminoacids, is able to carry out an ATP-dependent conformational change that is able to extrude intracellular substrates to the exterior of the cell.[51] It is able to transport a broad range of structurally expression -related or -unrelated compounds including anticancer drugs out of cells and as a result, decreases the intracellular accumulation of these compounds.[36]

Being able to extrude anticancer drugs from a cell, the P-gp can physiologically prevent cytotoxic compounds from staying in the cell by pumping them out to reduce their intracellular concentration.[36] In patients with tumors, P-gp is able to efflux various anticancer drugs such as doxorubicin and paclitaxel,[52] out of cancer cells. Overexpression of P-gp is a common feature of most acquired MDR in solid tumors.[36]

P-gp is synthesized and exported from the Endoplasmic Reticulum as a 150 kDa species that is N-glycosylated to a 170 kDa form which translocates to the cell surface as a mature drug efflux pump or, alternatively, the 150 kDa P-gp is cleaved to a 130 kDa proteolytic product by ER-resident proteases or it undergoes ubiquitylation and proteasomal degradation.[53] The upregulation of P-gp has been linked to evasion of apoptosis and hyperproliferation which promote tumorigenesis and the adaptive changes that characterize the MDR phenotype.[54]

1.8 The Endoplasmic Reticulum and Endoplasmic Reticulum Stress

In eukaryotic cells, the endoplasmic reticulum (ER) is a dynamic membranous organelle which plays a crucial role in protein folding, transport, and processing.[55] It is the cellular compartment where proteins enter the secretory pathway, undergo post translational modifications and acquire a correct conformation.[56] In addition,the ER also contains the molecular machines required to ensure the quality control and degradation of terminally misfolded secretory proteins, as well as the signaling components known to mediate communications with the rest of the cell.[56]

The ER is the organelle where protein folding occurs prior to transport to the extracellular matrix or to different intracellular sites.[57] This process depends on molecular chaperones that provide local environments favorable for protein folding. However, under a variety of conditions (ER stress), these folding reactions are compromized, and protein aggregation occurs.[58] When protein folding in the ER is disrupted by alterations in homeostasis in the ER lumen, eukaryotic cells have been found to activate a series of signal transduction cascades collectively termed the unfolded protein response (UPR).[59]

The UPR promotes normal and tumour cell survival by adjusting ER protein folding capacity during ERS. This protective and adaptive function of the UPR is reinforced by the endoplasmic reticulum-associated degradation (ERAD) pathway, a precision proteolytic and quality control machinery which decomposes proteins with abnormal or mutated conformations, preventing their toxic intracellular aggregation and accumulation.[60] Furthermore, the efficiency of UPR and ERAD in removing aberrant proteins is dependent on and coupled to the ubiquitin proteasome system (UPS, also referred to as the UPP - the ubiquitin proteasome pathway).[61,62] The UPS serves as a mono- or polyubiquitylation tagging system for many proteins implicated in cancer pathogenesis. Once proteins are covalently modified by ubiquitylation, their destruction by the 26S proteasome is imminent.[63] Therefore, malfunctioning of the UPS in relation to protein quality control, DNA repair mechanisms, cell growth and proliferation, apoptosis (programmed cell death, PCD) and the immune

response, can lead to cancer development.[61,62] Interestingly, but not surprising, ubiquitylation also regulates the stability of the MDR1 gene product, P-glycoprotein (P-gp), an ATP-dependent efflux pump that mediates multidrug resistance in cancer cells.[64] ERS also enhances MDR1 transcriptional induction and P-gp transport function in cancer cells.[65]

ERS activates many UPR target genes that encode protein folding enzymes (foldases) and molecular chaperones such as heat shock proteins (Hsps)[66,67] and glucose-regulated protein 78 (Grp78), also called immunoglobulin heavy chain binding protein (BiP).[68] Failure by the UPR transcriptional emergency response to establish cellular homeostatic or nontoxic levels of unfolded proteins invokes apoptosis, a cascade of PCD events that serves to protect the organism from harmful and fatal cellular insults.[69] Upregulation of the UPR generally confers a growth advantage on tumour cells,[69] although this may not always be the case, e.g, in mouse models of prostate tumorigenesis, the UPR appears to be selectively downregulated.[70] Thus, the ERS and UPR signalling pathways are finely tuned such that moderate ERS favours an anti-apoptotic UPR mode that will maintain tumour survival, metastasis, angiogenesis and chemoresistance, whereas chronic ERS will switch on an assertive pro-apoptotic UPR mode that will arrest tumour cell growth and induce apoptosis.[60] Dysregulated apoptosis is one of the hallmarks of cancer cells that not only allows them to sustain rapid growth and metastases, but also to develop resistance to radiation or cancer chemotherapeutic drugs. Cancer cells are by definition defective cells,[71,72] and their ability to evade apoptosis (the very genetic programme designed to eliminate them), and thus survive cytocidal purging, is orchestrated through specific cancer gene mutations and chromosomal abnormalities, many of which are unfolded protein response-responsive elements (UPRE) and ER-stress-responsive elements (ERSE).[69] Specific molecular cell stress sensors involved in ERS, UPR and apoptosis include the tumour suppressor protein p53 and its negative regulator, human/mouse double minute 2 (H/Mdm2, an E3 ubiquitin ligase),[73] growth arrest and DNA damage-inducible protein, also called CHOP (Gadd153/CHOP),[60] heat shock proteins (including Grp78/BiP, a member of Hsp70 family, Hsp27 and Hsp90),[74] the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA),[75] a transmembrane ER Ca^{2+}

pump whose overexpression induces the activation of ERS markers such as Xbp1 (X-box binding protein-1 which, in turn, regulates UPR genes that promote ERAD of misfolded proteins),[76] and nuclear factor kappa B (NF-κB) which is expressed in many cancer cells and implicated in the transcriptional activation of inhibitor of apoptosis proteins (IAPs).[77]

Though better known for their actions upon mitochondria, Bcl-2/Bax–family proteins also integrate into ER membranes, where they modulate ER Ca^{2+} homeostasis and control cell death induced by ER stress agents, including tunicamycin, brefeldin A (an inhibitor of ER-Golgi transport), thapsigargin, and oxidants.[78]

Common and well studied ER stressors include heat shock, proteasome inhibition (e.g., by MG132)[79,80] or Hsp90 inhibition (e.g., by geldanamycin),[81,82] ER homeostasis disruption (e.g., by tharpsigargin and tunicamycin)[83-89] or chemotherapeutic drugs (e.g., taxanes).[86,90]

1.9 Geldanamycin

Geldanamycin (GA) is a benzoquinone ansamycin antibiotic, known to manifest anti-cancer activity through the inhibition of Heat shock protein 90 (Hsp90) chaperone function,[91] and was the first benzoquinone ansamycin antibiotics identified from *Streptomyces hygrocopicus* in 1970.[91] Initially, GA was regarded as a tyrosine kinase inhibitor,[92] as it was found to block several signalling kinase activities.[91,93]

Hsp90 is a molecular chaperone that plays a key role in the conformational maturation of oncogenic signalling proteins, including HER-2/Er bB2, Akt, Raf-1, Bcr-Abl and mutated p53. Hsp90 inhibitors bind to Hsp90, and induce the proteasomal degradation of Hsp90 client proteins. Although Hsp90 is highly expressed in most cells, Hsp90 inhibitors selectively kill cancer cells compared to normal cells.[94]

GA targets the 90-kDa heat shock protein (Hsp90),[93,95,96] known to be an essential protein, expressed in both eukaryotic and prokaryotic cells, which serves to maintain the stability of “client proteins”

implicated in tumour growth and survival by regulating the physiology of cells exposed to environmental stress.[96,97]

GA inhibits the chaperone function of Hsp90 by competing with ATP for binding to the ATP-biding pocket or N-terminal ATPase site, a highly conserved nucleotide binding site near the N-terminus of the protein of Hsp90 and as a result, prevents Hsp90 protein binding causing the inhibition of ATP-dependent chaperone activities.[91,95,97] GA releases Hsp90 from its client proteins, thus destabilizing them. Once the Hsp90 is released from ErbB2, both the Hsp70 and the co-chaperone and E3 ubiquitin ligase, CHIP, are enlisted to the receptor. CHIP then ubiquitylates ErbB2, resulting in its intracellular accumulation and proteosome-mediated degradation.[91,98]

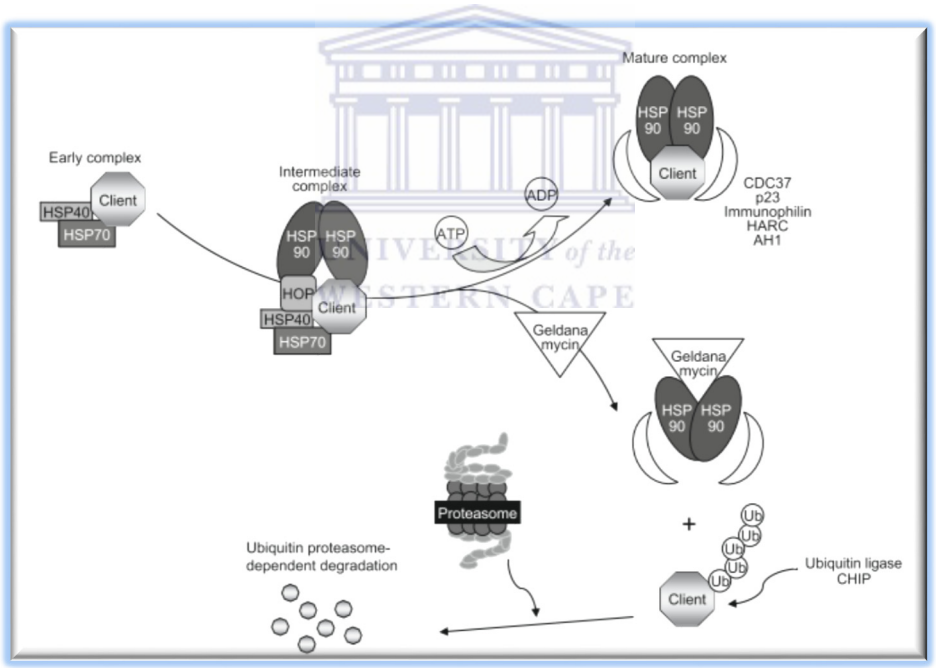


Figure 1.5: Heat shock protein 90 chaperone-client protein cycle and mechanism of action of geldanamycin[91]

Fig. 1.5 (above) Illistrates the Hsp90 chaperone–client protein cycle. Initially, the client protein is bound to the early complex (Hsp40/Hsp70), which then interacts with the Hsp90 homodimer through HOP (a Hsp90/Hsp70 organizing protein). By means of ATP hydrolysis, the release of Hsp40/Hsp70 and HOP from the intermediate complex occurs. In addition, the mature protein complex is then

formed by association between Hsp90 and cochaperones such as CDC37, p23, Immunophilin, HARC, AH1 or client proteins.

GA then acts by blocking the formation of the mature complex by binding to the ATP-binding site of Hsp90, leading to ubiquitin proteasome dependent degradation of the client proteins which are targeted by the CHIP E3 ligase. HOP, Hsp90/Hsp70 organizing protein; CHIP, carboxy-terminus of Hsp70-interacting protein.[91]

GA has exhibited potent antiproliferative activity in various cancer cell lines, however, no extensive clinical evaluation of GA has been undertaken because of its severe hepatotoxicity at therapeutic doses in animals and its poor water solubility. However, GA variants have been developed, leading to improvements in potency, tolerance, metabolic stability and water solubility, and are currently in various stages of clinical trials for the treatment of cancer.[91,95,96,99]

1.10 Thapsigargin

Thapsigargin (TG) is a sesquiterpene-γ-lactone extracted from the seeds and roots of *Thapsia garganica*. It inhibits the endoplasmic reticulum (ER) Ca^{2+} pump that leads to the depletion of the ER Ca^{2+} pool, which in turn activates the plasma membrane calcium channels resulting in an influx of extracellular calcium.[100]

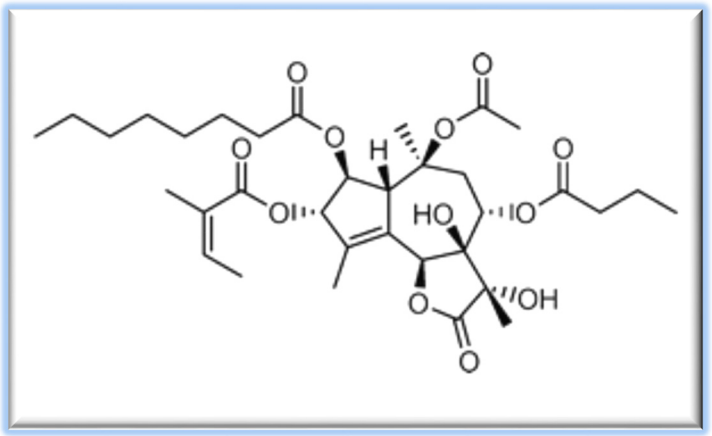


Figure 1.6: Chemical structure of thapsigargin[101]

Different mechanisms by which elevated Ca^{2+} levels may induce apoptosis has been proposed, such as: (i) a signal transduction pathway to activate Ca^{2+}-dependent protein kinases and phosphatases (ii) the activity of DNA catabolic enzymes may be catalyzed by sustained concentrations of Ca^{2+} in the nucleus (iii) the depletion of intracellular bound-calcium stores could trigger apoptosis by disrupting intracellular structures (iv) mitochondrial changes associated with the nitric oxide generation, the cytochrome c release and the activation of caspases.[100]

It has been suggested that this Ca^{2+} flux perturbant may be used to induce ER stress[102] and has been found not only to act on the ER and intracellular Ca^{2+} signalling pathways,[101] but also has the ability to diffuse across cell membranes and inhibit the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA).[103] It has also been reported that TG acts by directly inhibiting ER Ca^{2+}-ATPase resulting in the release of all sequestered Ca^{2+} as well as ATP-dependent Ca^{2+} from microsomal membrane vesicles.[104]

Intracellularly, TG rapidly increases cytosolic sequestered Ca^{2+} as a result of ER overstimulation, the Ca^{2+} permeates through Ca^{2+} specific pores and with it releasing inositol 1,4,5 trisphosphate and guanosine-5'-triphosphate (GTP).[105]

Due to TG's lipophilic nature, conversion to a structure with poor affinity for Ca^{2+} and ATP occurs. The weakened affinity for Ca^{2+} inhibits the sequestering of intracellular Ca^{2+} and subsequently, a secondary accumulation of extracellular Ca^{2+} occurs, thus initiating ER stress and as a result, apoptosis is induced.[101]TG inhibits SERCA, blocks the release of Ca^{2+} from the ER lumen and has been found to decreases the affinity of GRP78/BiP for Ca^{2+} as well as its ER chaperone anti-apoptotic effects[102].

As a result of Ca^{2+} increases, the accumulation of incompetent proteins occurs and by means of the ubiquitin proteasome system (UPS), caspase-3 mediated apoptosis occurs.[106]

1.11 Tunicamycin

Tunicamycin (TM) is a prototypic antibiotic. Figure .1.7 below depicts the chemical structure and numbering system, which has been discovered to play a role in, and has also been found to inhibit the biosynthesis of N-linked oligosaccharides[85] by the inhibition of UDP-N- acetylglucosamine: dolichol/polyprenol phosphate GlcNAc-1-P transferase (GPT) which inhibits the primary step of protein N-glycosylation.[107]

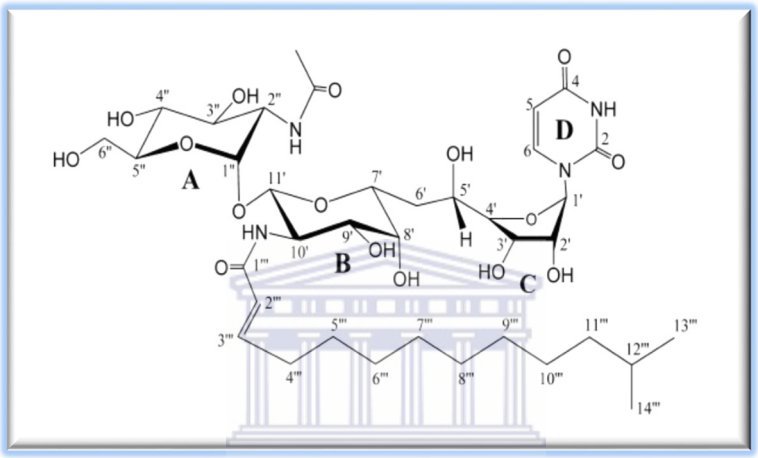


Figure 1.7: Chemical structure of tunicamycin and its relative numbering system[107]

Extensive research on the chemical composition and structure of TM reveals that it contains a four ring system (A, B, C, and D). Ring D contains uracil and ring C is linked to a D-pseudoribose in a N-glycosidically manner. Ring C is linked to D-N-acyl- pseudogalactosamine (Ring B) by C-5–C-6. Ring B is attached by an O-glycosidic bond to D-Nacetylglucosamine (Ring A).[107]

It was previously reported that TM induced apoptosis via tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).[108] It was then further substantiated by reporting that MCF-7 cells were resistant to TM-induced apoptosis due to the lack of caspase-3 expression,[109] however, both studies had reported that TM-induced apoptosis was enhanced with the addition of TRAIL and caspase-3.[108]

In TM-induced apoptosis, the inhibition of glycosylation and accumulation of unfolded and mis-folded proteins occurs in ER.[110] As a result, newly synthesized protein formation is inhibited, which

may lead to aberrant post translational modifications of important proteins, and ultimately perturbing normal cell function resulting in ER stress.[111] Following ER stress, a homeostatic mechanism is triggered, which facilitates the removal of those perturbant proteins known as the unfolded protein response (UPR).[112] As a result of inadequate UPR responses, cytosolic proteins increase and was found to induce apoptosis in various cell types.[113]

1.12 The Integrated Effects of Drugs

Due to the number of possible drug combinations being essentially limitless, a strategy for determining the most promising combinations and prioritizing their evaluation is vital.[114] The rapid development of hundreds of new agents that modulate an ever-growing list of cancer-specific molecular targets offers tremendous hope for cancer patients.[114] The introduction of the so-called "targeted therapies", predominantly those drugs that have been discovered to inhibit the activity of tyrosine kinases, has represented a remarkable progress in the treatment of cancer.[115] Although these drugs improve survival rates in cancer, significant cardiotoxicity, which could result in left ventricular dysfunction and/or heart failure, has emerged.[115]

Targeted therapies that inhibit the activity of tyrosine kinase receptors have shown activity against solid malignancies when used as single agents or in combination with chemotherapy.[116] The use of multiple drugs with different mechanisms or modes of action may also direct the effect against single target or a disease and treat it more effectively. The possible favourable outcomes for synergism include increasing the efficacy of the therapeutic effect, decreasing the dosage, but increasing or maintaining the same efficacy to avoid toxicity, minimizing or attenuating the development of drug resistance, and providing selective synergism against target (or efficacy synergism) versus host (or toxicity antagonism).[117]

1.13 Breast Cancer Cell Lines

Cancer cell cultures grown in vitro have been fundamental in delineating the complexities of tumour biology and the robust pursuit of identifying novel drug targets to develop a wide spectrum of

context-specific (proof-of-concept) anticancer drugs. Many cancer cell lines, including the National Cancer Institute (NCI) panel of cancer cell lines[118] and the widely used BC cell lines, closely resemble their primary tumours and CSCs—a property that has firmly secured their continued relevance in oncology, even though contentiously encouraging the use of primary cancer cells are undisputable.[119-127] Notwithstanding, several BC cell lines, including the MCF-7 breast carcinoma cell line used in this study, have been adequately validated to exhibit similar extensive genomic, transcriptional, and biological heterogeneity expressed by their corresponding primary tumours and are frequently used to distinguish various molecular BC diagnostic markers and as experimental model systems to study the effects of potential BC therapeutic modalities.[126,128-131]

The MCF-7 (Michigan Cancer Foundation—human cell line 7 derived from a pleural effusion of a primary breast carcinoma) was originally isolated in 1970 from a 69-year-old Caucasian American woman.[132,133] The main features of MCF-7 cells include their luminal epithelial nature, exemplification of invasive breast ductal carcinoma, ER^+/PR^+, marked proliferative response to oestrogen, ERBB2 gene amplification (with Her2/neu protein overexpression),[134] and tumorigenic potential in mice following oestrogen supplementation. This cell line also exhibits several characteristics of differentiated mammary epithelial cells, including the ability to metabolize oestradiol, a ligand for the cytoplasmic ER.

1.14 Research Proposal

1.14.1 Problem Statement

Approximately 90% of all cancer deaths arise from the metastatic spread of primary tumours.[135] Breast cancer is one of the leading causes of death among women worldwide and is the most frequently diagnosed form of cancer among females.[136] Many tumours commonly associated with poor prognosis, including breast cancer, have received a renewed focus and increased perspective with regard to drug discovery and innovation towards the development of rational combination regimens of first-line anticancer drugs and novel compounds that target diverse hallmarks of the cancer phenotype.[137] Cancer drug development is said to be leading the way in exploiting molecular,

biological and genetic information to develop "tailored" medicines.[138] The new paradigm is to design agents that target the precise molecular pathology that drive the progression of individual cancers.[139] Several molecularly targeted agents are now being developed in the hope that greater anticancer activity with fewer side effects might be attainable.[114,140]

Chemotherapy is a common, effective method for the treatment of malignant tumours. However, some tumours are inherently resistant to the majority of chemotherapeutic agents (i.e., intrinsic resistance), and many other tumours exhibit broad-spectrum or multidrug resistance (MDR) after several sessions of chemotherapy (i.e., acquired resistance).[141] MDR is a major concern for efficacious and successful treatment of cancer and a plethora of approaches that target the molecular mechanisms of this cancer hallmark are intensely pursued.[142-149] The overexpression of P-glycoprotein (P-gp), an ATP-binding cassette (ABCB1) transporter, which is usually associated with MDR cancer cells, is encoded by the mdr1 gene, has been found to correlate with the emergence of the MDR phenotype in malignant cells.[141]

In cancer cells, P-gp actively extrudes chemotherapeutic agents,[146,150] and is known to confer resistance to a variety of structurally and functionally unrelated antitumour drugs, such as daunorubicin, doxorubicin, etoposide, paclitaxel, vinblastine and vincristine.[151] P-gp, localized on the plasma membrane of resistant cancer cells, can bind and transport antitumor drugs in an ATP-dependent manner, and its mdr1 promoter activity is upregulated by various stimuli, including anticancer drugs, DNA-damaging agents, heat shock, serum starvation, ultraviolet irradiation and as a consequence of tumour progression, such as tumour suppressor gene p53 mutation and activation of ras oncogene.[152]

Many cellular stress conditions, such as alterations in glycosylation status, disturbances of calcium flux and hypoxia lead to accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen which results in ER stress.[153] The ER responses to such stress conditions are mediated by the activation of a range of stress-response signalling pathways which couple the ER

protein folding load with the ER protein folding capacity termed the unfolded protein response (UPR), a finely tuned transcriptional response.[59,154] Three important ER transmembrane proteins, i.e., activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and double stranded RNA-activated protein kinase-like ER kinase (PERK) are initiated by the UPR of mammalian cells.[154] During unstressed conditions in a cell, the domains within the lumen of these sensors are occupied by the ER chaperone glucose-regulated protein 78 (GRP78), variously known as immunoglobulin heavy chain binding protein (BiP) or Hsp5A. As a result of ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homo-dimerization of IRE1 and PERK, and also induces relocation and proteolytic cleavage of ATF6 in the cell.[154]

The UPR is activated in various solid tumours, such as breast cancer and prostate cancer, by nutrient deprivation, hypoxia and elevated expression of GRP78.[154-156] Overexpression of GRP78 is also associated with tumour development and growth, and correlates with resistance to certain forms of chemotherapy.[157] GRP78 is located predominantly in the ER, ensuring proper folding of aberrant proteins, preventing their aggregation and accumulation, targeting misfolded proteins for degradation and binding Ca^{2+} and regulating ER stress signalling.[157] It has been previously observed that GRP78 overexpression in tumour cells appears necessary for their survival during oncogenic stress.[157] Raised glucose metabolism results in glucose starvation, low pH and severe hypoxia, extreme conditions under which cancers are extraordinarily well adapted to survive.

All these factors induce ER stress via activation of the GRP78 promoter.[157] Known inhibitors targeting GRP78 activity or its induction have been derived from natural products such as genistein and (-)-epigallocatechin gallate (EGCG) that inhibit the pathophysiological activity of GRP78.[157] These agents selectively inhibit the growth of cancer cells or sensitize them to cytotoxic chemotherapy. Genistein and EGCG act on numerous cellular pathways and components, in addition to GRP78. The GRP78 down-regulator, versipelostatin, blocks the transcriptional activation of the UPR, targets the *GRP78* and *GRP94* genes and inhibits tumour xenograft growth. GRP78 is also

found in the cell membranes of cancer cells, and recent therapeutic approaches have targeted the membrane-attached substrate-binding domain of this chaperone.[157]

Heat shock proteins (Hsps) are evolutionarily conserved molecules which are synthesized by cells that are exposed to sub-lethal stresses.[156] Acting as molecular chaperones, Hsps protect cells from environmental stress damage by assisting in proper folding and stabilization of proteins and also help to sequester severely damaged proteins for degradation by the UPR. Owing to the nature of their function, Hsps are often found to be overexpressed in a wide range of cancers.[156] There are several major classes of Hsps (e.g., Hsp110, Hsp90, Hsp70, Hsp27, Hsp25) that are commonly found in mammalian cells and named in accordance with their molecular weights. Members of the Hsp family have been implicated in tumour promotion, uncontrolled cancer cell proliferation and inhibition of cell death pathways.[158-161] Hsp27 has recently been demonstrated to play a pivotal role in HER2-induced mammary tumourigenesis,[158] whereas its induced expression perturbs P-gp (ABCB1)-mediated drug efflux and *mdr1* gene expression in doxorubicin-resistant human breast cancer cells.[160] Moreover, inhibition of Hsp90 sensitizes HeLa human cervical cancer cells and MCF-7 human breast cancer cells to DNA damage by γ-ionizing radiation.[159]

The Hsp70 family of molecular chaperones, of which GRP78 is a member, represents one of the most evolutionarily conserved groups of chaperone proteins involved in protein folding.[157] In the Hsp90 chaperone–client protein cycle, the client protein is bound to the early complex (Hsp40/Hsp70), which then interacts with the Hsp90 homodimer through HOP (a Hsp90/Hsp70 organizing protein). ATP hydrolysis releases Hsp40/Hsp70 and HOP from the intermediate complex. In addition, the mature protein complex is then formed by association between Hsp90 and cochaperones [91] indicating that Hsp90 is yet another important molecular chaperone involved in the folding, assembly, maturation, and stabilization of several oncogenic client proteins (e.g., ErbB2, c-Raf, Cdk4, Akt, steroid hormone receptors, mutant p53, hypoxia-inducible factor-1-alpha (HIF-1α), survivin, and human telomerase (hTERT) that regulate the survival of malignant cells.[162] These proteins are involved in transcriptional regulation, signal transduction, cell cycle control and other

crucial steps that result in the expression of the malignant phenotype, invasion, angiogenesis, and metastasis of cancer cells.[162] Hsp90 inhibitors can bind to Hsp90, to induce the proteasomal degradation of Hsp90 client proteins.

ER stressors include heat shock, proteasome inhibition (e.g., by MG132)[79,163] or Hsp90 inhibition (e.g., by geldanamycin),[81,82] ER homeostasis disruption (e.g., by tunicamycin and tharpsigargin)[83-89] or chemotherapeutic drugs (e.g., taxanes)[86,90] can induce ER stress in cells. GA binds to the amino-terminal ATP-binding pocket of Hsp90 and inhibits ATP binding and hydrolysis. The binding of GA to Hsp90 interferes with Hsp-mediated target protein folding, leading to target aggregation and degradation.[164] GA and its synthetic derivatives have been shown to possess a higher affinity for Hsp90 in tumour cells compared to normal tissues and constitute a class of potential antitumour drugs.[91] TM is a prototype antibiotic that inhibits the biosynthesis of N-linked oligosaccharides[85] by the inhibition of UDP-N-Acetylglucosamine:dolichol/polyprenol phosphate GlcNAc-1-P transferase that catalyzes the primary step of protein N-glycosylation.[165] TG interferes with various cellular signal transduction pathways which rely on regulation of ER Ca^{2+} ion movements.[104] This Ca^{2+} flux perturbant may be used to induce ER stress since it inhibits the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA).[166] Intracellularly, TG rapidly increases the cytosolic sequestered Ca^{2+} by overstimulation of the ER, Ca^{2+} permeates through Ca^{2+} pores to stimulate the release of inositol 1,4,5-triphosphate and guanosine-5'-triphosphate, which then activate their signalling cascades.[104] Due to TG's lipophilic nature, it is able to inhibit intracellular Ca^{2+} sequestering which leads to ER stress and subsequent apoptosis.[167]

1.14.2 Reasearch Hypothesis

Inhibiting Hsp90 expression or even limiting its pharmacological activity in cells could have important therapeutic benefits for successful cancer treatment as previous studies linked high Hsp90 expression levels with decreased survival in breast cancer, [162] thus indicating that Hsp90 inhibition may be a favourable target for investigational therapy in breast cancer. Recently, the MDR phenotype, in particular the overexpression of P-gp, has been associated with increased resistance to

Hsp90 inhibitors. Hence, ablation of the resultant stress response induced by GA treatment via inhibition of P-gp may have implications for resistance to Hsp90-targeted therapy.[82] GA, a Hsp90 inhibitor, has exhibited potent antiproliferative activity in various cancer cell lines, however, no extensive clinical evaluation of GA has been undertaken because of its severe hepatotoxicity at therapeutic doses in animals and its poor water solubility.[91] Therefore, by combining the Hsp 90 inhibitor with other ER stress inducers such as TG and TM, as well as intergrating the effects of TG and TM, synergistic behaviour may occur to yeild an improved therapeutic effect, at a decreased dosage, by either maintaining or enhancing the efficacy to avoid toxicity, minimizing or attenuating the development of drug resistance such as MDR and P-gp expression and providing selective synergism against target (or efficacy synergism) versus host (or toxicity antagonism).[117] The integration of the ER stress inducers may also have positive implications in reducing the effects of P-gp-mediated drug efflux activity compared to the ER stress inducers alone.

1.14.3 Research Aims and Objectives

This study aimed to investigate the effects of Endoplasmic stress inducers GA, TG and TM alone, and in combination to observe implications that integrated effects of drugs may yield in terms of efficacy or synergism, with relevance to effects it may have on P-gp-mediated drug efflux activity.

The objectives of the present study were to:

- Investigate the normal growth pattern at which MCF-7 breast carcinoma cells proliferate under ideal conditions for up to 72 hours.
- Expose the MCF-7 breast carcinoma cells to incremental $\log_{10}$ concentrations of geldanamycin, thapsigargin and tunicamycin alone, and in combination to determine their respective 50% inhibitory concentrations (IC_{50} values) at 24, 48 and 72 hours.
- Examine cell proliferation patterns in the presence of relative IC_{50} concentrations of each drug alone for 24, 48 and 72 hours.

- Evaluate the MCF-7 breast carcinoma cell response in the presence of calculated IC_{20} and IC_{50} values of ER stress inducers alone and in combination with regard to its P-gp-mediated efflux pump at 4, 8 and 16 hour intervals using P-gp-specific modulators (e.g., cyclosporin A and verapamil) as controls.

- Determine cell viability with relation to cytotoxicity and early apoptosis via caspase-3 activation in the presence of the ER stress inducers alone at 6,12 and 24 hour intervals

- Morphologically confirm the early apoptosis and/or necrosis in the presence of ER stress inducers at relative IC_{50} values alone.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

The focus of this chapter outlines and describes the research methodology and design that has been chosen for the study. It summarizes the materials and methods used such as chemicals required, drugs tested and the maintenance of MCF-7 breast carcinoma cell cultures. Analyses of MCF-7 breast carcinoma cells exposed to geldanamycin (GA), thapsigargin (TG) and tunicamycin (TM) included growth and dose response curves (cytotoxicity assays), determining cellular drug efflux by means of calcein-AM assays, triplex assays focusing on cell viability, cytotoxicity and apoptosis caspase-3 activation and morphological staining of apoptotic cells making use of the Annexin V-FITC. The statistical methods used for data analysis are also described.

2.2 Approval to Conduct the Study and Ethical Considerations

The research described in this dissertation was approved by the University of the Western Cape Faculty Board Research and Ethics Committee and the Senate Research Committee (Registration/Ethical Clearance Numbers: Project Registration #: ScRIRC 2007/3/29; Funding Application #: ScRIRC 2007/3/44 and Senate Registration #: 07-3-37). This study involved *in vitro* cell culture work. The extensive use of continuous cancer cell lines, such as MCF-7 breast carcinoma cells, an authenticated cell line of known provenance (www.atcc.org), carries little risk to humans from routine cell culture.

However, risk of exposure to any form of infection was minimized by avoiding the use of "sharps" (such as needles and blades) and any items or processes likely to create aerosols. Aseptic technique and good cell culture practice and the recommended procedures for handling, use, storage, transportation and disposal of genetically modified organisms, including modified cell lines, have

been adhered to in this study as provided in the Genetically Modified Organisms (Contained Use) Regulations Health and Safety Commission. ISBN 0717611868 Guide to Genetically Modified Organisms (Contained Use) Regulations, 1992. HSE Guide, L29, HSE Books, PO Box 1999, Sudbury, Suffolk CO10 6FS, 1992. See also http://www.sigmaaldrich.com/Area_of_Interest/Life_Sciencesources/ECACC_Handbook/Cell_Culture_Techniques_2.html.

A level 2 containment, the minimum requirement for manipulating human cancer cell lines as described in the Advisory Committee on Dangerous Pathogens (ACDP) guidelines Advisory Committee on Dangerous Pathogens, 4th edition. Categorization of biological agents according to hazard and categories of containment, The Stationery Office Books, PO Box 276, London SW8 5DT) was applied in this study. Control of the disposal of laboratory waste to prevent exposure of staff and the environment to infectious hazards and to prevent contamination was executed according to recommended procedures (Health Services Advisory Committee. ISBN 0717604470 Safe Disposal of Clinical Waste. HSE Books, PO Box 1999, Sudbury, Suffolk CO10 6FS, 1992).

2.3 Drugs and Chemicals

Drugs and chemicals used in this study include GA from *Streptomyces hygroscopicus* (CAS: 30562-34-6;Sigma-Aldrich), TG (CAS:67526-95-8; Sigma, SA), tunicamycin (CAS: 11089-65-9; Sigma, SA), 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) (GibcoLife Technologies), penicillin/streptomycin (Gibco, Life Technologies), trypsin-EDTA (Gibco, Life Technologies), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), isopropanol, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) MTT (CAS: 298-93-1; Sigma, SA), Trypan Blue (CAS: 72-57-1; Sigma SA), Cayman Chemicals Multi Drug Resistance kit (Calcein-AM) (Item no. 600370), Annexin V-CY3 (Cat no: APOAC; Sigma-Aldrich), ApoTox-glo triplex assay kit (Cat no: G6320; Promega).

2.4 Maintenance of MCF-7 Breast Carcinoma Cells

All tissue culture operations were carried out in a model NU-5510E NuAire DHD autoflow automatic CO2 air-jacketed incubator (and an AireGard NU-201-430E horizontal laminar airflow table top workstation which provides a HEPA filtered clean work area (NuAire). The human breast cancer cell line, MCF-7, was kindly provided by Professor Maryna De Kock, Department of Medical Biosciences, University of the Western Cape, South Africa. The MCF-7 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 humidified incubator (Relative Humidity/RH 80%) in an atmosphere of 5% CO2:95% air.

Cryovials containing the MCF-7 breast carcinoma cells frozen away in 90% FBS and 10% DMSO were removed from -80°C storage and placed in a 37°C water bath and allowed to thaw. The caps were wiped with 70% ethanol and the contents of the vial transferred aseptically to a T-25 culture flask (surface area (SA) 2500 mM^2) containing 5 ml of complete medium (GIBCO® Dulbecco's Modified Eagles Medium/F-12 supplemented with 1% penicillin-streptomycin, and 10% HIFBS, all acquired from Invitrogen). 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% CO2 and 80% relative humidity, 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 24h, 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 MCF-7 cells.

Once confluency had been reached, cells were gently trypsinized. The medium was aspirated and the cells rinsed with 2 ml of PBS. After 1 minute, the PBS was aspirated and replaced with 1ml 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, 2 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, placed in a centrifuge and spun for 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-75 culture flask (SA 7500 mM^2) containing 12 ml complete medium to maintain stock cultures.

2.5 MCF-7 Mammary Carcinoma Growth Curve Analysis

MCF-7 breast carcinoma cells were trypsinized and transferred to a 15ml centrifuge tube and spun at 2500 rpm for 5 min. The supernatant was removed and the pellet resuspended in 5ml of complete medium. Cells were seeded into 24-well plates at 1 x 10^4 cells/ml per well. Following a 24 hour attachment period, Cells were exposed to approximate 24 hour IC_{50} inhibitory concentrations of each drug based on data generated from the MTT cell proliferation assay. The IC_{50} values were determined using the latest version 6.05 of GraphPad Prism®. The cells were exposed to 199.1 µM of GA, 12 µM TG, 100 µM of TM, including a control row containing no drug, just growth medium used to maintain cell cultures and plates were incubated for 24, 48 and 72h. After the relative time intervals elapsed, cells were trypsinized with 0.25% trypsin-EDTA and counted using the Bio-Rad TC-20 cell counter at a ratio 1:1 cell suspension: 0.4 µM trypan blue.

2.6 MTT Cell Viability Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is reduced to formazan, following cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria of metabolically active cells.[168,169] The inability of formazan to diffuse through the cell membrane allows its accumulation within healthy living cells. The insoluble formazan crystals that form within the cell can be dissolved in acidified isopropanol, dimethylsulfoxide (DMSO) or sodium dodecyl sulfate (SDS).

MCF-7 breast carcinoma cells were seeded at a density of 5 x 10^4 cell/ml into 96-well flat-bottom tissue culture plates of which a 100 µl cell suspension was added to each well and cells were allowed to attach for 24 h under normal incubation conditions. After 24 hours, medium was aspirated and replaced with 100 µl complete medium alone (served as control) and medium containing increasing $\log_{10}$ concentrations of GA, TG and TM alone (0.001 0.01, 0.1, 1, 10, 100 and 1000 µM) and in combination (0.005, 0.05, .0.5, 5, 50 and 500 µM) at equimolar concentrations in six replicate wells. Plates were incubated for 24, 48 and 72 h, respectively. After the elapsed incubation period, 10 µl of MTT solution (5 mg/ml MTT in PBS) was added to each well and the plate was covered with aluminium foil and incubated for a further 2 hours. Thereafter, the supernatant was aspirated and 200 µl of neat isopropanol was added to each well, incubated at room temperature but placed on a vortex shaker for 25 min, still enclosed in foil. Plates were read at 560 nm using the Promega GloMax™ multiscan plate reader.

2.7 Measurement of P-glycoprotein-mediated Efflux

Cayman's Multi-Drug Resistance Assay Kit provides a convenient tool for studying MDR protein modulators.[170] 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. Hoechst dye is used as a marker of cell nuclei. 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 was carried out in black, clear bottom 96-well tissue culture treated plates. MCF-7 breast carcinoma cells were seeded at a density of 1 x 10^4 cells/well in 100 µl of complete DMEM cell culture medium and incubated allowing for cells to attach and grow over night (24 hours). Following the elapsed time period, medium was aspirated and replaced with 100 µl of test compounds GA, TG and TM alone and in combination at both IC_{20} and IC_{50} values based on MTT data analysis using the latest GraphPad Prism® software. Included in the kit was cyclosporin A and verapamil which were used as positive controls, which were diluted 1:1000 into culture medium.

At the end of the specified treatment interval, 100 µl of the calcein-AM/Hoechst dye staining solution was added to each well, and the plate was gently shaken, to allow the calcein-AM/Hoechst dye staining solution to mix with the culture medium. The plate was then placed in a CO_2 incubator at 37°C for 30 minutes to allow sufficient uptake of the calcein-AM to occur. Cells were then analysed immediately and Cells taking up calcein-AM displayed strong fluorescence intensity with λex 355 nm and λem 465 nm, respectively. Cell number/density was reflected by the fluorescence intensity with Hoechst dye staining that was measured using λ_{ex} 355 nm and λ_{em} 465 nm, respectively using the Promega GloMax™ multiscan plate reader.

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

2.8.1 Principle of the Apotox-Glo™ Triplex Assay

The ApoTox-Glo™ Triplex Assay combines three Promega 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 the Caspase-Glo ® Assay Technology by providing a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, 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.

2.8.2 Assay Conditions for the ApoTox-Glo™ Triplex Assay

All Apotox-GloTM Triplex assays were carried out in white opaque bottom 96-well plates. 1 x 104 cells were seeded into each well in a final volume of 100 µl per well and allowed to attach for 24 hours. After the attachment period, culture medium was removed and replaced with vehicle controls and test compounds GA, TG and TM at log doses (0.01. 0.1, 1, 10, 100, 1000 µM) to the appropriate wells. Plates were placed in a CO_2 incubator at 37°C for the appropriate time intervals 6 hours, 12 hours and 24 hours. After the exposure time intervals have elapsed, 20 µl of Viability/ Cytotoxicity reagent containing both GF-AFC Substrate and bis-AAF-R110 Substrate was added to all wells, plate covered in foil and briefly mixed by orbital shaking at 300 rpm for 30 seconds. After the reagents were added and allowed to mix, plates were placed in the CO_2 incubator at 37°C for 1 hour. Following the incubation period, plates were removed from the incubator, the foil was removed, and fluorescence was measured at 400 Ex/505Em and 485 Ex/520Em for viability and cytotoxicity respectively using the Promega GloMax™ multiscan plate reader. To determine apoptosis, 100 µl 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.

2.9 Annexin V Cy3™ Apoptosis Assay

MCF-7 breast carcinoma cells were seeded into 24 well plates, at $2x10^5$ cells per well in 1 ml of culture medium. Cells were then incubated and allowed to attach for 24 hours. After 24hours, cells were exposed to relative IC_{50} concentrations of GA, TG and TM alone for 24 hour time intervals in order to induce apoptosis. After inducing apoptosis using the specified concentrations of ER stress inducers, cells were washed in phosphate buffered saline (PBS). Thereafter, cells were trypsinized and resuspended in PBS. 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 µl) of cell suspension was placed inside the circle and cells were allowed to attach to the slide by incubating at room temperature.

The cells were washed with binding buffer (10 mM Hepes/NaOH, pH 7.5, containing 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1.8 mM $CaCl_2$)[171] and a double label staining solution (Sigma-Aldrich; Annexin V Cy3.18 and 6-carboxyfluorescein diacetate) added onto each circle and covered with foil. Cells were incubated at room temperature for 10min. Slides were washed with 1X binding buffer in order to remove excess unbound staining solution. A cover slip was added onto the slide and results were viewed using a fluorescent microscope.

Cells starting the apoptotic process were stained with stains-Annexin Cy3.18 (red) and 6-carboxyfluorescein diacetate (green). Annexin-Cy3.18 (AnnCy3) binds to phosphatidylserine 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 a green fluorescence (Source: Sigma-Aldrich Annexin V-Cy3™ Apoptosis DetectionKit. (http://www.sigmaaldrich.com /content/dam/sigmaaldrich/docs/Sigma/Bulletin/apoacbul.pdf).

2.10 Statistical Analysis

Regression analysis of dose-response curves, IC_{50}s and p values using One-Way ANOVA or Student's t-test were calculated using GraphPad Prism (GraphPad Prism version 6.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Data obtained for the growth inhibitory effects The ER stress inducers TG, TM and GA on MCF-7 breast carcinoma cells were also analysed by One-Way ANOVA using GraphPad Prism (GraphPad Prism version 6.04 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com. All pairwise multiple comparisons were performed according to the Holm-Sidak method and the overall significance level was set at 0.05. Actual p-values are indicated in the text. Best-fit IC_{50} values and corresponding 95% confidence intervals were obtained from non-linear regression analysis of sigmoidal dose-response curves, using the three-parameter logistic equation of GraphPad Prism (GraphPad Prism version 6.04 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com. All data were derived from 2-3 independent experiments where sample size ($n \geq 6$).

CHAPTER 3

RESULTS

3.1 Introduction

In this chapter, the results of the research undertaken in this study are presented. Details of the research methodology used to obtain and analyze the data have been presented in Chapter 2. In this study, MCF-7 breast carcinoma cells were exposed to 3 Endoplasmic Reticulum stress inducers, geldanamycin (GA), thapsigargin (TG) and tunicamycin (TM) alone, and in combination. Initially, growth curves for MCF-7 breast carcinoma cells were generated to establish their behaviour under normal culture conditions and their proliferation in response to 24 hour IC_{50} values of GA, TG and TM for 24, 48 and 72 hour time intervals, once it had been determined via dose response curves. Dose response curves were generated to determine relative IC_{20} and IC_{50} values of each drug at 24, 48 and 72 hour time intervals.

A carefully designed time interval study was conducted to ascertain the effects of the ER stress inducers on the effect of the P-gp cellular efflux pump activity of the cells. The triplex assay was performed at different time intervals, which include intervals lower than and 24 hours to determine the log dose effect of the ER stress inducers on the MCF-7 breast carcinoma cells. This was then followed by the by Annexin V-Cy3 fluorescent staining of the cells exposed to their relative 24 hour IC_{50} concentrations. The results are presented and briefly discussed in the sections that follow.

3.2 MCF-7 Cell Growth Curve Analysis

MCF-7 breast carcinoma cells were seeded at $1x10^4$ cells/ml, allowed to attach for 24 hours, and replaced with cell medium alone, which served as the control (i.e. cells not treated with inhibitor) and in the presence of the endoplasmic reticulum stress inducers GA (199.1 μM), TG (12 μM) and TM (100 μM). Results obtained after 24, 48 and 72 hours is depicted in Figure 3.1. These

concentrations were based on the actual 24 hour IC_{50} values generated from the MTT cell proliferation assays performed (Figure 3.3).

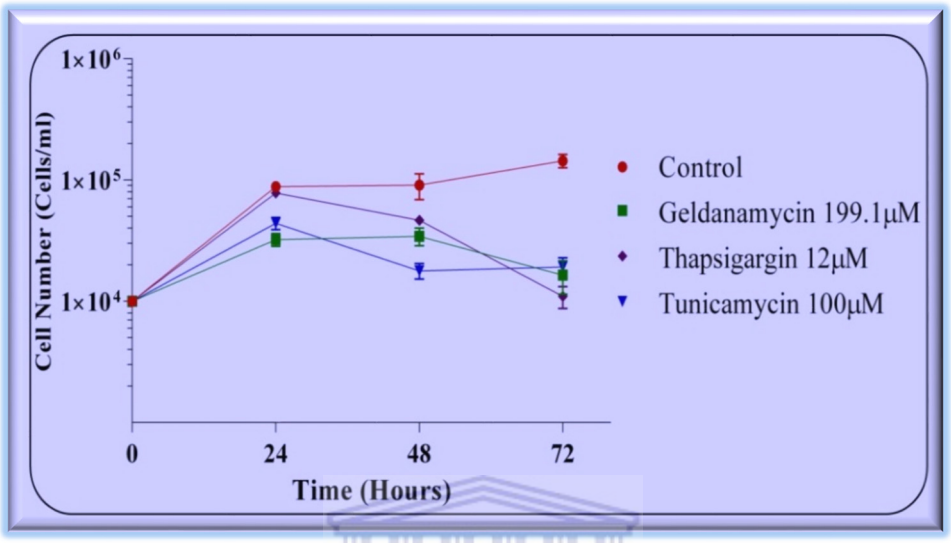


Figure 3.1: MCF-7 breast carcinoma cell Growth curve at 24 hour intervals

Cells seeded at a constant density and exposed to geldanamycin (199.1 µM), TG (100 µM) and tunicamycin (12 µM)) constantly for the different time intervals relative to control. Data were analyzed by One-way ANOVA using Graphpad Prism®. All pairwise multiple comparisons were performed according to the Holm-Sidak method and the overall significance level was set at 0.05. Actual p- values are indicated in the text.

In Figure 3.1, a visible increase in cell proliferation has been obtained between 0-24 hours (initial seeding and exposure interval), whereby a significant increase in cell proliferation ($p<0.001$) in the control was seen, as well as in the presence of TG, but no significant increase in cell number in the presence of GA and TM ($p=0.9305$ and $p=0.2383$ respectively). Although there was an increase in cell number, responses in the presence of the stress inducers do not surpass the cell number obtained by the control group.

As cell number increases with time duration in the control group, a lag phase was observed between 24 and 48 hours with no significant difference in cell proliferation ($p>0.05$), and a log growth phase was visible with a significant increase in cell growth between the 48 and 72 hour time interval

(p=0.0015). Cell proliferation inhibition in the presence of GA is evident with no significant increase in cell number between 24 and 48 hours (p>0.05) and no significant difference (p>0.05) but a sustained decrease in cell number after 72 hours. In the presence of TG, a decrease in cell proliferation is observed between 24 and 48 hours and a significant (p<0.0001) sustained decrease in cell number is clearly visible at 72 hours. TM was found to hinder cell proliferation between 24 and 48 hours but not significantly (p=0.7537), with a minor but not significant (p>0.999) increase in proliferation between 48 and 72 hours.

3.2.1 Cell Growth Analysis of MCF-7 cells exposed to Geldanamycin, Thapsigargin and Tunicamycin

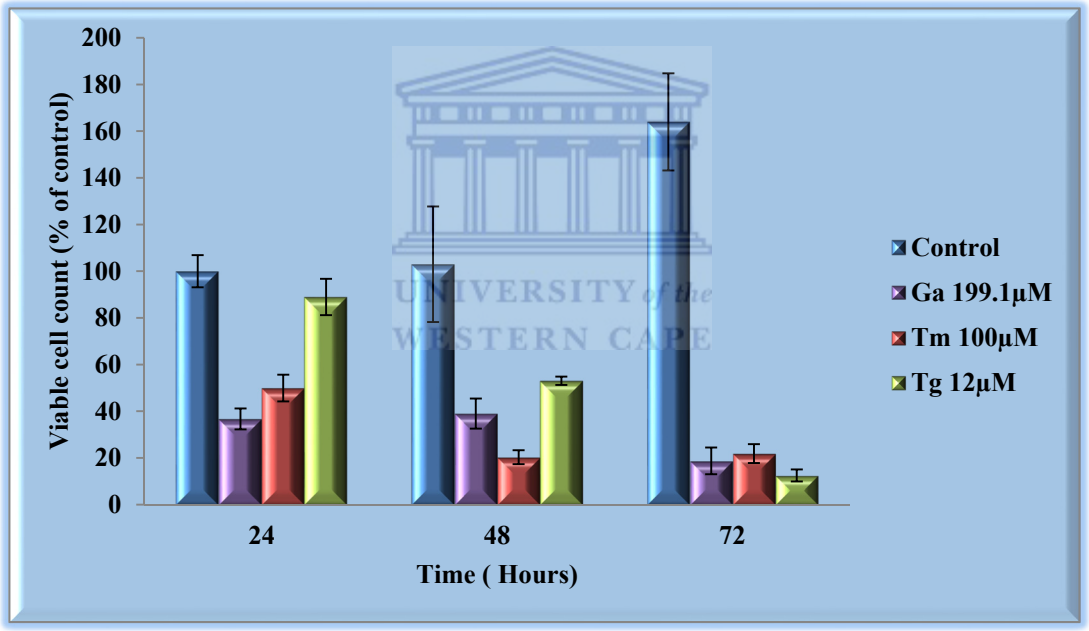


Figure 3.2: Growth inhibitory effects of geldanamycin , tunicamycin and thapsigargin on MCF-7 breast carcinoma cells at 24, 48 and 72 hour intervals. Data were analysed by One-way ANOVA using the latest Graphpad prism® software version 6.05. All pairwise multiple comparisons were performed according to the Holm-Sidak method and the overall significance level was set at 0.05. Actual p- values are indicated in the text.

In Figure 3.2, a significant inhibition of cell viability was obtained after 24 hours in the presence of GA and TM relative to control (p=0.0008 and p=0.0219 respectively). Although there was a decrease in cell viability in the presence of TG, it was not significant (p>0.05). After 48 hours of exposure to

the endoplasmic reticulum stress inducers GA, TG and TM, a significant decrease in cell viability was achieved ($p=0.0007$,$p=0.224$ and $p<0.0001$ respectively). Exposure for 72 hours resulted in a significant decreased cell viability relative to control ($p<0.0001$ in all cases) and maintained a constant decrease in viability as time progressed, apart from that found in the presence of TM, where there was a negligible yet non-significant decrease in cell viability ($p>0.05$).

3.3 Potencies of ERS Inducers Geldanamycin, Thapsigargin and Tunicamycin Alone and in Combination

Figure 3.3 summarizes the effects of the endoplasmic reticulum stress inducers GA, TG and TM alone, on the MCF-7 breast carcinoma cell line. The IC_{50} values determined are indicated on the graphs in conjunction with their relative 95% Confidence Interval (95% CI) values and regression line (R^2) values.

The IC_{50} values determined for GA were 199.1 µM, 113 µM and 38, 60 µM for 24, 48 and 72 hours where R^2 = 0.80,0 .93 and 0.92 respectively. A dose dependant decrease in cell viability was obtained, and the IC_{50} value was found to decrease as time intervals increased. A visible dose and time dependent response from the cells in the presence of the drug was achieved as the IC_{50} value decreased as time intervals increased and cell viability decreased as the log dose of the drug increased.

The IC_{50} values determined for TG were 11.93 µM, 1.655 µM and 8, 96 µM for 24, 48 and 72 hours where R^2 = 0.80,0 .92 and 0.93 respectively. A dose dependant decrease in cell viability was obtained, and the IC_{50} value at 48 hours was found to be lower than found at 24 and 72 hours, however at 72 hours, the IC_{50} value was lower (1.655 µM) than that at 24 hours (11.93 µM). A clearly visible dose and time dependent response from the cells in the presence of the drug was achieved as the IC_{50} value decreased as time intervals increased and cell viability decreased as the log dose of the drug increased.

The IC_{50} values determined for TM were 115.2 µM, 108.2 µM and 26.64 µM for 24, 48 and 72 hours where R^2 = 0.85,0 .90 and 0.88 respectively. A dose dependant decrease in cell viability was obtained, and the IC_{50}

value at 72 hours was found to be prominently lower than found at 24 and 48 hours. A dose and time dependent response from the cells in the presence of the drug was achieved as the IC_{50} value decreased as time intervals increased and cell viability decreased as the log dose of the drug increased.

Figure 3.4 summarizes the effects of the endoplasmic reticulum stress inducers GA, TG and TM in combination, on the MCF-7 breast carcinoma cell line. The IC_{50} values determined are indicated on the graphs in conjunction with their relative 95% Confidence Interval (95% CI) values and regression line (R^2) values.

The IC_{50} values determined for the combination of the GA and TG were 11.01 µM, 7.798 µM and 18.13 µM for 24, 48 and 72 hours where R^2 = 0.95,0 .96 and 0.81 respectively. A dose dependant decrease in cell viability was obtained, and the IC_{50} value was found to decrease as time intervals increased. A visible dose and time dependent response from the cells in the presence of the drug was achieved as the IC_{50} value decreased as time intervals increased and cell viability decreased as the log dose of the drug increased.

The IC_{50} values determined for the combination of the GA and TM were 91.66 µM, 4.648 µM and 0.372 µM for 24, 48 and 72 hours where R^2 = 0.92,0 .88 and 0.78 respectively. A dose dependant decrease in cell viability was obtained, and the IC_{50} value was found to decrease as time intervals increased. A visible dose and time dependent response from the cells in the presence of the drug was achieved as the IC_{50} value decreased as time intervals increased and cell viability decreased as the log dose of the drug increased.

The IC_{50} values determined for the combination of the TG and TM were 20.81 µM, 15.32 µM and 0.622 µM for 24, 48 and 72 hours where R^2 = 0.92,0 .93 and 0.90 respectively. A dose dependant decrease in cell viability was obtained, and the IC_{50} value was found to decrease as time intervals increased. A visible dose and time dependent response from the cells in the presence of the drug was achieved as the IC_{50} value decreased as time intervals increased and cell viability decreased as the log dose of the drug increased.

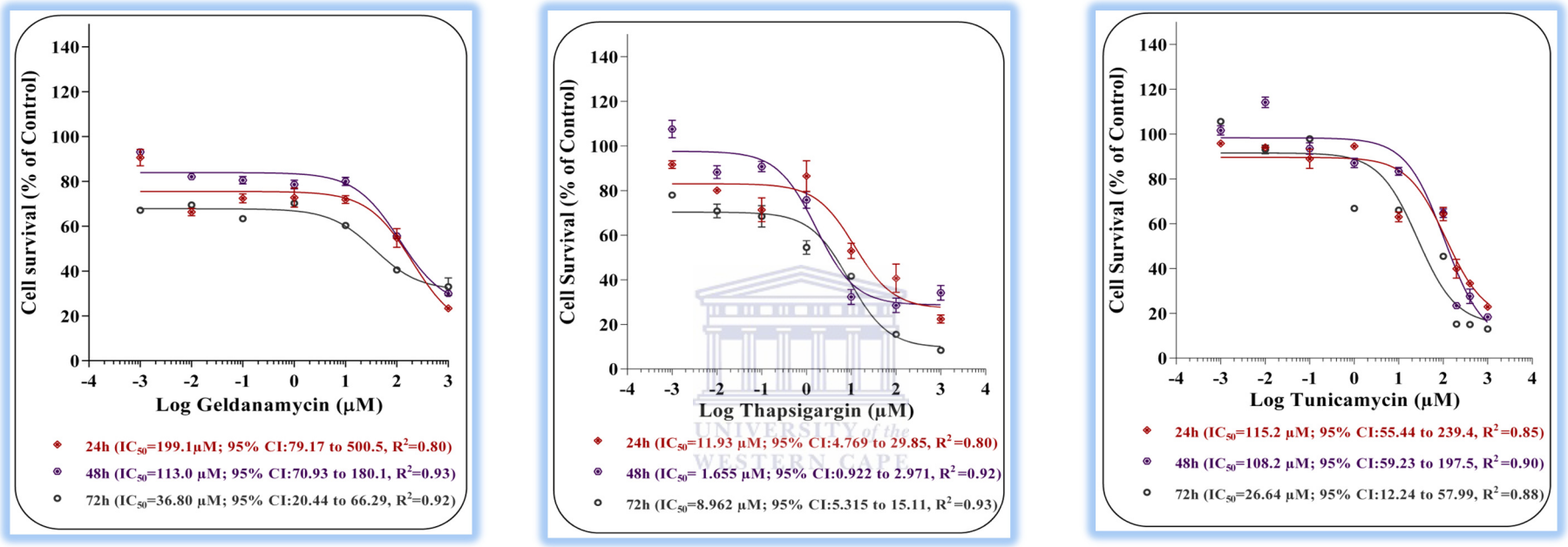


Figure 3.3: Dose response curves obtained by nonlinear regression analysis following exposure of MCF-7 breast carcinoma cells to geldanamycin thapsigargin and tunicamycin for 24, 48 and 72h, respectively. Indicated are the 50% inhibitory concentrations (IC_{50}s) and 95% Confidence Intervals (CI) and Regression line analysis (R^2)

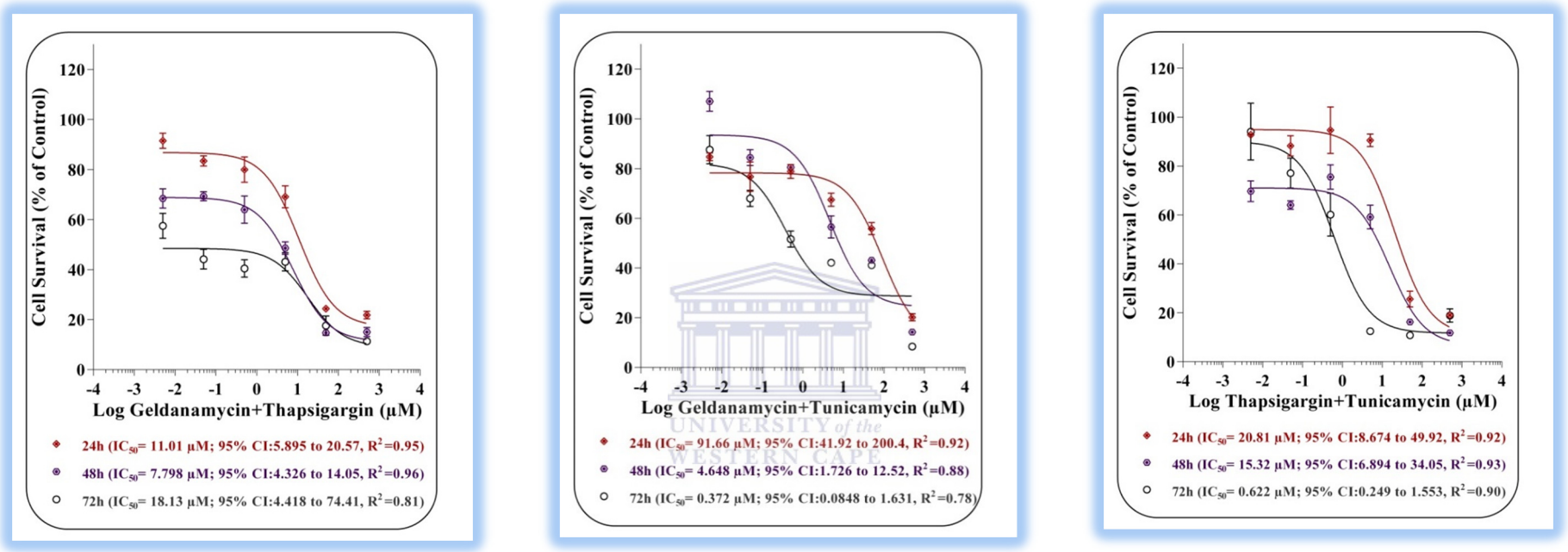


Figure 3.4: Dose response curves obtained by nonlinear regression analysis following exposure of MCF-7 breast carcinoma cells to decreased but equimolar concentrations of geldanamycin thapsigargin and tunicamycin combined for 24, 48 and 72h, respectively. Indicated are the 50% inhibitory concentrations (IC_{50}s) and 95% Confidence Intervals (CI) and Regression line analysis (R^2).

3.4 Measurement of P-gp-Mediated Efflux via Calcein-AM

3.4.1 Measurement of P-gp-Mediated Efflux in the Presence of Geldanamycin

Figures to 3.5 to 3.7 summarize the effects of calculated IC_{20} (50 µM) and IC_{50} (199.1 µM) values of the endoplasmic reticulum stress inducer GA alone, on the MCF-7 breast carcinoma cell line with regard to its response to P-gp-mediated efflux after 4, 8 and 16 hour intervals. Decrease in calcein-AM substrate indicated an increased cellular efflux, as less calcein-AM was retained in the cells after the specified time interval. Actual p values are indicated in the text.

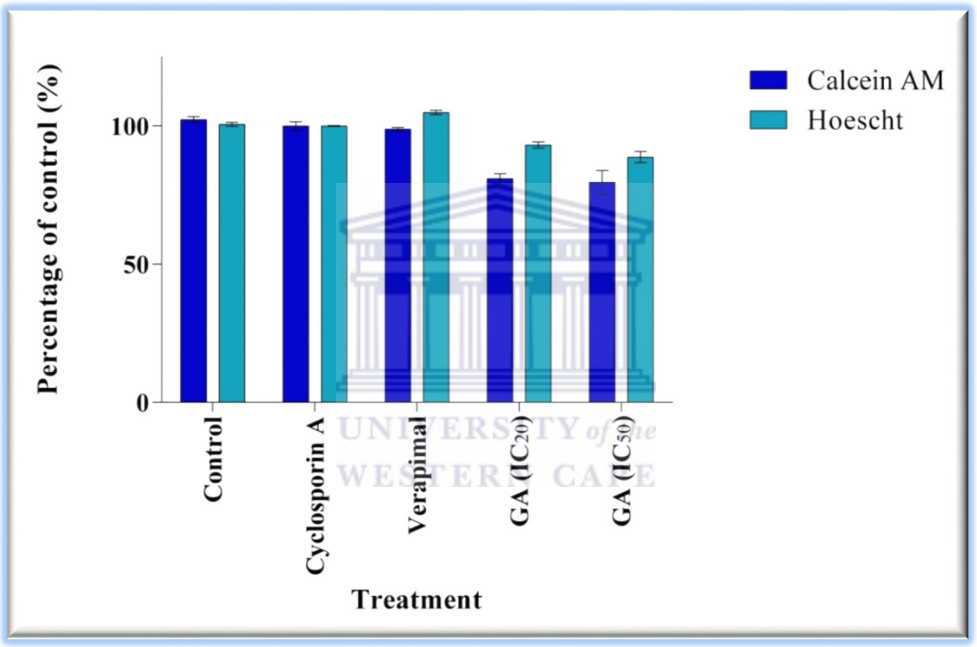


Figure 3.5: The effects of geldanamycin on P-glycoprotein-mediated efflux after 4 hours

After 4hours (Figure 3.5), a minor insignificant increase in cell viability in the presence of verapamil was observed compared to control ($p>0.05$) with no significant difference found in the presence of Cyclosprorin A or varapimal compared to control. A significant increase in cellular efflux was observed in the presence of GA at both IC_{20} and IC_{50} concentrations compared to control, verapamil and cyclosporin A ($p<0.0001$) as a decrease in cellular influx was present. A significant decrease in cell viability only in the presence of geldanamycins' IC_{50} concentration was found ($p=0.005$) relative

to control. A significant increase in cellular efflux in the presence of the both IC_{20} and IC_{50} concentrations was also seen relative to its viability ($p<0.05$ in both cases).

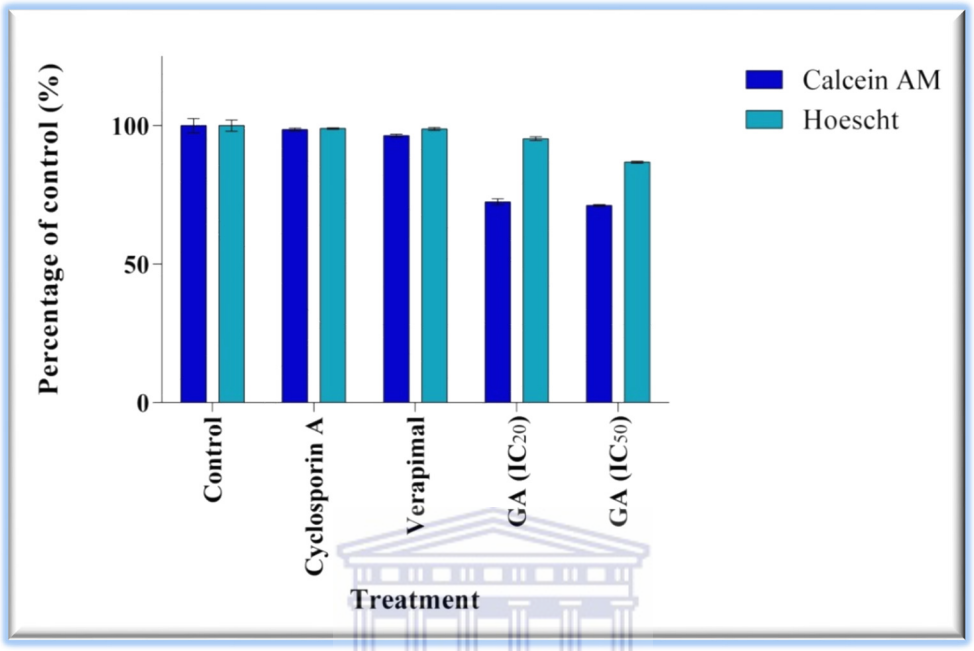


Figure 3.6: The effects of geldanamycin on P-glycoprotein-mediated efflux after 8 hours

After 8 hours (Figure 3.6), no significant differences in either viability or calcein-AM retention were seen in the presence of cyclosporin A and verapamil compared to control ($p>0.05$). An insignificant decrease in viability is present at GAs IC_{20} concentration ($p=0.0914$) and a significant decrease in cell viability was seen in the presence of GAs IC_{50} concentration ($p<0.0001$), however in both cases, a significant increase in cellular efflux was seen relative to control ($p<0.0001$) and relative to its respective viability percentages ($p<0.0001$).

Following 16 hours of exposure (Figure 3.7), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. A significant decrease in cell viability was seen in both IC_{20} and IC_{50} concentrations ($p=0.0198$ and 0.0118 respectively) compared to control, however both concentrations showed a decreased cellular efflux, where by the IC_{20} concentration was insignificantly lower ($p=0.3167$) and the IC_{50}

concentration significantly lower compared to control (p=0.0118), however at the IC_{20} concentration, an insignificant decrease in cellular efflux was seen relative to its viability (p>0.05) and a significant decrease in cellular efflux was seen at the IC_{50} concentration relative to its viability (p<0.05).

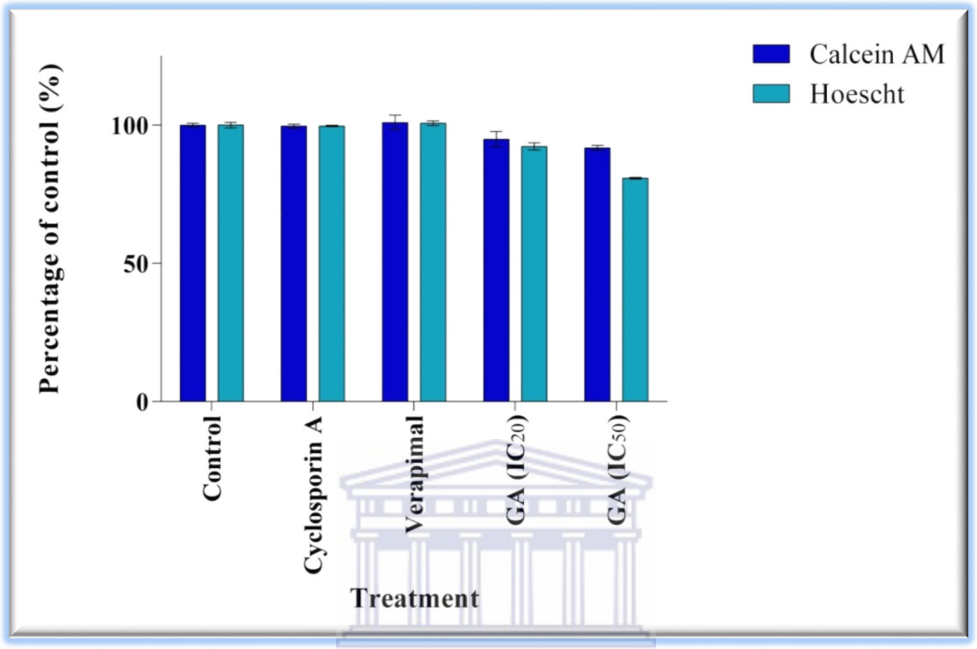


Figure 3.7: The effects of geldanamycin on P-glycoprotein-mediated efflux after 16 hours

3.4.2 Measurement of P-gp-Mediated Efflux in the Presence of Thapsigargin

Figures to 3.8 to 3.10 summarize the effects of calculated IC_{20} (3 µM) and IC_{50} (12 µM) values of the endoplasmic reticulum stress inducer TG alone, on the MCF-7 breast carcinoma cell line with regard to its response to P-gp-mediated efflux after 4, 8 and 16 hour intervals. Decrease in calcein-AM substrate indicated an increased cellular efflux, as less calcein-AM was retained in the cells after the specified time interval. Actual p values are indicated in the text.

Following a 4 hour exposure period (Figure 3.8), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil (p>0.05) compared to control. A minor insignificant increase in cell viability was seen the presence of verapamil. The only significant

difference present was a weak significant decrease in cellular influx in the presence if TGs IC_{50} concentration (p=0.0483) relative to control.

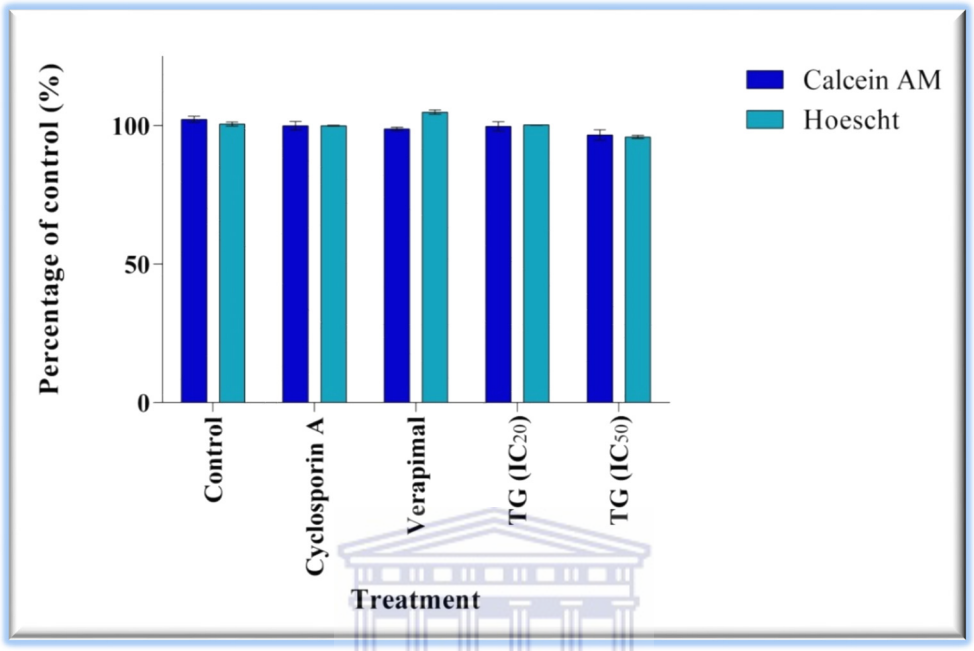


Figure 3.8: The effects of thapsigargin on P-glycoprotein-mediated efflux after4 hours

Following an 8 hour exposure period (Figure 3.9), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil (p>0.05) compared to control. No significant differences in cell viability was found, however significant increases in cellular efflux was noticeable at both IC_{20} and IC_{50} concentrations (p=0.0004 and <0.0001 respectively) compared to control. At both concentrations there was a significant increase in cellular efflux relative to its relative viability (p<0.05 in both cases).

After a 16 hour exposure period (Figure 3.10), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil (p>0.05) compared to control. No significant differences in cell viability or cellular efflux were found in the presence of TG at both concentrations, however, at both concentrations, insignificant decreases in both viability and efflux was noticeable (p>0.05) relative to control.

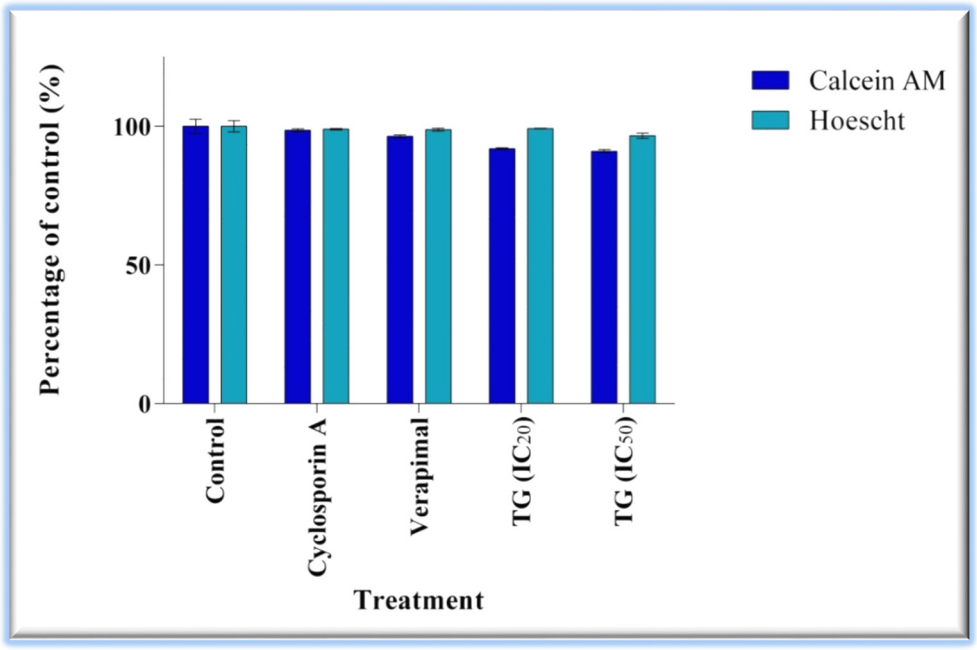


Figure 3.9: The effects of thapsigargin on P-glycoprotein-mediated efflux after 8 hours

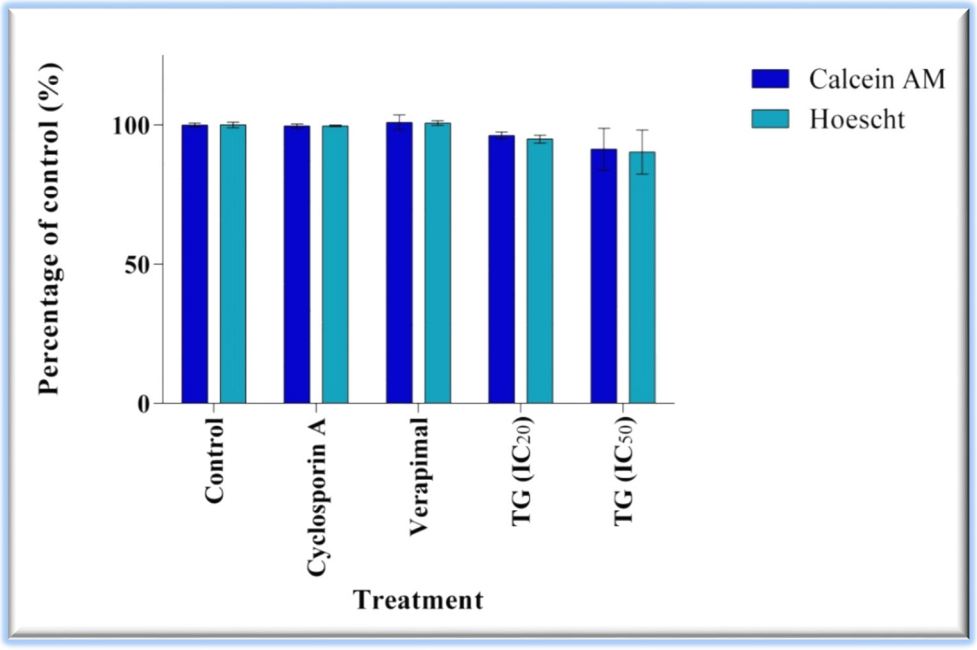


Figure 3.10: The effects of thapsigargin on P-glycoprotein-mediated efflux after 16 hours

3.4.3 Measurement of P-gp-Mediated Efflux in the Presence of Tunicamycin

Figures to 3.11 to 3.13 summarize the effects of calculated IC_{20} (28.8 µM) and IC_{50} (115 µM) values of the endoplasmic reticulum stress inducer TM alone, on the MCF-7 breast carcinoma cell line with regard to its response to P-gp-mediated efflux after 4, 8 and 16 hour intervals. Decrease in calcein-AM substrate indicated an increased cellular efflux, as less calcein-AM was retained in the cells after the specified time interval. Actual p values are indicated in the text.

Following a 4 hour exposure period (Figure 3.11), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. A minor insignificant increase in cell viability was seen the presence of verapamil No significant difference in viability or cell efflux was seen at both concentrations.

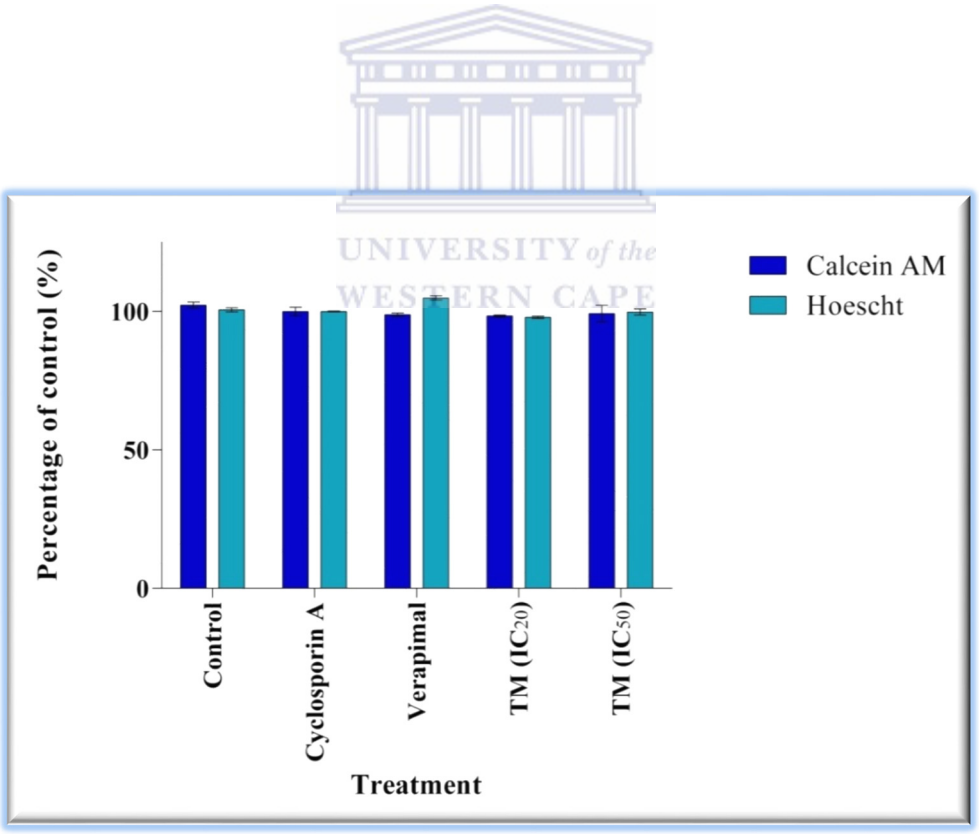


Figure 3.11: The effects of tunicamycin on P-glycoprotein-mediated efflux after 4 hours

Following an 8 hour exposure period (Figure 3.12), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control.

No significant differences in cell viability was observed ($p>0.05$), however significant increases in cellular efflux was noticeable at both IC_{20} and IC_{50} concentrations ($p=0.0005$ and <0.0001 respectively) compared to control. It was also visible that a significant decrease in cellular efflux is visible at both concentrations relative to each concentrations viability percentage ($p<0.05$).

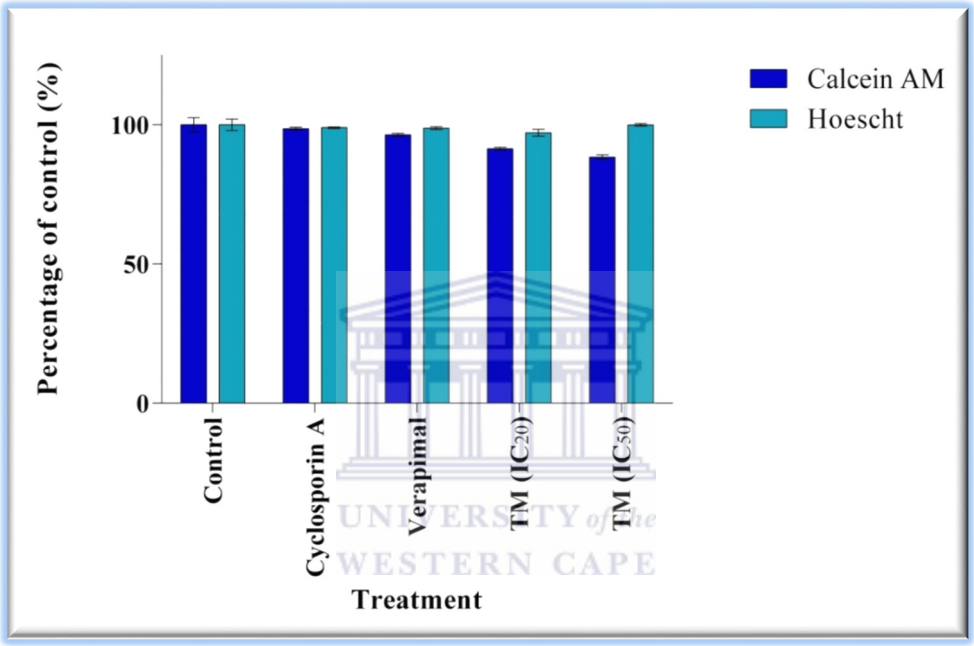


Figure 3.12: The effects of tunicamycin on P-glycoprotein-mediated efflux after 8 hours

Following a 16 hour exposure period (Figure 3.13), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. No significant differences in cell viability or cellular efflux were observed at both IC_{20} and IC_{50} concentrations compared to control. In the presence of TM at its IC_{20} and IC_{50} concentration, an insignificant decrease in calcein-AM accumulation was seen relative to its viability percentage ($p<0.05$.

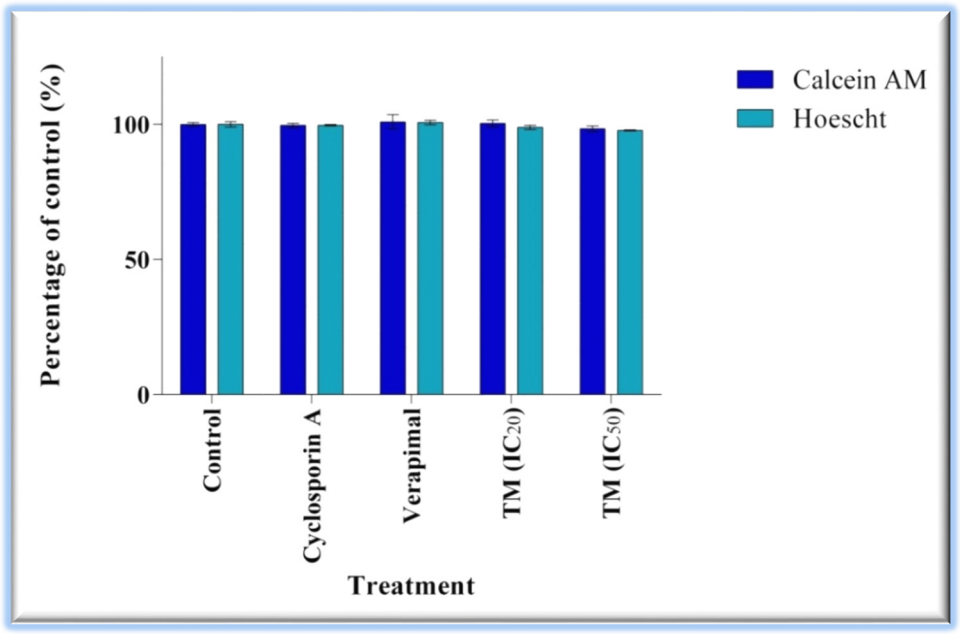


Figure 3.13: The effects of tunicamycin on P-glycoprotein-mediated efflux after 16 hours

3.4.4 Measurement of P-gp-Mediated Efflux in the Presence of Geldanamycin and Thapsigargin

Figures to 3.14 to 3.16 summarize the effects of calculated IC_{20} (11 µM) and IC_{50} (28.8 µM) values of the endoplasmic reticulum stress inducers GA and TG in combination, on the MCF-7 breast carcinoma cell line with regard to its response to P-gp-mediated efflux after 4, 8 and 16 hour intervals. Decrease in calcein-AM substrate indicated an increased cellular efflux, as less calcein-AM was retained in the cells after the specified time interval. Actual p values are indicated in the text.

Following a 4 hour combination exposure (Figure 3.14), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil compared to control ($p>0.05$), with an insignificant increase in cell viability in the presence of verapamil. No significant differences in cell viability or cellular efflux were observed at both IC_{20} and IC_{50} concentrations compared to control with an insignificant decrease in cell viability in the presence of the IC_{50} concentration. However, a minor but insignificant increase in calcein-AM was seen in the presence of the IC_{50} concentration compared to its relative viability percentage ($p>0.05$).

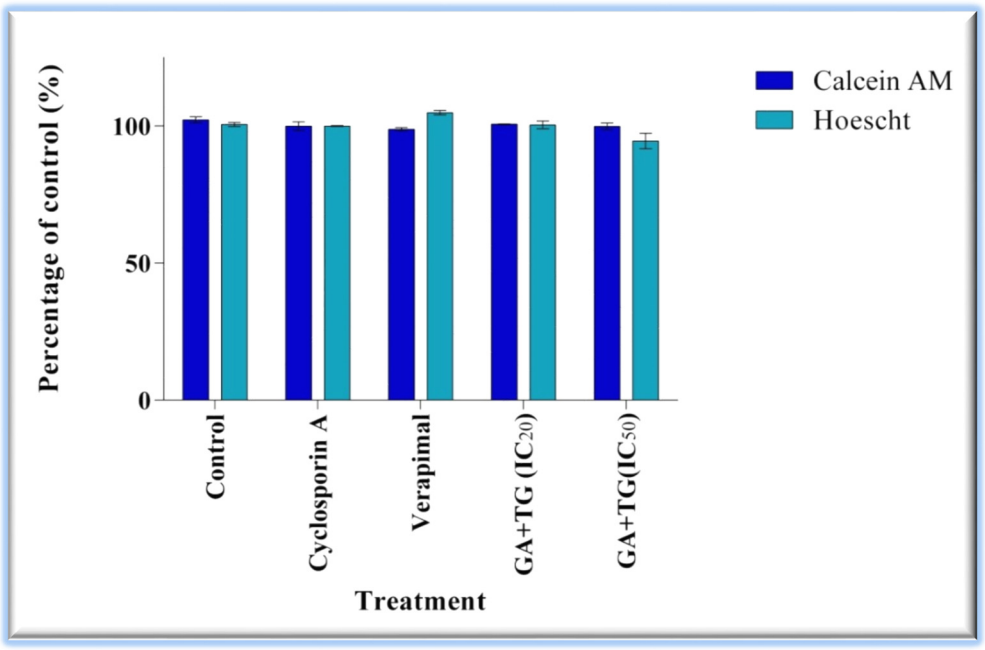


Figure 3.14: The combined effects of geldanamycin and thapsigargin on P-glycoprotein-mediated efflux after 4 hours

Following an 8 hour exposure period (Figure 3.15), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. No significant differences in cell viability was observed in all cases ($p>0.05$), apart from the IC_{50} concentration where a minor significant decrease in cell viability was seen relative to control ($p=0.038$).

After 16 hours (Figure 3.16), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. No significant differences in cell viability or cellular efflux were observed at both IC_{20} and IC_{50} concentrations compared to control as well ($p>0.05$), however, minor but insignificant increases in cellular influx is seen at both concentrations relative to their viability percentages ($p>0.05$).

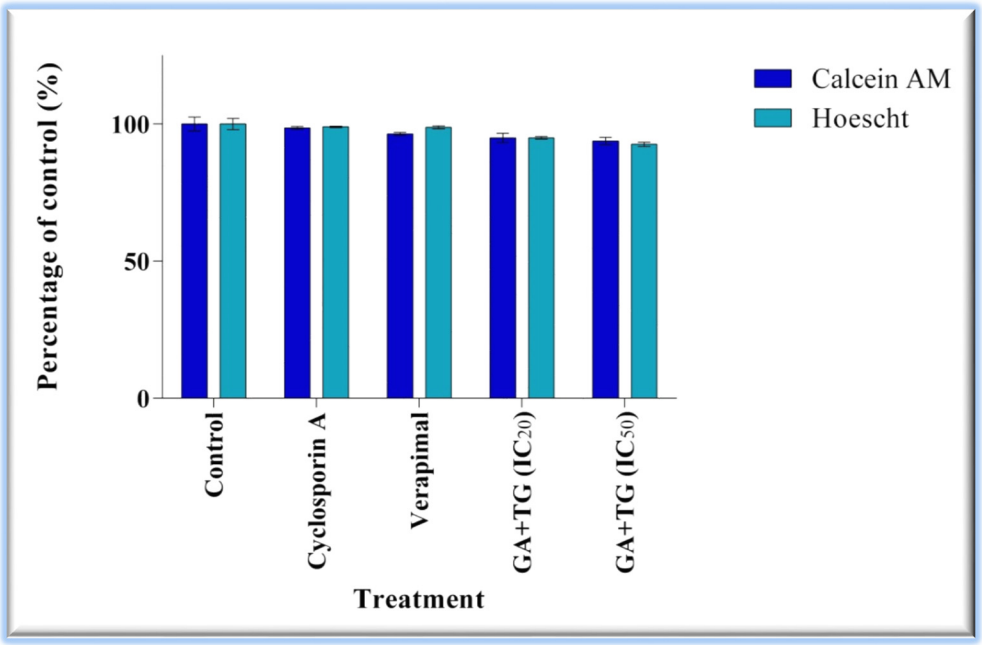


Figure 3.15: The combined effects of geldanamycin and thapsigargin on P-glycoprotein-mediated efflux after 8 hours

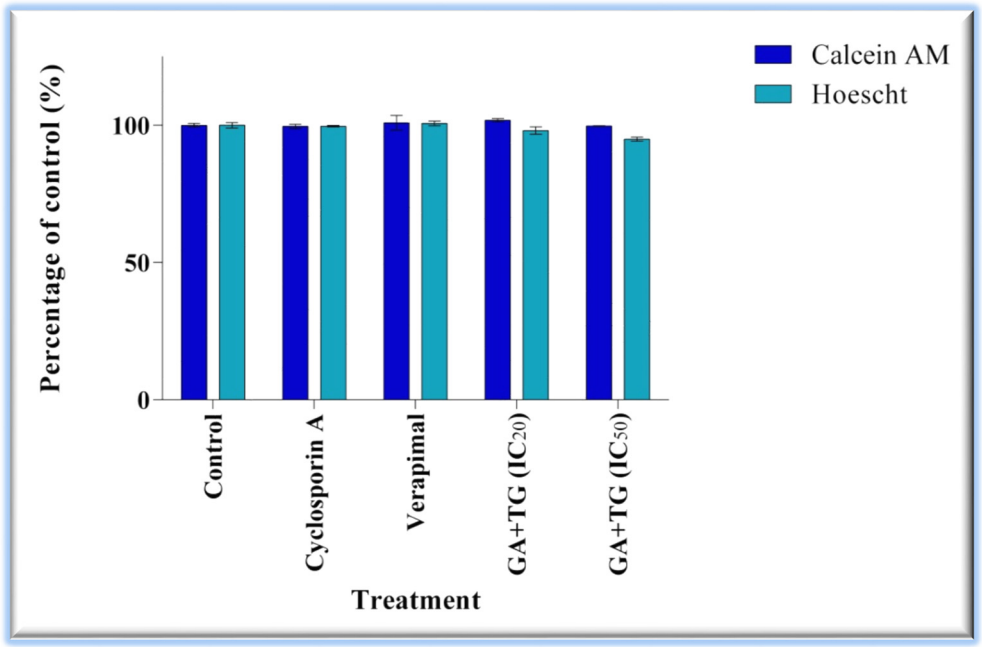


Figure 3.16: The combined effects of geldanamycin and thapsigargin on P-glycoprotein-mediated efflux after 16 hours

3.4.5 Measurement of P-gp-Mediated Efflux in the Presence of Geldanamycin and Tunicamycin

Figures to 3.17 to 3.19 summarize the effects of calculated IC_{20} (22.92 µM) and IC_{50} (91.66 µM) values of the endoplasmic reticulum stress inducers GA and TG in combination, on the MCF-7 breast carcinoma cell line with regard to its response to P-gp-mediated efflux after 4, 8 and 16 hour intervals. Decrease in calcein-AM substrate indicated an increased cellular efflux, as less calcein-AM was retained in the cells after the specified time interval. Actual p values are indicated in the text.

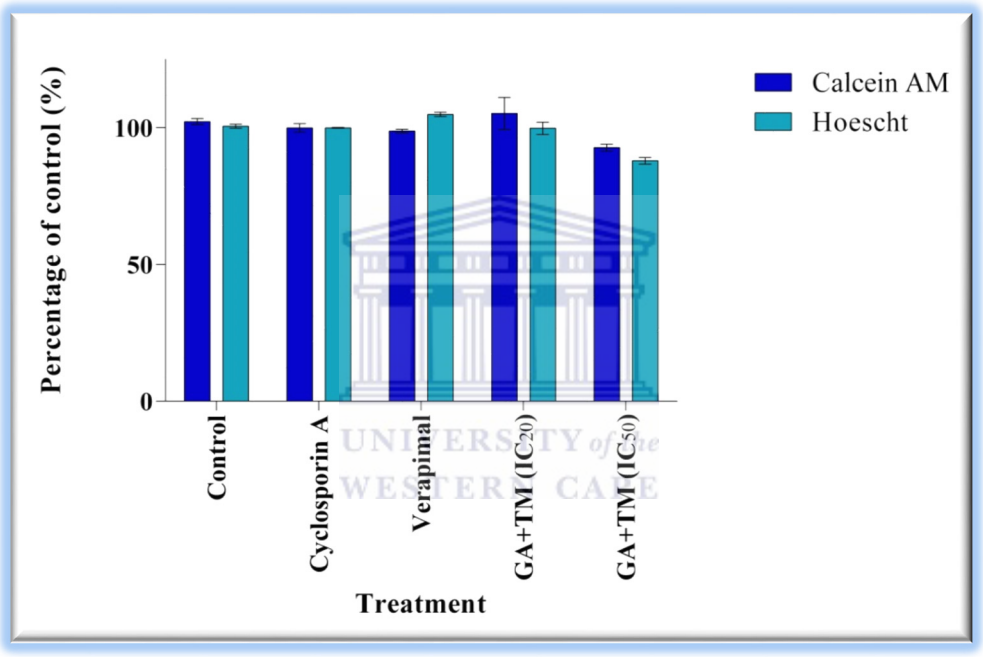


Figure 3.17: The combined effects of geldanamycin and tunicamycin on P-glycoprotein-mediated efflux after 4 hours

Following a 4 hour combination exposure of GA and TM (Figure 3.17), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil compared to control ($p>0.05$), with a minor but insignificant increase in cell viability in the presence of verapamil ($p>0.05$) and a minor insignificant decrease in cellular efflux relative to control seen at the IC_{20} concentration ($p>0.05$). A significant decrease in viability was seen at the IC_{50} concentration relative to control, cyclosporin A and verapamil ($p=0.0218$, 0.0315 and 0.0009 respectively) with an

insignificant increase in cellular efflux compared to control ($p>0.05$), however displaying a minor but insignificant decrease in cellular efflux relative to its viability ($p>0.05$).

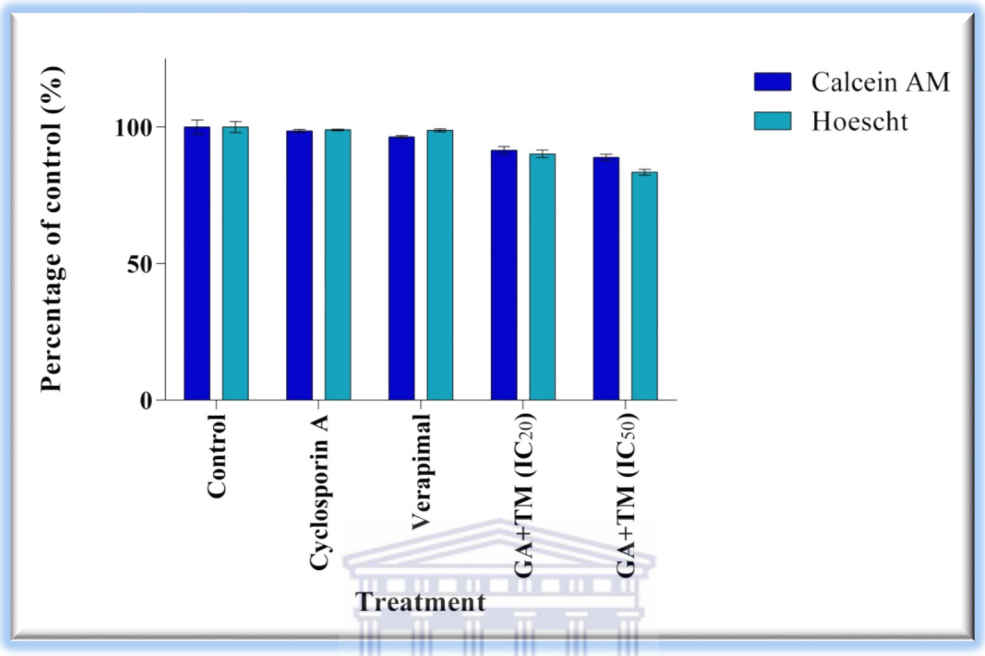


Figure 3.18: The combined effects of geldanamycin and tunicamycin on P-glycoprotein-mediated efflux after 8 hours

Following an 8 hour exposure period (Figure 3.18), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. In the presence of both, the IC_{20} and IC_{50} concentrations, a significant decrease in cell viability ($p=0.0017$ and <0.0001 respectively) and increase in cellular efflux ($p=0.007$ and 0.0004 respectively) was seen relative to control, however an insignificant decrease in cellular efflux was seen compared to its viability at the IC_{50} concentration ($p>0.05$)

After 16 hours (Figure 3.19), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. In the presence of the IC_{50} concentration, a significant decrease in cell viability relative to control, cyclosporin A and verapamil was observed ($p<0.0001$, <0.0001 and 0.0292 respectively), and a significant decrease in cellular

efflux was seen relative to its viability ($p<0.0001$). A significant decrease in cell viability of the IC_{50} compared to the IC_{20} concentration was also observed ($p=0.0005$).

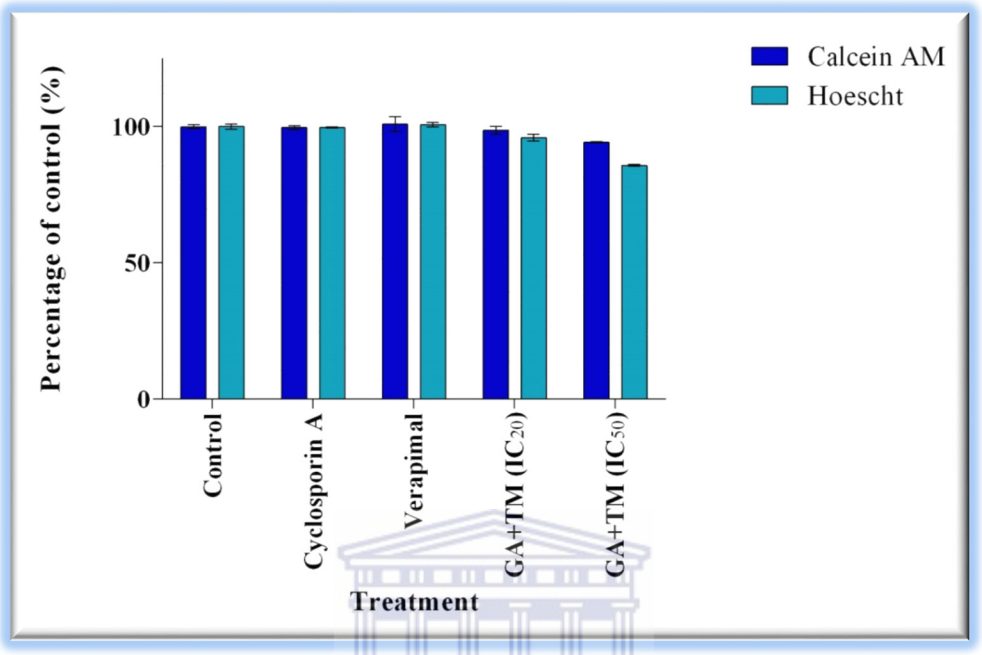


Figure 3.19: The combined effects of geldanamycin and tunicamycin on P-glycoprotein-mediated efflux after 16 hours

3.4.6 Measurement of P-gp-Mediated Efflux in the Presence of Thapsigargin and Tunicamycin

Figures to 3.20 to 3.22 summarize the effects of calculated IC_{20} (5.2 µM) and IC_{50} (20.81 µM) values of the endoplasmic reticulum stress inducers TG and TM in combination, on the MCF-7 breast carcinoma cell line with regard to its response to P-gp-mediated efflux after 4, 8 and 16 hour intervals. Decrease in calcein-AM substrate indicated an increased cellular efflux, as less calcein-AM was retained in the cells after the specified time interval. Actual p values are indicated in the text. After a 4 hour combination exposure (Figure 3.20), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil compared to control ($p>0.05$), with an insignificant increase in cell viability in the presence of verapamil. No significant differences in cell viability or cellular efflux were observed at both IC_{20} and IC_{50} concentrations compared to control with an insignificant decrease in cell viability in the presence of the IC_{50} concentration.

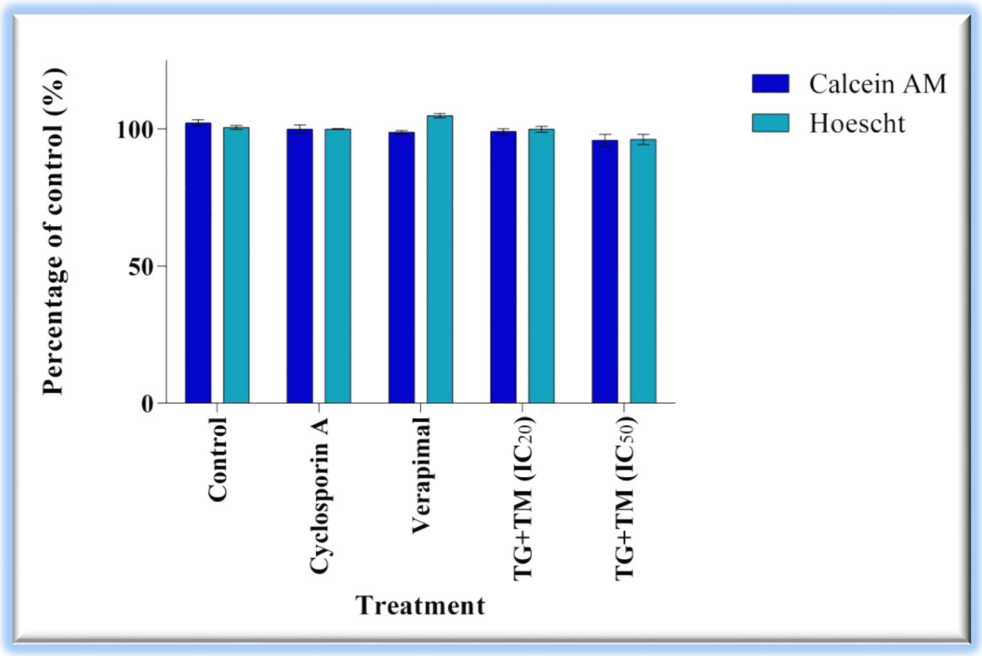


Figure 3.20: The combined effects of thapsigargin and tunicamycin on P-glycoprotein-mediated efflux after 4 hours

Following an 8 hour exposure period (Figure 3.21), no significant differences in cellular efflux or viability was observed in the presence of cyclosporin A and verapamil compared to control ($p>0.05$). In the presence of the IC_{50} concentration, a significant decrease in viability was observed ($p=0.0007$) compared to control and cellular efflux was significantly increased at both IC_{20} and IC_{50} concentrations compared to control ($p=0.0049$ and <0.0001 respectively). A significant increase in efflux was also seen in the presence of both concentrations ($p=0.0336$ and 0.0006 respectively) compared to cyclosporin A. No significant differences in cellular efflux were observed in the presence of both concentrations relative to its viability ($p>0.05$).

After 16 hours (Figure 3.22), no significant differences in cellular efflux or viability was found in the presence of cyclosporin A and verapamil ($p>0.05$) compared to control. In the presence of the IC_{50} concentration, significant decreases in viability were observed compared to control, cyclosporin A, and verapamil ($p=0.0161$, 0.0251 and 0.0055 respectively). No significant differences in cellular efflux was observed, however at the IC_{50} concentration, a minor but insignificant decrease in cellular efflux was observed relevant to its viability ($p>0.05$).

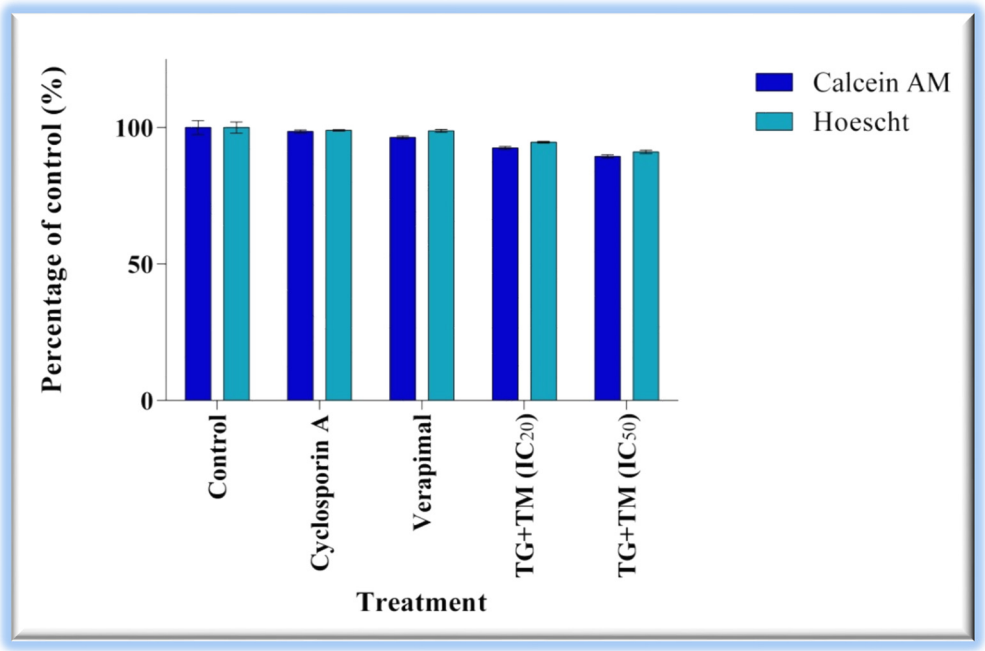


Figure 3.21: The combined effects of thapsigargin and tunicamycin on P-glycoprotein-mediated efflux after 8 hours

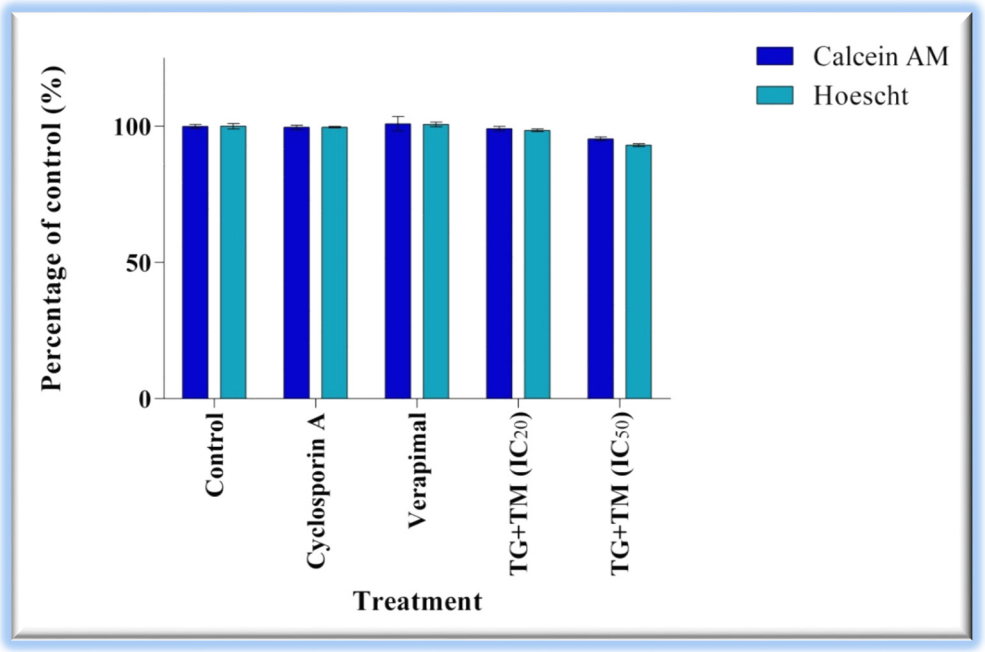


Figure 3.22: The combined effects of thapsigargin and tunicamycin on P-glycoprotein-mediated efflux after 16 hours

3.5 Measurement of MCF-7 Cell Viability, Cytotoxicity and Caspase-3 Activation

3.5.1 Effects of Geldanamycin

Figures 3.23 to 3.25 summarize the effects of the MCF-7 breast carcinoma cells in response to the endoplasmic reticulum stress inducer GA alone with regards to viability (Panel A), cytotoxicity (Panel B) and early apoptosis via caspase-3/7 activation (Panel C) after exposure over 6, 12 and 24 hour intervals.

In Figure 3.23, following a 6 hour exposure period, cell viability patterns (Panel A) remained similar at lower doses, with a minor significant decrease at 1 and 100 µM ($p=0.0482$ for both cases) relative to control and at 1000 µM, there was a highly significant decrease in cell viability ($p<0.0001$).

Cell cytotoxicity levels (Panel B) were significantly lower at 0.01, 0.1 and 10 µM ($p=0.019$, 0.0009 and 0.019 respectively), and a highly significant increase was found at 1000 µM ($p<0.0001$), with an increasing trend of cytotoxicity as the drug concentrations increased. At lower doses, a decreasing trend in caspase-3/7 activation (Panel C) relative to control was present, with minor, but insignificant increases between 0.01 and 100 µM ($p>0.05$ in all cases), however at 1000 µM there was a highly significant ($p<0.0001$) 3 fold increase in caspase-3/7 activation measuring apoptosis.

In Figure 3.24, following a 12 hour exposure period to GA, no significant decrease in viability (Panel A) patterns were visible between 0.01 and 10 µM ($p>0.05$), however at 100 and 1000 µM, a significant decrease in cell viability was found ($p=0.0021$ and <0.0001 respectively) relative to control. Cell cytotoxicity (Panel B) was found to be significantly lower at 0.01, 1and 10 µM ($p=0.0392$, 0.0392 and 0.0219 respectively) relative to control, and a noticeable but insignificant trend to an increase in cytotoxicity (Panel C) at 100 µM with a highly significant ($p<0.0001$), almost 2 fold increase in cell cytotoxicity at 1000 µM. No significant differences were present relative to control at concentrations ranging from 0.01 to 100 µM, however, at 1000 µM a highly significant ($p<0.0001$) increase in apoptosis (Panel C) was found.

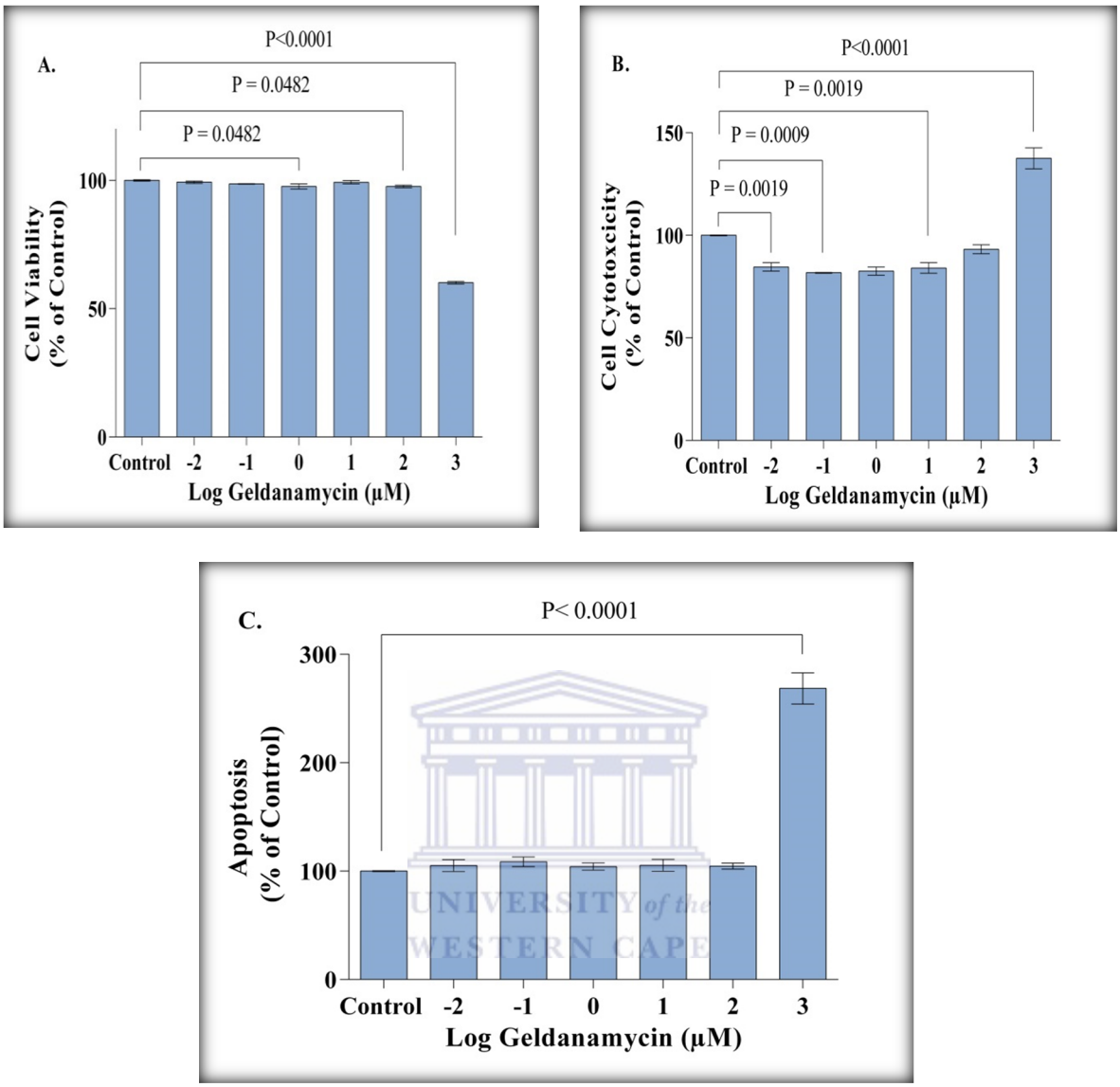


Figure 3.23: Treatment of MCF-7 breast carcinoma cells with geldanamycin following a 6 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

In Figure 3.25, a dose dependant trend after 24 hour exposure is noticeable in Panel A. With an increase in log dose concentrations of GA, a decrease in cell viability is present, however only significant decreases are present at 100 and 1000 µM ($p<0.0001$ for both cases) relative to control. A highly significant ($p<0.0001$) increase in cell cytotoxicity (Panel B) was only achieved at 1000 µM. At 0.01 µM an insignificant ($p>0.05$) increase in cell cytotoxicity was present, and at concentrations 0.1, 1, 10 and 100 µM, cell cytotoxicity was found to be lower than that of control, but not significantly ($p>0.05$). Apoptosis expression via caspase-3/7 activation (Panel C) significantly decreases at 100 and 1000 µM ($p=0.0038$ and $p<0.0001$ respectively).

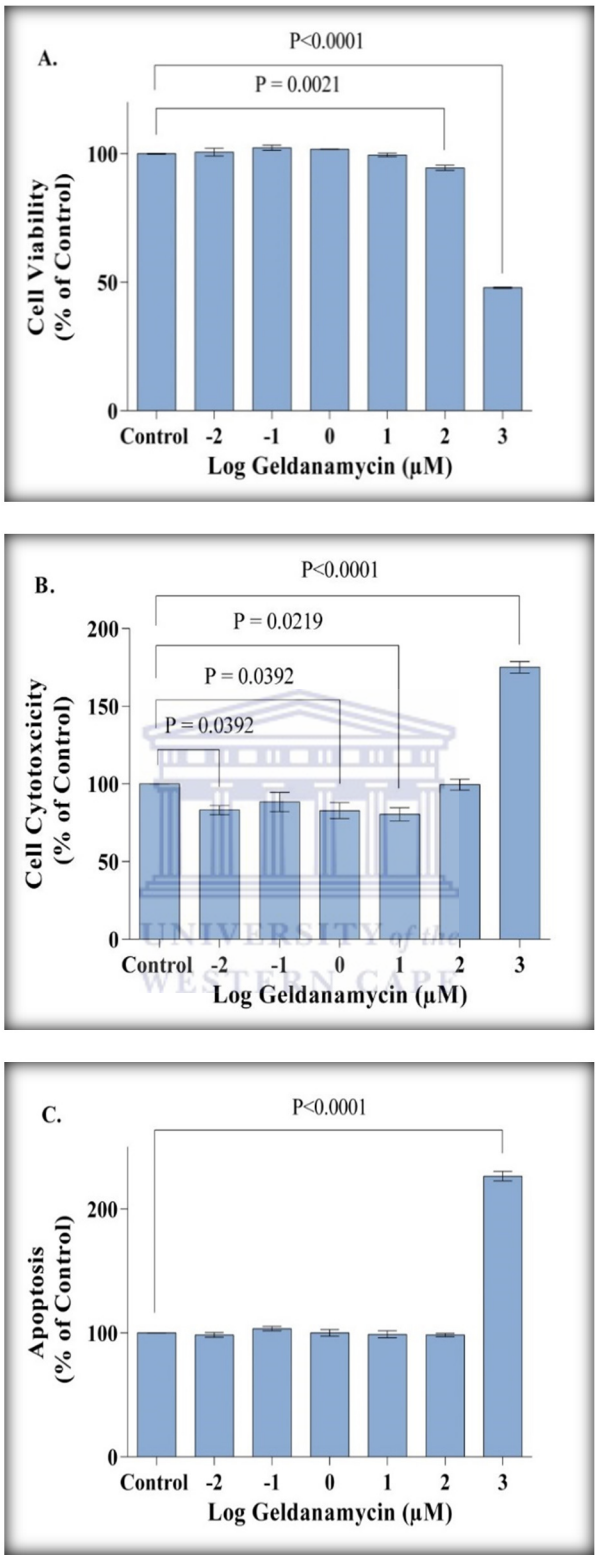


Figure 3.24: Treatment of MCF-7 breast carcinoma cells with geldanamycin following a 12 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

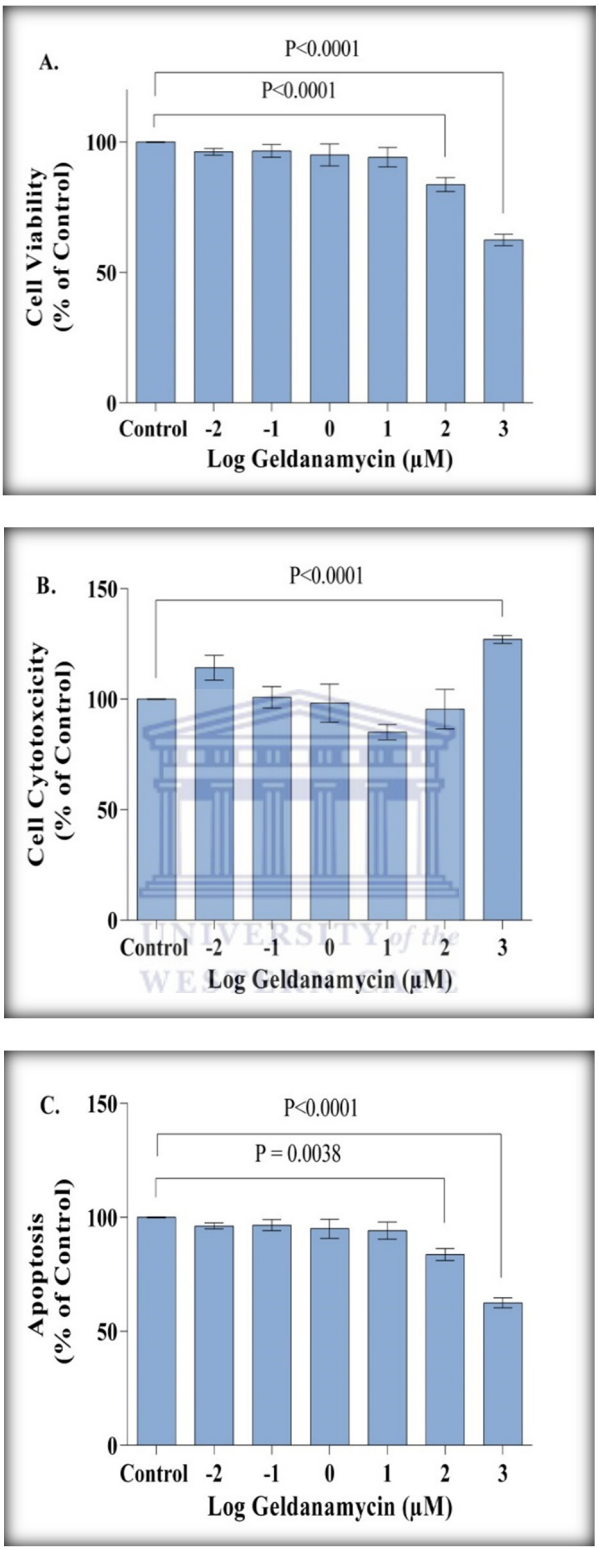


Figure 3.25: Treatment of MCF-7 breast carcinoma cells with geldanamycin following a 24 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

3.5.2 Effects of Thapsigargin

Figures 3.26 to 3.28 summarize the effects of the MCF-7 breast carcinoma cells in response to the endoplasmic reticulum stress inducer TG alone with regards to viability (Panel A), cytotoxicity (Panel B) and early apoptosis via caspase-3/7 activation (Panel C) after exposure over 6, 12 and 24 hour intervals.

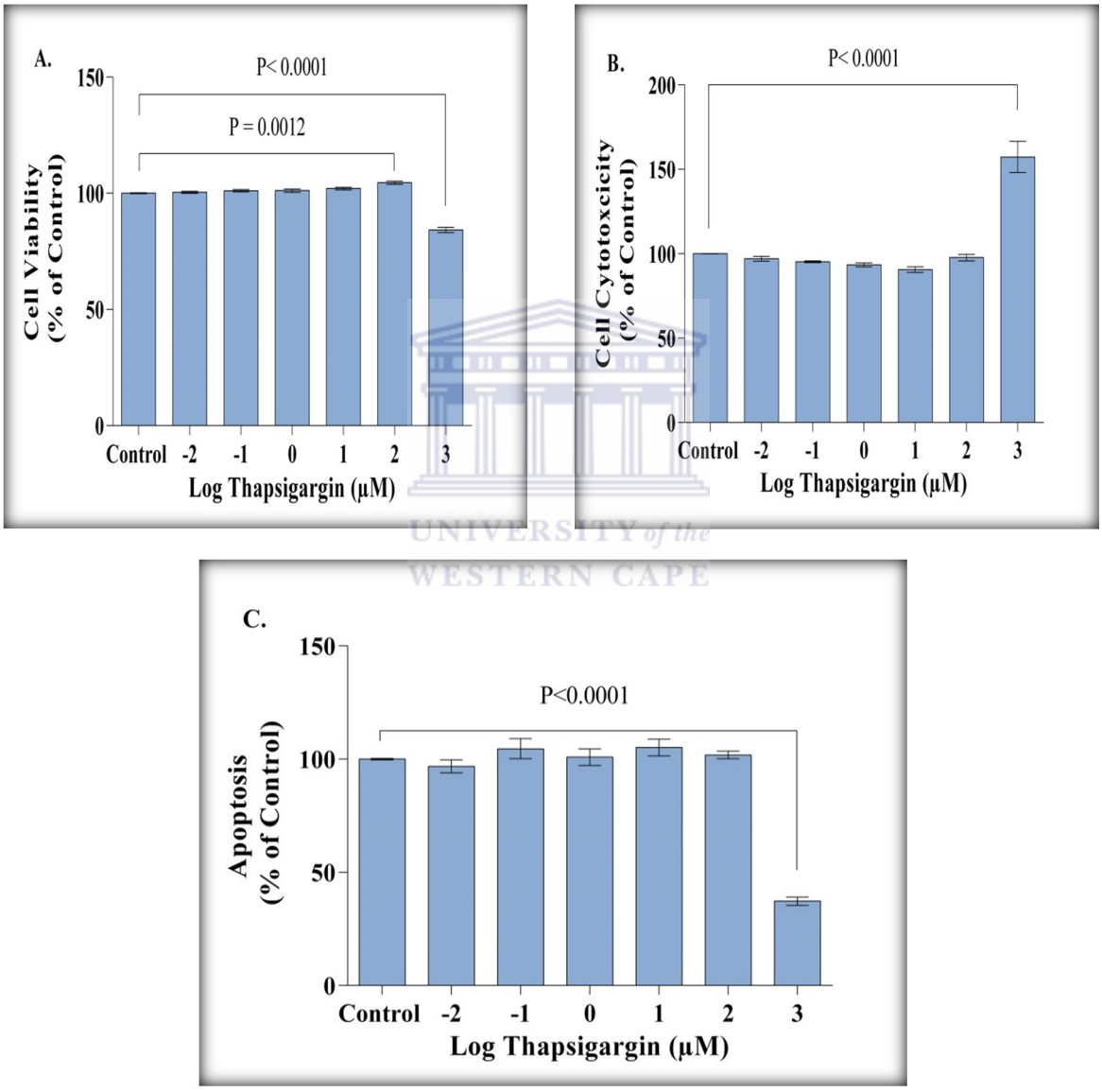


Figure 3.26: Treatment of MCF-7 breast carcinoma cells with thapsigargin following a 6 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

In Figure 3.26, the trend in cell viability (Panel A) remained unchanged after 6 hour exposure to TG, and a significant increase in cell viability was found at 100 µM ($p=0.0012$), however at 1000 µM, a highly significant ($p<0.0001$) decrease in cell viability was present. No significant changes in cell cytotoxicity (Panel B) was present in concentrations ranging from 0.01 to 100 µM ($p>0.05$ in all cases), however at 1000 µM, a significant increase in cell cytotoxicity is visible. Caspase-3/7 activity (Panel C) was higher than control, however not significantly higher at concentrations ranging from 0.1 to 100 µM. At 1000 µM there was a significant decrease in caspase-3/7 activity.

In Figure 3.27, the effects of TM on viability at 12 hours (Panel A) show a significant increase in cell viability when exposed to lower doses 0.01 to 1 µM ($p= 0.0013,0.0020,0.003$ and 0.003 respectively), however at 1000 µM, a significant decrease in cell viability was seen ($p<0.0001$) relative to control. Cell cytotoxicity (Panel B) was found to be insignificantly lower than that of control at lower doses 0.01, 0.1,1 ($p>0.05$) and a weakly less significant than control at 10 µM ($p=0.0412$). At 1000 µM, a highly significant ($p<0.0001$) 3 fold increase in cell cytotoxicity was observed. An increasing but insignificant trend in apoptosis (Panel C) was seen at concentrations 0.01 and 0.1 µM ($p>0.05$), however as the log drug concentrations increased from 1 to 100 µM, a decrease but not significant in caspase-3/7 activity was seen ($p>0.05$). Clearly visible at 1000 µM is the significant decrease in cell apoptosis protein expression.

Following a 24 hour exposure period in Figure 3.28, cell viability (Panel A) was found to be higher than control, with no significant increase between concentrations 0.01 to 10 µM ($p>0.05$ in all cases), however at 100 and 1000 µM, a highly significant decrease in cell viability was found relative to control ($p<0.0001$). It was found that at concentrations lower than 100 µM, cell cytotoxicity (Panel B) was lower but no significant decrease was found relative to control. There was a highly significant increase in cell cytotoxicity with a 1.5 fold increase at 100 µM, and more than a 2 fold increase at 1000 µM ($p<0.0001$ for both cases respectively). As concentrations increased, there was a significant decrease in early apoptotic protein expression (p values indicated in Panel C) for all concentrations with an increase in significant differences as concentrations increased.

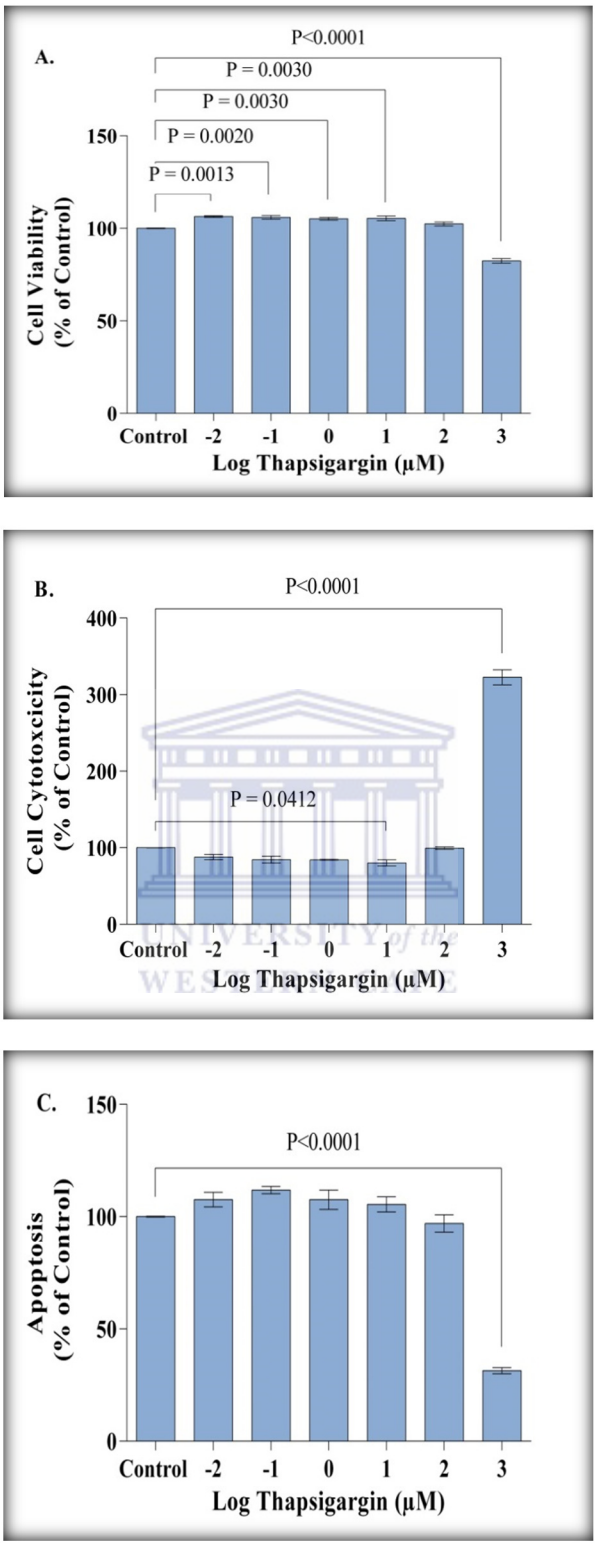


Figure 3.27: Treatment of MCF-7 breast carcinoma cells with thapsigargin following a 12 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

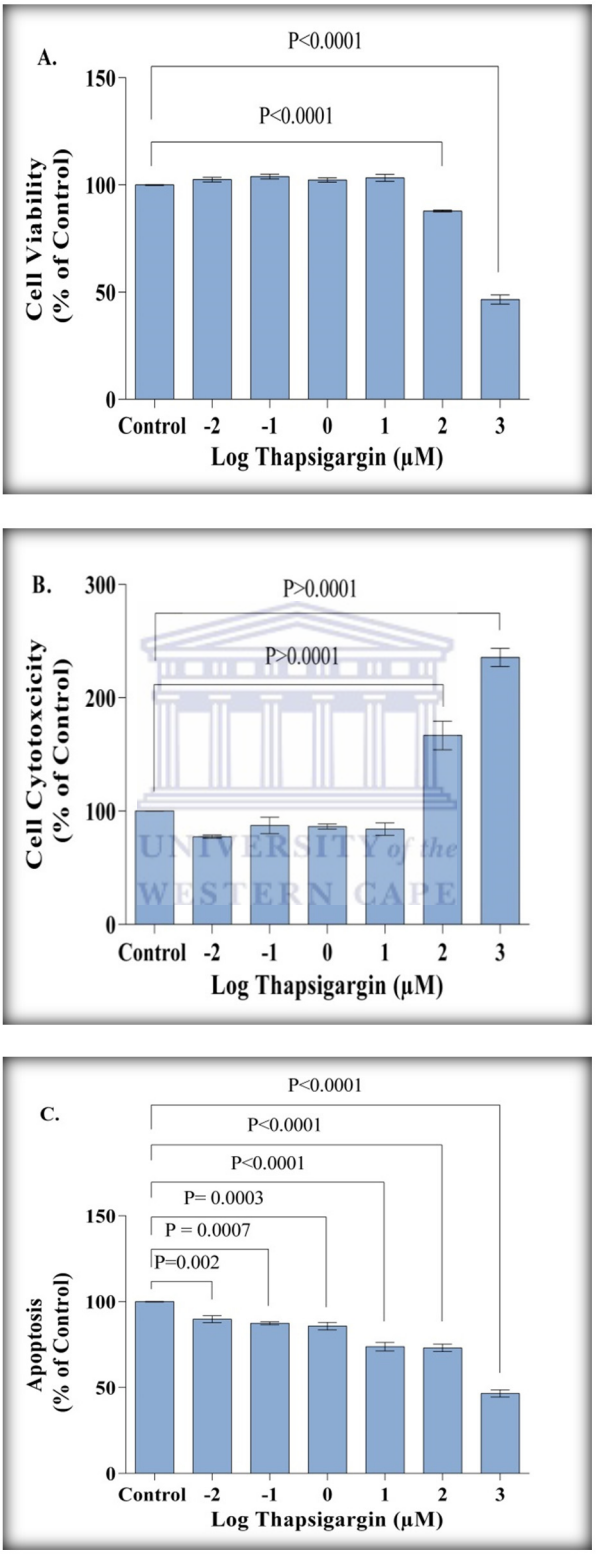


Figure 3.28: Treatment of MCF-7 breast carcinoma cells with thapsigargin following a 24 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

3.5.3 Effects of Tunicamycin

Figures 3.29 to 3.31 summarize the effects of the MCF-7 breast carcinoma cells in response to the endoplasmic reticulum stress inducer TG alone with regard to viability (Panel A), cytotoxicity (Panel B) and early apoptosis via caspase-3 activation (Panel C) after exposure over 6, 12 and 24 hour intervals.

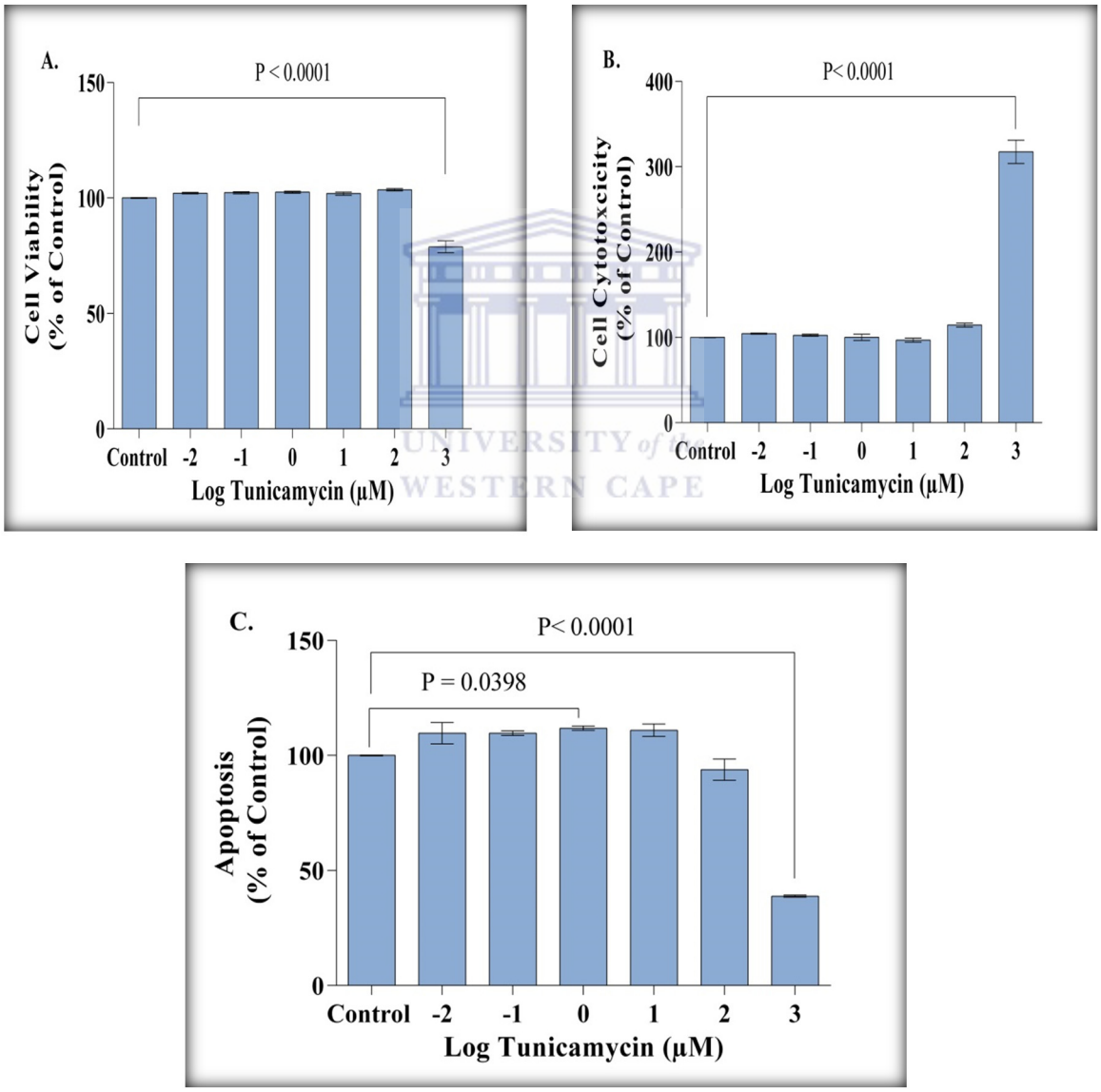


Figure 3.29: Treatment of MCF-7 breast carcinoma cells with tunicamycin following a 6 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

In Figure 3.29, after a 6 hour exposure period to TM, there was a minor but insignificant increase in the viability (Panel A) trend between 0.01 µM and 100 µM ($p>0.05$), however at 1000 µM, there was a significant decrease in cell viability ($p<0.0001$). Cell cytotoxicity (Panel B) gradually increased with no significant difference ($p>0.05$) relative to control whereas at 1000 µM, a highly significant 3 fold increase was seen. An increase in apoptosis expression was found at concentrations ranging from 0.01 µM to 10 µM with a significant increase only found at 1 µM ($p=0.0398$). At concentrations 100 µM and 1000 µM, a decreasing trend in apoptosis caspase3/7 expression (Panel C) was seen, with a highly significant decrease at 1000 µM relative to control ($p<0.0001$).

In Figure 3.30, after 12 hours, a noticeable but insignificant increase in cell viability (Panel A) was found at lower concentrations 0.01 to 10 µM ($p>0.05$), however at higher concentrations 100 and 1000 µM, a decrease trend was found with a highly significant decrease in cell viability at 1000 µM. Cytotoxicity levels (Panel B) remained unchanged at concentrations 0.01 to 10 µM with no significant increase or decrease in cell viability relative to control ($p>0.05$), however it was found that at higher concentrations (100 and 1000 µM), an increase trend in cell cytotoxicity was present, with a highly significant more or less 3 fold increase in cell cytotoxicity relative to control ($p<0.0001$).

A highly significant ($p<0.0001$) increase in caspase-3/7 early apoptotic proteases (Panel C) activity were present at concentrations 0.01, 1 and 10 µM with an increasing trend. As concentrations increased, a decreasing trend was found, however at 10 µM, apoptotic caspase activity were still significantly higher than that of control. At 100 and 1000 µM, there was a significant decrease in early apoptosis relative to control ($p=0.0154$ and <0.0001 respectively).

In Figure 3.30, after 24 hours exposure to TM, an insignificant increasing trend in cell viability (Panel A) was found at concentrations 0.01 to 1 µM following an insignificant decrease in cell viability at 10 and 100 µM, with a highly significant decrease in cell viability at 1000 µM relative to control ($p<0.0001$).

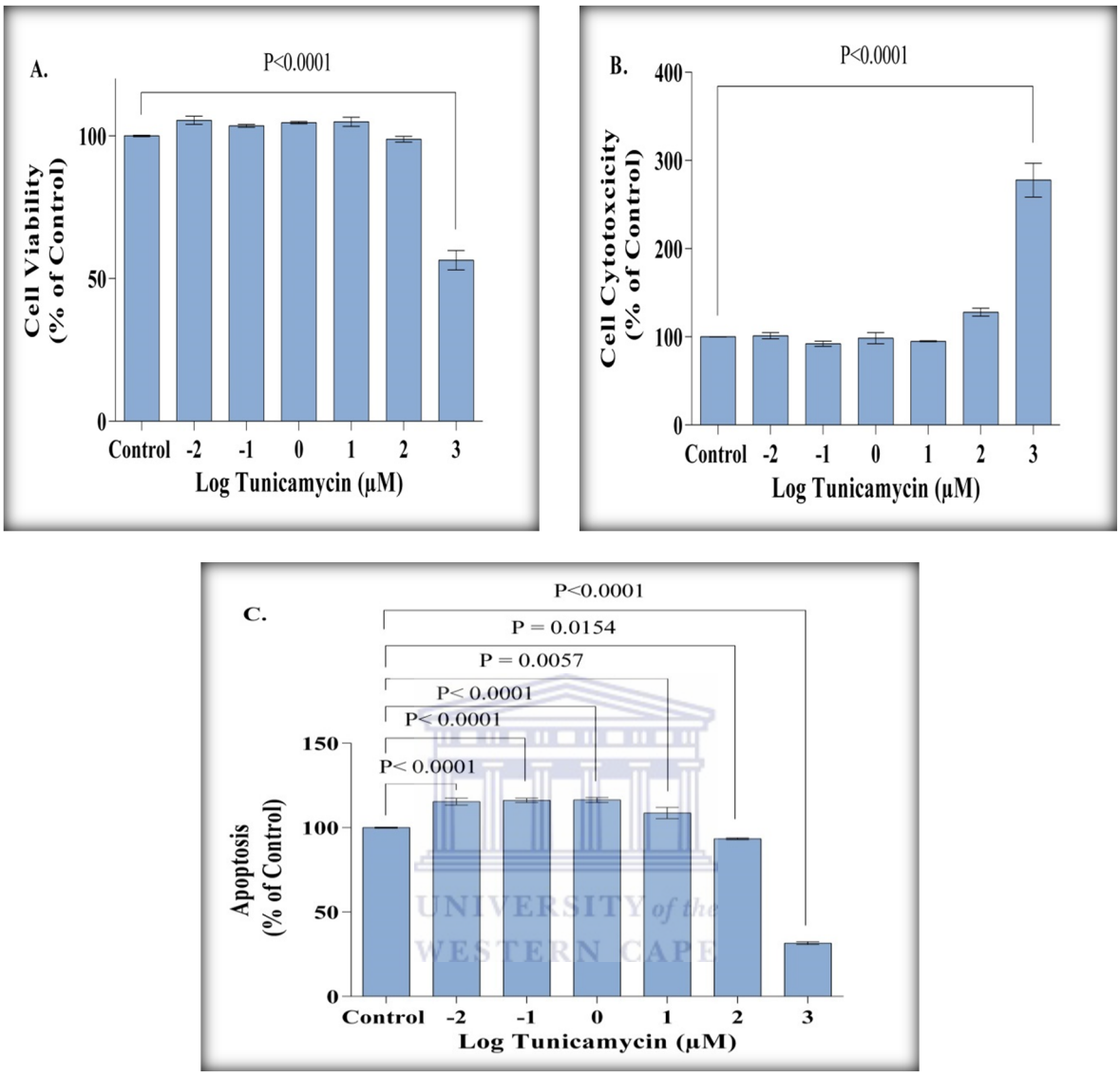


Figure 3.30: Treatment of MCF-7 breast carcinoma cells with tunicamycin following a 12 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

Cell cytotoxicity (Panel B) levels at all concentrations lower than 1000 µM were insignificantly lower relative to control, however at 0.01 and 1 µM cell cytotoxicity was found to be significantly lower relative to control ($p=0.0340$ and 0.0434 respectively). At 1000 µM, cell cytotoxicity was significantly higher relative to control ($p<0.0001$) with almost a 2 fold increase in cell cytotoxicity relative to control. As log concentrations of TM increased, an insignificant decreasing trend in caspase 3/7 protein expression (Panel C) was visible relative to control, with significant decreases in caspase activity at log concentrations surpassing 10 µM ($p<0.0001$) to 1000 µM.

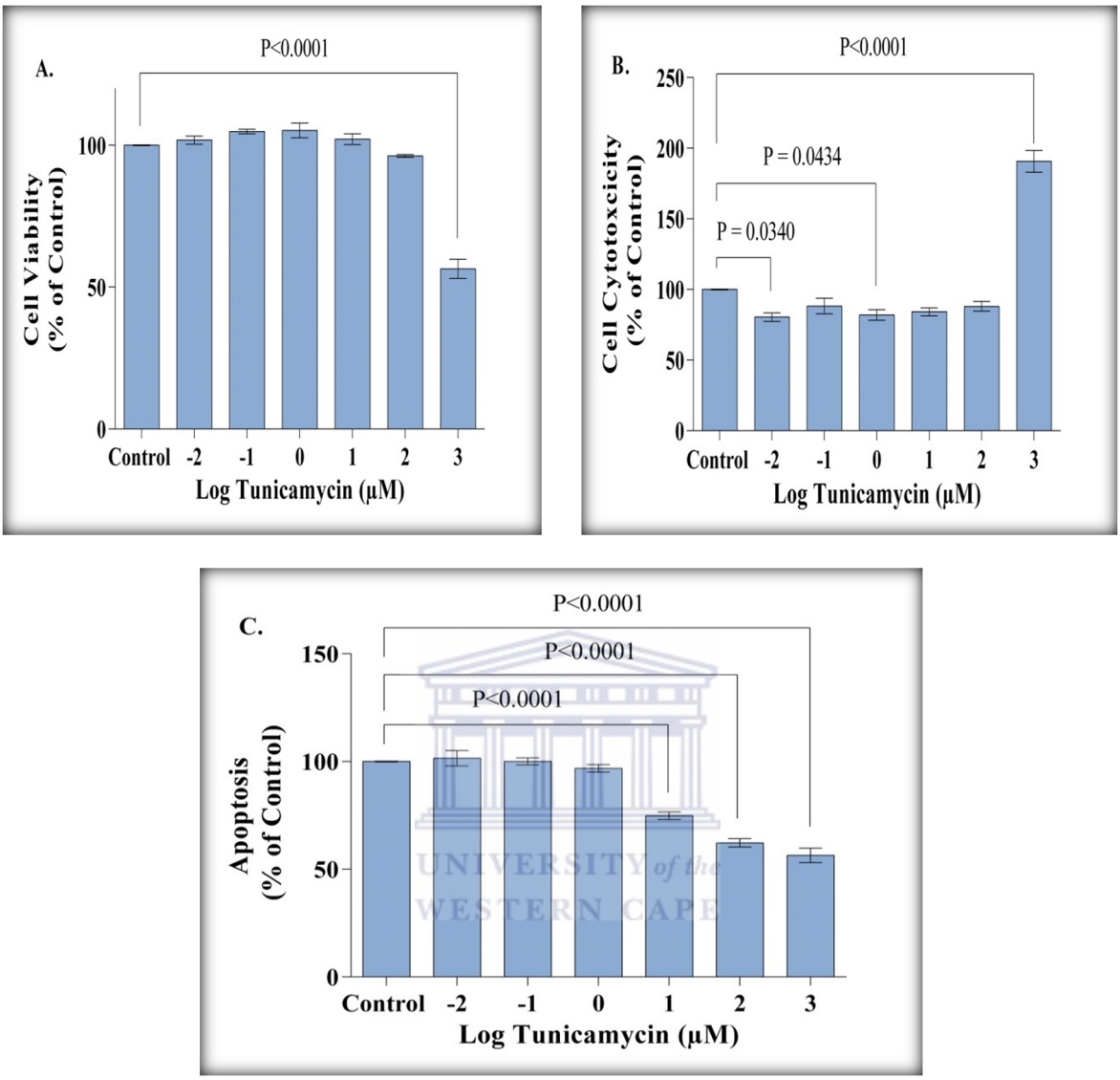


Figure 3.31: Treatment of MCF-7 breast carcinoma cells with tunicamycin following a 24 hour exposure period. The 3 parameters measured were cell viability (Panel A), Cytotoxicity (Panel B) and apoptosis (Panel C)

3.6 Annexin V-Cy3 Fluorescent Staining Analysis of ERS-Induced Apoptosis in MCF-7 Breast Carcinoma Cells

MCF-7 breast carcinoma cells were exposed to endoplasmic reticulum stress inducers GA, TG and TM, at 1991 µM, 12 µM and 115 µM respectively which were their relative IC_{50} values determined by Graphpad prism for 24 hours. In Figures 3.32A-E, viable cells (green fluorescence), early apoptotic cells (outer membrane stained red) and cell necrosis (majority of cell stained red) are visible.

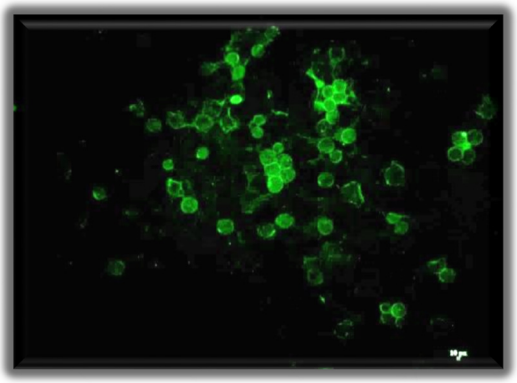


Figure 3.32-A: Untreated apoptosis negative control MCF-7 breast carcinoma cells stained with Annexin V-Cy3 fluorescent dye. Green fluorescence indicates viable cells

Figure 3.32-B: Staurosporine (1 µg/ml) induced apoptosis as positive control. MCF-7 breast carcinoma cells stained with Annexin V-Cy3 fluorescent dye. Blue arrow indicates early apoptotic cells

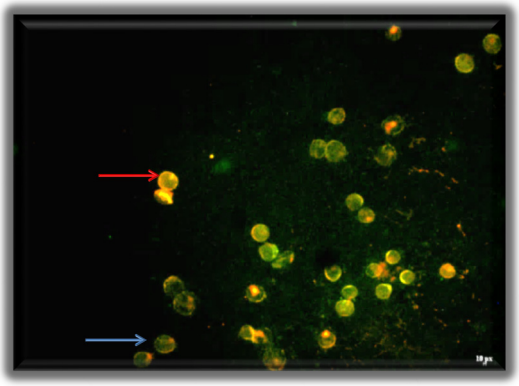


Figure 3.32-C: Annexin V-Cy3staining with 199.1 µM geldanamycin following 24 hours. Blue arrow indicates early apoptotic cells and the red arrow indicates cell necrosis

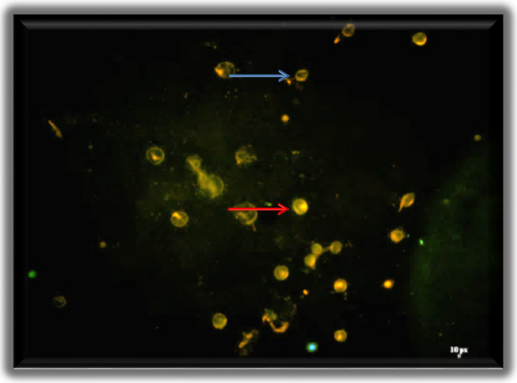


Figure 3.32-D: Annexin V-Cy3staining with 12 µM thapsigargin following 24 hours. Blue arrow indicates early apoptotic cells and the red arrow indicates cell necrosis

Figure 3.32-E: Annexin V-Cy3staining with 119 µM tunicamycin following 24 hours. White arrow indicates viable cells, blue arrow indicates early apoptotic cells and the red arrow indicates cell necrosis

CHAPTER 4

DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 Introduction

In this chapter, the results of the research undertaken in this study are discussed. Results obtained were presented in chapter 3 in which MCF-7 breast carcinoma cells were exposed to 3 Endoplasmic Reticulum stress inducers, geldanamycin (GA), thapsigargin (TG) and tunicamycin (TM) alone and in combination to determine cellular response to various log dose, IC_{20} and IC_{50} concentrations with reference to viability, P-gp-mediated efflux pump activity, and apoptosis.

4.2 Discussion

Ordinary cells undergo a variety of growth phases such as alterations within the cell in order to proliferate, repair or segregate the genome,[172] however, tumorgenesis due to the down regulation of tumor suppressor genes, occurs in cancer cells that have been mutated and either genetically or phenotypically altered.[173] Although the implementation of screening/prevention programs and novel treatment strategies are decreasing breast cancer mortality rates, more than 120,000 estimated deaths due to breast cancer are expected annually in the US and Europe combined.[174] This emphasizing the fact that the need to further elucidate breast cancer mechanisms, circumventing multidrug resistance and discovering methods to reduce host cytotoxicity and increasing efficacy of treatments is crucial.

GA binds to the molecular chaperone protein Hsp90, inhibiting the chaperone molecule, which leads to the ubiquitination of its client proteins,[175] thus activating the unfolded protein response (UPR). As a result of prolonged and inadequate UPR, cells undergo apoptosis.[113] Cell proliferation was negatively affected and sustained in a time dependent manner, as shown in the results obtained in this study when MCF-7 breast carcinoma cells were exposed to a constant IC_{50} concentration over 24, 48 and 72 hours. It was also observed that cell sensitivity increased with a decrease in

proliferation in a dose-and time-dependent manner in the presence of GA the same trend which was also previously reported in a study conducted by Hartmann and colleagues.[92]

Endoplasmic Reticulum stress (ERS) has been found to enhance MDR1 transcriptional induction and P-gp transport function in cancer cells.[65,176] This was investigated by exposing MCF-7 breast cancer cells to determined 24 hour less (IC_{20}) and more cytotoxic (IC_{50}) concentrations of GA in order to induce ERS over shorter time intervals (4, 8 and 16 hours) to determine effects (if any) on P-glycoprotein (P-gp) mediated drug efflux. Although it was previously reported that MCF-7 breast carcinoma cells do not readily express P-gp and that it is overly expressed in resistant cell lines such as MCF-7/Adriamycin resistant cell lines,[177] tumors generally develop significant resistance to repeated anticancer treatment with one kind of agent and often become resistant to similar or completely different drugs which can be intrinsic or acquired through chemotherapeutic drug exposure in which multiple mechanisms are likely to contribute to Multidrug Resistance (MDR).[36] It was observed in the study that at 4 and 8 hour intervals, cellular efflux was increased in the presence of the IC_{20} and IC_{50} concentrations compared to control, and relative to its viability, which may indicate that cellular stress was evoked, and as a result, an increase in efflux activity of the cells was observed. Surprisingly after a 16 hour exposure interval, it was found that cellular influx was significantly higher compared to its relative cell viability, which may indicate that due to prolonged ERS, cellular efflux and P-gp activity and was perturbed.

In this study, it was observed that as the log dose concentration of GA increased, cell viability decreased (consistent with previous findings),[92] with both an increase in cytotoxicity and early apoptosis at 6 and 12 hour intervals, whereas at 24 hours, significantly higher cytotoxic concentrations induced a decrease in apoptosis expression proteins, suggesting cell necrosis.[178] This was further validated by Annexin staining in the presence of the IC_{50} concentration.

TG inhibits the endoplasmic reticulum (ER) Ca^{2+} pump that leads to the depletion of the ER Ca^{2+} pool, which in turn activates the plasma membrane calcium channels resulting in an influx of

extracellular calcium[100]. In this study MCF-7 breast carcinoma cells were exposed to a constant 24 hour IC_{50} concentration of TG over 24, 48 and 72 hours.

It was observed that after 24 hours, cell viability had decreased, but not significantly, whereas at longer time intervals, a highly significant 2 and 8 fold decrease in cell viability was seen at 48 and 72 hours respectively compared to cellular response in the presence of TG at 24 hours, indicating that cells failed to recover and remained sensitive to the drug exposure over prolonged periods of time. These findings are consisitant with previous reports,[179] whereby MCF-7 cells were treated with TG,following a >4 fold increase in Ca^{2+} levels after treatment which returned to baseline following a few hours, however they discovered that a second elevation occurred with prolonged periods of exposure to the ERS inducer followed by cell death.

In the present study, a dose- and time-dependant pattern was observed when cells were treated with increasing log concentrations of TG, the same patten was observed previously and it was found that the IC_{50} concentration following 120 hours was decreased to 3 nM from 100 nM after a 1 hour exposure period,[179] indicating that cells responded in both a time and dose dependant manner. In this study, TG at 72 hours was more potent in the presence of most concentrations compared to the viability seen at 24 and 48 hours at the same concentrations. After 48 hours, the IC_{50} concentration determined in this study was 1.655 µM, which was relatively consistant with previous findings, as it was established that 1 µM of TG was found to induce the delayed secondary rise in Ca^{2+} after 36 hours, and temporarily precede the onset of apoptotic morphological changes and DNA fragmentation in MCF-7 cells.[179]

TG has been found to inhibit the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA).[103,180]Previous studies have also shown that TG not only increased intracellular Ca^{2+}, but also increased P-gp expression in human colon cancer cells by inducing Doxorubicin resistance via P-gp induction.[180] In this study, stress was induced at 24 hour IC_{20} and IC_{50} concentrations for 4, 8 and 16 hours. At 4 and 16 hour interval exposures, no effect on P-gp activity was presented,

however, following an 8 hour exposure period, results yielded a significant increase in cellular efflux, which may indicate that as a result of SERCA inhibition, increased Ca^{2+} concentrations and possible UPR mechanisms, P-gp activity in ER stressed MCF-7 cells may have either been activated or purturbed, which may relate to previous findings.[180]

In the present study, following 6, 12 and 24 hours, a time and dose dependant relationship was observed with reference to cell viability and cytotoxicity, whereas increasing log doses at higher concentrations resulted in significant decreases in cell death with increased cell cytotoxicty. At lower log doses, early apoptotic proteins are expressed over 6 and 12 hour exposure periods, however at 24 hours, cell necrosis occured in response to doses increases. Apoptosis and necrosis was further validated by means of Annexin staining in the presence of the IC_{50} concentration after 24 hours.

Previous studies reported that TM induced apoptosis via tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).[108] It was then further substantiated by reporting that MCF-7 were found to tolerate long term treatments of TM, due to high levels of cells were resistant to TM-induced apoptosis due to the lack of caspase-3 expression and high levels of calnexin expression, [109] which is a type 1 integral membrane chaperone that binds to glycosylated glycoproteins thereby promoting their folding and oligomerization. Calnexin has been reported to be involved in the late apoptotic processes following prolonged ERS.[109]. These findings could provide an understanding as to why the cellular response in the presence of 100 µM yielded significant death after 24, 48 and 72 hours, however at 72 hours cells, cell sensitivity to TM was reduced, which may be due to calnexin activity in response to prolonged ERS and may also suggest possible explanations behind the non-cytotoxic effects of the drug on MCF-7 cells to lower log doses and yielding a high IC_{50} concentration (115.2 µM) at 24 hours, however, based on present findings, also reveals that TM acts in both a dose and time dependant manner as IC_{50} concentration values decreased as exposure time to TM increased

TM is generally known to inhibit the protein N-glycosylation process within the endoplasmic reticulum (ER) by blocking the transfer of N-acetylglucosamine-1-phosphate from uridine diphosphate Nacetyl-glucosamine to dolichol phosphate.[88] However, tunicamycin also induced an elevation of P-gp expression (at both the mRNA and protein levels) including efflux activity in Fao hepatoma cells.[88] In the present study, no significant effect on P-gp efflux pump activity was seen at 4 and 16 hours, which may be due to lack of expression of P-gp in MCF-7 cells,[181] however after exposure to the drug for 8 hours, significant increase in cellular efflux was seen in both concentrations relative to its viability.

In a recent study, it was reported that the inhibition of P-gp glycosylation by tunicamycin was associated with increased ubiquitination and subsequent degradation of P-gp, thus stating that tunicamycin may induce either a decrease or increase in drug resistance associated with an improvement or impairment of P-gp function, respectively and reported that TM induced the inhibition of N-glycosylation P-gp without altering its function as a plasma membrane drug efflux pump in L1210 resistant cells.[88]

The present study demonstrates that after 6, 12 and 24 hours, cells were less sensitive to TM at lower doses, with increased but insignificant changes cell proliferation at those doses compared to control, and significant differences in cell death and cell cytotoxicity was only present at higher concentrations (100-1000 μM) relative to control, however after a 6 and 12 hour exposure, at lower doses, expression of apoptotic proteins were significantly higher compared to control, whereas at 24 hours, lack of apoptotic proteins and increased cell cytotoxicity suggests cell necrosis.[178] Apoptosis and necrosis was further validated by means of Annexin staining in the presence of the IC_{50} concentration after 24 hours.

The combination studies carried out in the present study have not been performed previously, therefore, this is the first study to investigate the integrated effects of GA combined with TG, GA

combined with TM and TG combined with TM on cell viability, and cellular responses based on P-gp efflux activity of MCF-7 breast carcinoma cells.

GA binds to the Hsp90 chaperone inhibiting the molecule, which leads to the ubiquitination of its client proteins,[175] thus activating the unfolded protein response (UPR) and as a result of prolonged and inadequate UPR, cells undergo apoptosis.[113] TG is a sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) pump inhibitor.[103,180] The combination of these two ER stress inducers have proved more potent in combination, then when used alone. The IC_{50} concentration of GA alone was 199.1 µM and the IC_{50} concentration of TG alone was 11.93 µM. The combined effect at lower concentration log doses yielded an IC_{50} concentration of 11.01 µM. The IC_{50} concentration yeilded by the combination of the 2 ER stress inducers was substatially lower then GA alone, and slightly lower than TG had yielded alone after 24 hours, and it was observed that the response in combination at the lower concentrations had more of a cytotoxic effect on the cells compared to the cytotoxicity seen in the presence of the drugs alone indicating that in combination, drug potency was increased compared to the drug potency alone. This may suggest synergism amongst the drugs when used in combination, as a decreased dose maintained the same efficacy where as compared to the drugs used alone Cell viability was decreased in a dose and time dependant manner as IC_{50}. Values at 48 and 72 hours were drastically decreased in combination, as apposed to when drugs were used alone which suggests that when combined, efficacy was drastically increased at lower and/ same doses.

When assessing the P-gp efflux activity of the cells in the presence of the drugs in combination, a significant decrease in cellular efflux in the presence of the IC_{50} concentration was seen after 4 hours. No significant differences were present at after 8 hour exposure and a significant decrease in cellular efflux at 16 hours in the presence of the IC_{50} concentration was seen, with an insignificant decrease in efflux pump activity in the presence of the IC_{20} concentration. When assessed alone, GA and TG showed no cellular efflux at 4 hours relative to its viability. Following an 8 hour exposure period, GA alone had shown no effect, however an increase in cellular efflux was seen in the presence of TG

relative to its viability. Following 16 hour exposure periods, a decreased efflux activity was seen in the presence of GA, and no effect was seen in the presence of TG. This may suggest that by integrating GA and TG, mechanisms of action with regard to P-gp pump activity may act synergistically to decrease cellular efflux activity.

The combined effects of GA and TM causes in cellular stress and as a result of the UPR, apoptosis occurs. This mechanism of action combines the inhibition of the Hsp90 chaperone activity,[175] and inhibition of protein N-glycosylation process within the endoplasmic reticulum (ER) by blocking the transfer of N-acetylglucosamine-1-phosphate from uridine diphosphate N-acetyl-glucosamine to dolichol phosphate.[88] The integrated effects of the 2 ERS inducers combined yielded a lower IC_{50} concentration (91.66 µM) compared to GA (199.1 µM) and TM (115.2 µM) alone after 24 hours. This suggests that by combining the 2 mechanisms of action, they may work synergistically to yield a higher potency at a lower dose. At both 48 and 72 hours, the combination yielded lower IC_{50} concentrations (4.648 µM and 0.032 µM respectively) compared to GA (113 µM and 36.89 µM respectively) and TM (108.2 µM and 26.64 µM respectively) alone, decreasing cell viability in both a dose and time dependant manner, but with increased efficacy.

By integrating the mechanisms of action on the P-gp activity of both GA and TM, minor, yet insignificant decreases in cellular efflux activity was seen after 4 (IC_{20} and IC_{50} concentrations) and 8 hours (IC_{50} concentration), whereas in the presence of GA alone, cellular efflux activity was higher compared to its viability, and no effect was seen in the presence of TM after 4 hours. Following 8 hours of exposure, no change in efflux pump activity was seen in the presence of GA and was increased in the presence of TM alone, however after 16 hours, efflux activity was decreased in the presence of the combination of GA and TM, whereas, alone, GA showed a decreased efflux and TM showed no effect alone. These results may suggest that both GA and TM work in a synergistic manner with regard to decreasing P-gp efflux pump activity in ERS induced cells.

TG is known to inhibit the SERCA of a cell,[103,180] TM inhibits the protein N-glycosylation process within the endoplasmic reticulum (ER) by blocking the transfer of N-acetylglucosamine-1-phosphate from uridine diphosphate N-acetyl-glucosamine to dolichol phosphate.[88] Integrating both mechanisms of action with regard to its effect on cell viability yielded IC_{50} concentrations 20.81 µM, 15.32 µM and 0.622 µM after 24, 48 and 72 hour exposure periods respectively. IC_{50} concentrations yielded by the ERS inducers alone yielded 11.93 µM, 1.655 µM and 8.962 µM in the presence of TG alone, and 115.2 µM, 108.2 µM and 26.64 µM in the presence of TM after exposure periods of 24,48 and 72 hours respectively. Although The IC_{50} values of TM were drastically decreased, the IC_{50} values of TG were slightly increased, however after 72 hours, the IC_{50} value of TG was drastically decreased. This may suggest that weak levels of antagonism or synergism can exist between the 2 ERS inducers when in combination, however at longer time exposures, strong levels of synergism may be suggested.

In terms of its effects on P-gp-mediated efflux activity, the combination of TG and TM, No changes in perturbing pump the activity was seen after 4 hours, this same result was obtained in the presence of the ERS inducers alone. Following an 8 hour exposure period, no change in the presence the ERS in combination had been obtained compared to the ERS inducers alone. All resulted in an increased P-gp-mediated efflux activity. Following a 16 hour time exposure, no effect on P-gp-mediated efflux was seen in the presence of the ERS inducers alone, however after 16 hours the integrated effects yielded a decrease in P-gp-mediated efflux pump activity relevant to its viability.

4.3 Conclusion

It can be concluded that GA, TG and TM alone yielded a dose- and time- dependant response with regard to effects on cell viability. When combined, GA yielded enhanced cell cytotoxic effects when integrated with ERS inducers TG and TM, as relative IC_{50} concentrations at 24, 48 and 72 hours were drastically reduced when compared to their IC_{50} concentrations alone, as well as increasing and/or maintaining efficacy of combined drugs at lower doses. The combination of TG and TM had drastically reduced the IC_{50} concentrations of TM alone; however IC_{50} concentrations of TG alone

were slightly lower than that found in combination. Interestingly enough, The IC_{50} concentration after 72 hours was reduced compared to the ERS inducers alone, thus suggesting that an increase in time may have yielded an increase in synergistic effects of the ERS inducers when combined. It can also be concluded that all ERS inducers decreased cell viability in both dose-and time-dependant manners With regard to P-gp-mediated efflux pump activity, the integrated effects of GA and TG reduced efflux pump activity at 4 and 16 hours however not significantly. GA and TM combined, reduced efflux pump activity at 4 and 8 hours however not significantly, whereas after 16 hours at its IC_{50} concentration, significantly reduced efflux pump activity and TG and TM in combination had no effect on P-gp efflux mediated activity after 4 and 8 hours, however after 16 hours, an insignificant decrease in cellular efflux was yielded. The combination of GA with TG and with TM alone drastically decreased its IC_{50} inhibitory concentration, which may be an alternative treatment solution to drug use alone, as severe hepatotoxicity at therapeutic doses in animals and its poor water solubility were the reasons behind the drug alone being a unsuccesful theapeutic agent in the treatment of cancer.

4.4 Limitations and future perspectives of the study

This study is limited to MCF-7 breast carcinoma cells. Every effort was made to study all possible combinations with the ERS inducers in terms of viability and P-gp-mediated efflux responses. Due to funding constraints, apoptosis by means of caspase-3/7 activity was not be carried out as well as a Western Blot analysis to verify P-gp presence once cellular stress was induced. Therefore this study did not measure all possible integration effects on the MCF-7 cell line. Although MCF-7 breast carcinoma cells do not readily express P-gp, its effects were still tested to see the UPR response and activity. The comparison of these findings in conjunction with cells known to overly express P-gp in resistant cell lines such as MCF-7/Adriamycin resistant cell lines,[177] would be a good future perspective as well as determining levels of synergism and/or antagonism at which these drugs in combination may act given specific doses and time intervals via dose reduction indices .

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