The In Vitro Effects of Nicotine and Selected Antibiotics, Tunicamycin and Thapsigargin on Human Breast Carcinoma (MCF-7) Cells

by

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DECLARATION

I declare that "**The *In Vitro* Effects of Nicotine and Selected Anti-biotics, Tunicamycin and Thapsigargin on Human Breast Carcinoma (MCF-7) Cells,**" is my own work, that has not been submitted before for any degree or assessment in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Rabia Isaacs August 2012

Signed

DEDICATION

To my parents, for their sacrifices and

challenges faced, in order to afford me

an education and a better life,

Shukran

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My greatest gratitude is to the Almighty for bestowing upon me, my accomplishments. “All talents I have come from Thee, as Thy art the fundamental source of all knowledge”.

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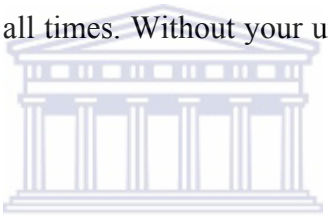

To my family and friends who encouraged me to continue even when I had no fight left, my sincerest thanks is to you, for believing in me and for pushing me to explore my capabilities and reaching my potential.

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ABSTRACT

Cancer is defined as the abnormal growth of genetically mutated or perturbant cells. Nicotine is a known cancer promoter and an apoptotic suppressor. This alkaloid acts on the nicotinic acetylcholine receptors which affects the ubiquitin-proteasome protein degradation pathway and ultimately hinders apoptosis. The endoplasmic reticulum (ER) is an interconnecting organelle which synthesises proteins and its quality control processes ensures the proper protein folding, post-translational modifications and conformation of secretory and trans-membrane proteins. Studies demonstrated that the antibiotic, Tunicamycin (Tm) and the sesquiterpene lactone, Thapsigargin (Tg) causes ER stress and consequently cellular arrest. Tm interferes with N-glycosylation of newly synthesised proteins triggering the unfolded protein response, while Tg inhibits intracellular Ca^{2+} ATPases resulting in increased cytosolic Ca^{2+}. Studies showed that these compounds have potential pro-apoptotic effects. The combinatorial effects of nicotine, Tm and Tg may produce antagonistic or synergistic effects and provide a therapeutic tool against breast cancer.

The aim of the study was to determine the apoptotic effects of nicotine, Tm, and Tg on human breast carcinoma (MCF-7) at various time intervals and further to elucidate whether selected ratios of their combinations resulted in synergistic or antagonistic effects.

MCF-7 cells were exposed to various concentrations of nicotine (0.1 nM to 1 mM), Tm (0.1 nM to 1 μM), and Tg (0.1 nM to 1 μM) at 24, 48 and 72 hrs to determine cell viability, ATP activity, DNA proliferation and early apoptosis. The combinatorial concentrations of nicotine with Tm and nicotine with Tg were at selected 1:1 ratios for the analysis of the above mentioned parameters. Data was analysed using

GraphPad Prism 5 and the MedCalc, Mann-Whitney test for not-normally distributed unpaired samples. Statistical significances was denoted as $p<0.05$.

Results in this study demonstrated that:

(1) MCF-7 cell viability was unaffected by the selected concentrations of nicotine at 24, 48 and 72 hrs. Results showed consistently high levels of ATP and DNA proliferation and nicotine did not induce early apoptosis.

(2) Tm exposure resulted in a significant decrease ($p\leq0.0159$) in viability. There was a noticeable decrease in ATP production ($p\leq0.005$), a marked elevation ($p\leq0.0286$) in DNA proliferation and also induction in early apoptosis between 24 and 72 hrs.

(3) Tg resulted in a noticeable decrease ($p\leq0.001$) in viability and ATP concentrations ($p\leq0.009$) between 24 and 72 hrs. There was a significant increase in DNA proliferation ($p\leq0.0286$) and Tg induced early apoptosis at 24 hrs only at 0.4 μM. Significant apoptotic effects were observed at 48 and 72 hrs.

(4) Combinatorial effects of nicotine and Tm (1:1 ratios) resulted in a significant decrease ($p\leq0.05$) in viability between 24 and 72 hrs. The ATP levels noticeably decreased ($p\leq0.0286$) while % DNA significantly increased ($p\leq0.0286$) between 24 and 72 hrs. The nicotine and Tm combination induced apoptosis in MCF-7 cells.

(5) Combinatorial effects of nicotine and Tg (1:1 ratios) resulted in the marked decrease ($p\leq0.0001$) in viability and ATP concentrations ($p\leq0.0286$) while DNA proliferation significantly increased ($p\leq0.0286$) between 24 and 72 hrs. Apoptosis was induced only at 48 hrs. The combination Tg and nicotine could not induce apoptosis at 24 and 72 hrs, except at 1:1 ratio of 5 μM at 72 hrs.

(6) Selected concentrations of Tg in combinations with 0.105 μM Tm resulted in a significant decrease ($p \leq 0.0022$) in viability, ATP levels ($p \leq 0.0286$) and lowered DNA concentrations ($p \leq 0.05$) between 24 and 72 hrs. Increased Tg concentrations resulted in greater inhibition of viability. Results also showed that the combinations could induce apoptosis in MCF-7 cells.

(7) Selected concentrations of Tm in combination with 0.06 μM Tg resulted in a marked decrease ($p \leq 0.0022$) in cell viability, ATP levels ($p \leq 0.0286$) and lowered DNA concentrations ($p \leq 0.00238$) between 24 and 72 hrs. Increased Tm concentrations resulted in a lowered inhibition of viability. Results also showed that the combinations could induce apoptosis after 48 hrs in MCF-7 cells.

It was concluded from this study that nicotine supported MCF-7 cell growth while Tm and Tg displayed apoptotic effects. Nicotine suppressed the apoptotic effects of Tm and also reduced its cytotoxicity. Nicotine and Tg combinations only induced apoptosis at 48 hrs, suggesting that nicotine could suppress the apoptotic effects of Tg after 72 hrs. This finding implies that the apoptotic effects of Tg were reversible. This is the first study to report on the combinatorial effects of Tm and Tg which demonstrated their synergistic apoptotic effects in MCF-7 cells.

Keywords: Nicotine, tunicamycin, thapsigargin, endoplasmic reticulum, apoptosis

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LIST OF ABBREVIATIONS

3HC	3'hydroxycotinine
3HC-Glu	3'hydroxycotinine glucuronide
ADP	Adenosine diphosphate
AOX	Aldehyde oxidase
ATF6	Activating transcription factor 6
ATP	Adenosine trisphosphate
ATP	Adenosine trisphosphate
ATPase	Adenosine triphosphatase
Ca^{2+}	Calcium ion
CCT	Chaperone containing TCP-1 protein
CYP	Cytochrome
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERAD	ER-associated protein degradation
ER-positive cancer	Estrogen receptor-positive cancer
FMO3	Flavin containing monooxygenase 3
GADD153/CHOP	Growth arrest and DNA damage-inducible protein

GPT	GlcNAc-1-P transferase
Grp78/BiP	Glucose regulating protein 78/ immunoglobulin heavy chain binding protein
GTP	Guanosine-5'-triphosphate
Hsp	Heat shock protein
IRE1	Inositol requiring enzyme
JNK	c-Jun N-terminal kinase
LCIS	Lobular carcinoma in situ
MCF-7	Breast Cancer Cell Line
nAChr	Nicotinic acetylcholine receptor
nAChRs	Nicotinic acetylcholine receptors
NF-κB	Necrosis Factor - κB
Nic	Nicotine
Nicotine-Glu	(S)-nicotine-N-glucuronide
NNK	4-(methylnitros-amino)-1-(3-pyridyl)-1-butone
NNO	Nicotine N'-oxide
NNO	nicotine-N'-oxide
PERK	PRK-like ER resistant kinase
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
Tg	Thapsigargin
Tm	Tunicamycin

TRAIL	Tumour necrosis factor related apoptosis-inducing ligand
TSGs	Tumour suppressor genes
UCHL1	Ubiquitin carboxyl-terminal esterase L1
UGT	Uridine diphosphate
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
UV	ultraviolet

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CHAPTER 1

1. Introduction

1.1 Cancer

Cancer is defined as the abnormal growth of mutated cells (Fig. 1). These cells may be altered genetically or result from a perturbation of normal cell function. Malignant cancer cells spread readily through the lymphatic system and have the ability to proliferate uncontrollably and invade other tissue sites to establish secondary tumours or metastasis, while benign tumours do not spread through the lymphatic system and remain in their site of origin (Dervan, 2001 and Löwy, 2010).

Cancers are divided into subtypes which are dependent on either the cell type or the region it occurs in. The main categories of cancer include: (i) Carcinoma which is a cancer that begins in the skin or in tissues that line or cover internal organs, (ii) Sarcoma, a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue, (iii) Leukemia which starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood, (iv) Lymphoma and myeloma, cancers which originate from cells of the immune system, and (v) Central nervous system cancers that begin in the tissues of the brain and spinal cord (Dervan, 2001 and Löwy, 2010).

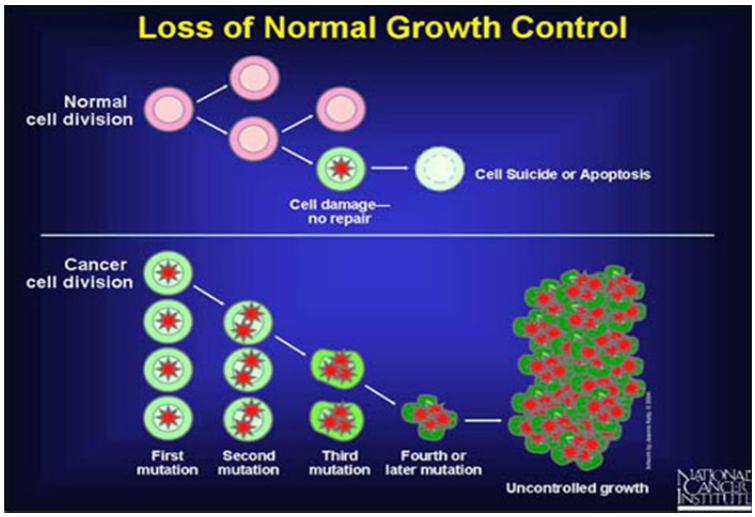

Fig. 1. Comparison of cell proliferation in normal and cancerous cells (National Cancer Institute:http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer).

During tumourigenesis, tumour suppressor genes (TSGs) are downregulated due to anomalous promoter methylation, and may be used as a marker for early diagnosis of numerous carcinomas. It has been reported that protein ubiquitylation is a crucial aspect in a variety of biological processes as well as cell proliferation, cell cycle, apoptosis, signal transduction, especially in breast cancer (Xiang et al., 2012).

Ubiquitin carboxyl-terminal esterase L1 (UCHL1), serves as a dual-regulator of the ubiquitin proteasome pathway, in which it controls cytosolic protein stability by transferring ubiquitin directly to protein substrates and releasing ubiquitin from repeatedly conjugated ubiquitin monomers (Xiang et al., 2012). Xiang and coworkers (2012) also recognized that UCHL1 displayed tumour suppressor characteristics by deregulating ubiquitylation of p53 while additionally stimulating p53, consequently preventing cell replication and bringing about apoptosis in cancerous cells.

Amongst the most common cancers to affect females, breast carcinoma is the leading cancer associated with mortality. While major advancements have been achieved in attempting to understand, diagnose and manage breast cancer, early detection methods are still severely restricted (Xiang et al., 2012) and thus still leaves clinicians with the challenge of effective and non-invasive therapy.

1.2 Breast cancer

Jemal and associates (2011) reported that the leading cause of cancer deaths in females in developing countries had shifted from cervical to breast cancer in recent years. Mortality associated with breast cancer accounts for 14% of all diagnosed cancer cases. In 2008, the World Health Organization (WHO, Globocan) reported that breast cancer statistics were at an 8.4% mortality rate and accounted for 11.3% of all cancer cases reported.

Characteristically, there are two types of breast cancer of which ductal carcinoma is the most common. This type of cancer originates in the milk ducts of breast tissue. The other form, lobular carcinoma, may originate in the breast lobules, which produces the milk. Rarely does the cancer originate from other areas in the breast tissue. Breast cancer may be classified either as invasive or non-invasive depending on the nature of the tissue. Non-invasive carcinomas refer to cancerous cells which have not invaded surrounding healthy tissues and are referred to as “in situ”. In situ carcinomas, if left untreated, have the ability to progress into an invasive cancer. The most aggressive of the breast cancer types is lobular carcinoma in situ (LCIS), which is also a marker for an increased risk of malignancy. However, ductal carcinoma in

situ (DCIS), found in the lining of the milk ducts, only become progressive if left untreated (Dervan, 2001and Löwy, 2010).

Breast cancer progression is also characterized by surface receptors exhibiting hormonal sensitivity, or an increase in gene expression by the cancer cells. Estrogen receptor-positive cancer (ER-positive cancer), are cancers with an increased expression of estrogen receptors on the cell surface. This results in an increased affinity for estrogen and thus increases the rate of progression of the cells. HER2-positive cancer is an aggressive form of breast carcinoma due to the upregulation of the HER2 gene expressed in the cell. HER2 is a gene which assists in cellular replication and cellular repair. Women who suffer from this form of breast cancer have the highest incidence for recurrence of disease (Liu et al., 2012).

Breast cancer is diagnosed in stages, each stage signifying the progression of the cancer and consequently affects the treatment options. Diagnoses at stages 0, I, II, or III, are usually prescribed with treatment to eradicate and prevent relapses. At stage IV, practitioners are only able to assist in prolonging and increasing the current quality of the patients life (Dervan, 2001 and Löwy, 2010).

Therapy is usually dependent on the stage of invasion and may include surgery, lumpectomy or mastectomy, radiation and chemotherapy. Radiation and surgery are considered local treatments in which only a designated area is targeted. Chemotherapy however, is a systemic treatment option and affects the whole body (Dervan, 2001 and Löwy, 2010).

A hormonal therapeutic approach is employed in ER-positive cancers, in which Tamoxifen is the hormone used to inhibit estrogen from binding to the receptor,

thereby decreasing progression of the cancer. Another estrogen inhibitory drug prescribed for ER-positive cancer is exemestane, a class of aromatase inhibitors, which is prescribed for post-menopausal women (Gross et al., 2011). As for HER2 positive cancers, trastuzumab (Herceptin®) is used in their treatment. Chan and co-workers (2005) reported that patients who had no prior chemotherapy treatment had a response rate of 23-26% when treated with trastuzumab.

1.3 Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a large membranous organelle that interconnects the plasma membrane, the cytoplasm and the nucleus (Fig. 2). The ER has multiple major physiological functions and is the site for the biosynthesis of proteins, sterols, lipids and glycoproteins (Yoshida, 2007 and Xu et al., 2005). Newly synthesised polypeptide chains are folded and assembled in the ER lumen with the aid of molecular chaperones (Wang et al., 2010). These quality control processes ensure the proper protein folding, post-translational modifications and conformation of secretory and trans-membrane proteins. Unfolded or improperly folded proteins are restricted from exiting and held within the ER for proteasomal degradation via ER-associated protein degradation (ERAD). The ER lumen also stores intracellular Ca^{2+} needed for many cellular signalling processes (Schröder, 2007) such as signal transduction (Takadera et al., 2007).

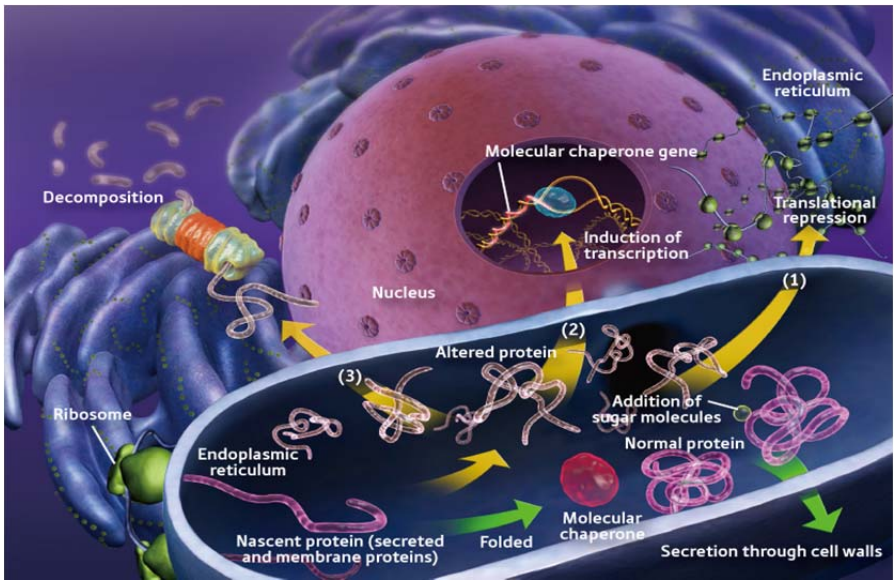

Fig. 2. Endoplasmic reticulum activities and responses to stress (http://www.rikenresearch.riken.jp/eng/frontline/6085).

1.3.1 ER stress

ER stress is triggered when any of the ER functions fail to ensure homeostatic (steady-state) levels between properly folded and misfolded proteins. Accumulation of inadequately folded proteins interferes with the ER folding capacity leading to cellular proteotoxicity. The protein folding apparatus has multiple chaperones, e.g., Hsp70, Hsp90 and lectin. Each chaperone works in a hierarchical manner in which, for example, Hsp70s are involved with completely unfolded or unstructured polypeptide chains, while Hsp94 and lectin are more focused on partly folded proteins (Schröder, 2007). Hsp70 chaperone, such as glucose-regulated protein 78/GRP78, immunoglobulin heavy chain binding protein/BiP (GRP78/BiP) has a higher affinity for unfolded polypeptides bound to ADP than those bound to ATP. Thus, ATP-ADP exchange is required during these mechanisms, or unfolded chains will accumulate and cause ER stress (Schröder, 2007). GRP78 is a protein released due to the chronic

stimulus of ER stress and promotes cell survival, proliferation and shelters cells from certain chemotherapeutic drugs (Hsu et al., 2009).

As ER proteins increase during stress, GRP78 expression is also upregulated. This upregulation can function as an anti-apoptotic trigger of GRP78 formation, prevent the activation of caspase cascades and function as a protector against apoptosis (Hsu et al., 2009). The accumulation of perturbant ER proteins leads to stress conditions within the organelle and persistence of this stress may result in the ultimate suicide of the cells (Nakayama et al., 2009). Unfolded proteins may also accumulate when disruptions to the Ca^{2+} gradient occurs. ER stress, in turn, activates the unfolded protein response (UPR) (Schröder, 2007), which attempts to alleviate ER stress (He et al., 2009). Eukaryotic cells, in particular mammalian cells, have receptors that monitor ER stress and protein accumulation. These receptors are PRK-like ER resistant kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1) (Wang et al., 2010). The ER stress triggers a homeostatic mechanism to relieve the stress caused by the accumulation of misfolded proteins, by facilitating the removal of perturbant proteins from the ER via the UPR (He et al., 2009).

1.4 Nicotine

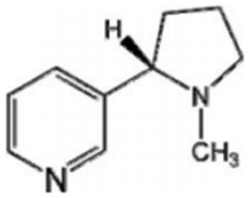

Fig. 3. Molecular structure of (-).(S)-nicotine (Clayton et al., 2010)

Nicotine is the principal toxic alkaloid in tobacco and cigarette smoke. This alkaloid ($C_{10}H_{14}$ N_2) has a molecular weight of 162.23 g/mol and functions as a tumour promoter by disturbing cellular progression though the cell cycle (Zeidler et al., 2007). This compound is extracted from the tobacco plant which is part of the *Solanoceous* plant family, also included in this family are the potato, green pepper and tomato plants (Yildiz, 2004). Nicotine is known for stimulating transcription factors, such as *c-fos and c-Jun*, suppressing apoptosis, promoting cell proliferation and expressing heat shock proteins (Hsp60, Hsp70 and Hsp90) (Klein et al., 2001, Dasgupta et al., 2009). It has also been reported to exert multiple effects on normal and tumour tissues, including lipid peroxidation, depletion of antioxidant status via reactive oxygen and nitrogen species and upregulation of matrix metalloproteinases (Balakrishnan and Menon, 2007).

This addictive and well documented notorious component of cigarettes is considered to be a ‘tumour enhancer’. It down-regulates important biological functions such as angiogenesis, and cell-mediated immunity, it also displays ‘protective’ characteristics such as anti-apoptosis. Nicotine binds to the nicotinic acetylcholine receptor (nAChR)

on neural and non-neuronal tissues. nAChRs are ligand-gated ion channels abundantly found within the brain (Zeidler et al., 2007). Nicotine has the ability to cross the blood-brain barrier (Yildiz, 2004) and, therefore, is regularly associated with studies regarding neural diseases such as Alzheimer's and Parkinson's.

Stereoisomeric forms of nicotine are important for the effectiveness and potency of the drug in biological systems. Nicotine has two subtypes, (2)-(S)-nicotine (Fig. 3), and (1)-(R)-nicotine, of which the former is the prevalent and potent alkaloid in tobacco (Clayton et al., 2010).

1.4.1 Metabolism of Nicotine

Nicotine is absorbed into the bloodstream in the pharmacologically active (S)-nicotine form. In the liver, it is converted to its major metabolites, namely; cotinine, nicotine N'–oxide (NNO), nicotine glucuronidate, (S)-nicotine-N-glucuronide (nicotine-Gluc) and nornicotine. This compound is converted by P450 cytochrome (CYP) enzymes to iminium ion, which is subsequently transformed to cotinine by means of the enzyme aldehyde oxidase (AOX). It can further be altered into NNO via flavin containing monooxygenase 3 (FMO 3), which can be converted back to nicotine and reused by the body (Benowitz et al., 2009). Nicotine is also converted to Nicotine-Glu from N-quaternary glucuronide, via the enzymatic reaction between nicotine glucuronidation and uridine diphosphate-glucuronosyltransferase (UGT) (Benowitz et al., 2009). Cotinic metabolites are readily excreted in the urine (Klein et al., 2001). The primary and secondary identified metabolites excreted by smokers are 3'-hydroxycotinine (3HC), and glucuronide conjugate (3HC-Gluc), respectively (Klein et al., 2001).

Between 40 and 60% of the nicotine intake is detected as 3HC and 3HC-Gluc in the urine and only 1% from norcotinine (Klein et al., 2001).

1.4.2 Nicotine and cancer

Nicotine oxidation is primarily catalysed by CYP P450 enzymes (Fig. 4) via 5'hydroxylation to yield an unstable intermediate metabolite 5′-hydroxynicotine that exists in equilibrium with nicotine-1′(5′)-iminium ion (Benowitz and Jacobs III, 1994). However, Hecht and co-workers (2000), demonstrated that CYP2A6 catalyses 2′-hydroxylation of nicotine to yield a significant amount of a lung cancer-precursor, aminoketone. Amino-ketone is the direct precursor to the tobacco-specific lung carcinogen 4-(methylnitros-amino)-1-(3-pyridyl)-1-butanone (NNK) (Caldwell et al., 1993). These findings provided the potential relationship between nicotine metabolism and cancer in mammals.

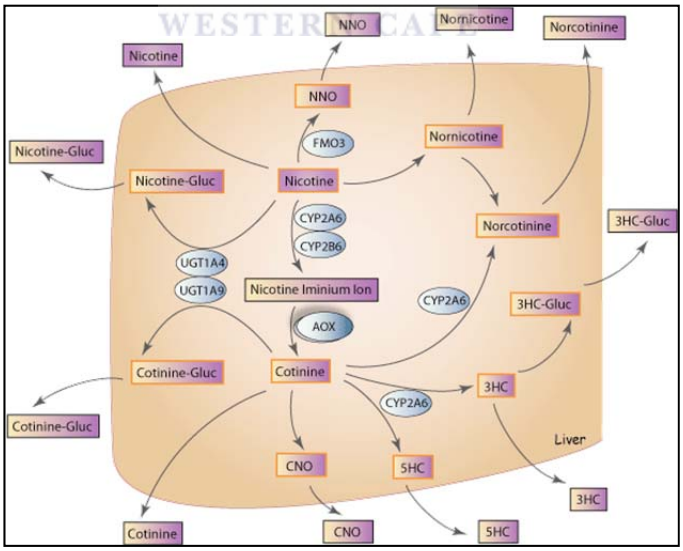

Fig. 4. Schematic representation of nicotine metabolism within the liver (http://www.pharmgkb.org/pathway/PA2011)

1.4.3 Basic mechanism of action

As mentioned previously, this compound binds to the nAChRs on neuronal and non-neuronal cells (Zeidler et al., 2007). It also (as part of its mechanism of action) affects the ubiquitin-proteasome protein degradation pathway components such as ubiquitin-conjugating enzymes, 20S and 19S proteasomal subunits, and chaperonin-containing TCP-1 protein (CCT) complexes (Kane et al., 2004) involved in reduction of the cytotoxic effects of DNA-damaging agents, such as cisplatin, UV and gamma radiation. It decreases the c-Jun N-terminal kinase (JNK) and GADD153/CHOP (growth arrest and DNA damage-inducible protein, also called CHOP) cell death signalling pathways (Onoda,et al., 2001), activates NF-κB and GRP78/BiP, and induces apoptosis via Hsp90 (Paleari et al., 2009 and Crowley-Weber et al., 2003).

1.4.4 Synergistic and antagonistic effects of nicotine

Recent studies reported that nicotine, when combined with various chemotherapeutic drugs, inhibited the toxicity of the drugs it was combined with (Zhou et al., 2007). Chronic exposure to nicotine inhibits arachidonic acid-, glutamate-, and b-amyloid- and tunicamycin (Tm)-stimulated neurotoxicity (Zeidler et al., 2007). Multiple nAChRs subtypes, such as, a4b2 and a7, have been proposed to be involved in these protective characteristics of nicotine (Zeidler et al., 2007). Due to the anti apoptotic properties of nicotine, diseases such as Alzheimer's and Parkinson's that are dependent on unfolded protein accumulation and ER stress, benefit from nicotine as an adjunct treatment (Sasaya et al., 2008).

1.5 Tunicamycin

Tunicamycin (Tm) is a prototypic antibiotic (Fig. 5) which inhibits biosynthesis of N-linked oligosaccharides (Zhang et al., 2009) by the inhibition of UDP-N-acetylglucosamine: dolichol/polyprenol phosphate GlcNAc-1-P transferase (GPT) which inhibits the primary step of protein N-glycosylation (Price and Tsvetanova, 2007). It has a molecular weight of 718.9 g and a molecular formula: $C_{30}H_{46}N_4O_{16}$.

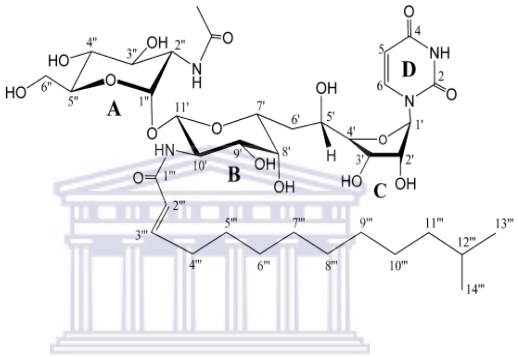

Fig. 5. Structure of Tm numbering system (Price and Tsvetanova, 2007)

Tm is composed of a four ring system (A, B, C, and D), with ring D containing uracil and ring C N-glycosidically linked to a D-pseudoribose. 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) (Price and Tsvetanova, 2007).

1.5.1 Metabolism of Tunicamycin

The biosynthesis of Tm lies at tunicamine, the 11 carbon dialdose sugar (link BC) and the αβ1- 11″-glycosidic bound GlcNac (link B and A) (Tsvetanova et al., 2002). The bound GlcNAc resembles the GPT, since Tm is a transition state analog of GPT

(Price and Tsvetanova, 2007). Rings BCD (*N*-acetyl-tunicamine-uracil) is prospectively produced by the ligation (incorporation) of uridine-5-aldehyde (I) with an unsaturated 5,6-ene sugar nucleotide, UDP-4-keto-Glc(Gal)NAc-5,6-ene (II). However, uridine-5-aldehyde (I) is not synthesized during primary metabolism of Tm, which presumably occurs either as an intermediate during the biosynthesis of *Streptomyces sp.* antibiotics or from oxidation of uridine via 5-dehydrogenase or oxidase activity (Price and Tsvetanova, 2007).

1.5.2 Tunicamycin and ER stress

Hasegawa et al. (2008) and Jung et al. (2012) reported that Tm induced apoptosis via tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Delom et al. (2007) further substantiated these findings by reporting that MCF-7 cells were resistant to Tm-induced apoptosis due to the lack of caspase-3 expression. However, they all reported that apoptosis induced by Tm was enhanced with the addition of TRAIL and caspase-3. Apoptosis in Tm-induced cells were as a result of the inhibition of glycosylation and accumulation of unfolded and misfolded proteins causing the cell to undergo ER stress (Sasaya et al., 2008). It is this inhibition of newly synthesised proteins that may lead to aberrant post translational modifications of important proteins, and ultimately creating ER stress (Yaun et al., 2007). The ER stress triggers a homeostatic mechanism by facilitating the removal of those perturbant proteins. This mechanism is known as the unfolded protein response (UPR) (He et al., 2009). Inadequate UPR may lead to increased cytosolic proteins and cause apoptosis in various cell types (Nakayama et al., 2009) (Fig. 6).

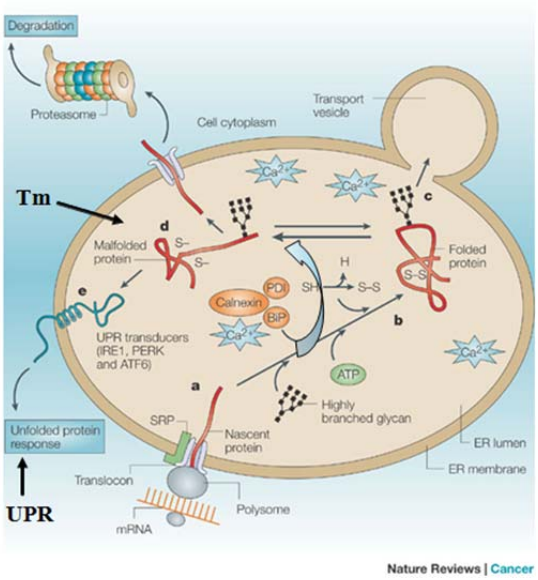

Fig. 6. The effect of tunicamycin in the ER

(http://www.nature.com/nrc/journal/v4/n12/fig_tab/nrc1505_F1.html)

1.6 Thapsigargin

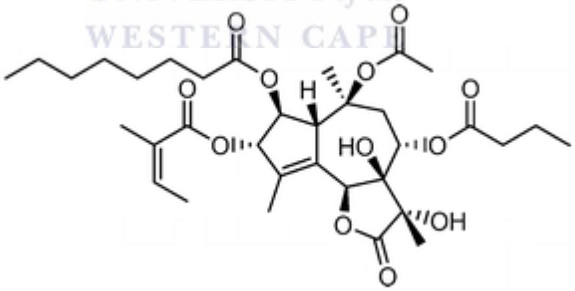

Fig. 7. Chemical structure of Thapsigargin (Ball et al., 2007)

Thapsigargin (Tg) has a molecular weight of 650.75 g and the molecular formula, $C_{34}H_{50}O_{12}$ (http://pubchem.ncbi.nlm.nih.gov). Important cellular functions such as signal transduction rely on regulation of calcium ions by the ER. Tg inhibits the Ca^{2+}ATPase and causes an increase in cytosolic Ca^{2+} (Takadera et al., 2007, Thastrup et al., 1990).

Thus, this Ca^{2+} flux perturbant may be used to induce ER stress (Zhang et al., 2009, Shu et al., 2008 and Sasaya et al., 2008). Tg does not only act on the ER and intracellular Ca^{2+} signalling pathways (Ball et al., 2007), but has the ability to diffuse across cell membranes and inhibit the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) (Shimoke et al., 2005). Intracellularly, Tg rapidly increases cytosolic sequestered Ca^{2+} by overstimulation of the ER, Ca^{2+} permeates through Ca^{2+} pores and with it releasing, inositol 1,4,5 trisphosphate and guanosine-5'-triphosphate (GTP) (Thastrup et al., 1990). Due to Tg's lipophilic nature, it converts into a structure with poor affinity for Ca^{2+} and ATP. The weakened affinity for Ca^{2+} inhibits the sequestering of intracellular Ca^{2+} and consequently, a secondary accumulation of extracellular Ca^{2+} would occur, thus causing ER stress and leading to apoptosis (Ball et al., 2007). Some neurological diseases may be linked to uncontrolled Ca^{2+} secretion such as stroke and Alzheimer's disease (Takadera et al., 2007).

1.6.1 Metabolism of Thapsigargin

Thapsigargin is a sesquiterpene lactone isolated from the umbelliferous plant *Thapsia garganica L.* (*Apiaceae*), which is largely distributed in the Mediterranean, on the Spanish peninsula, and in North Africa. Tg was identified as its principle skin-irritating compound. Interestingly, Tg is reported to have been used in Arabian folk medicine as a counter-irritant (Christensen and Norup, 1985). Sesquiterpene lactones belong t a class of chemical compounds which contain a lactone ring, found in many plants, known to cause allergic reactions and toxicity at high doses, particularly in grazing livestock

(http://www.ansci.cornell.edu/plants/toxicagents/sesqlactone/sesqlactone.html).

The complete biosynthesis of thapsigargin has not as yet been elucidated. However, there is a proposed biosynthetic pathway (Drew et al., 2009). Biosynthesis is initiated with common terpene farnesyl pyrophosphate controlled by the enzyme, germacrene B synthase. Subsequently, the C(8) position is activated for an allylic oxidation due to the position of the double bond. This is followed by the addition of the acyloxy moiety by a CYP450 acetyltransferase. The lactone ring is then formed by a cytochrome P450 enzyme using $NADP^{+}$. However, because of the butyloxy group on the C(8), the formation will only generate the 6,12-lactone ring. An epoxidation step that initiates the last step of the base guaianolide formation uses a CYP450 enzyme that closes the 5 + 7 guaianolide structure. The ring closing is critical, in order to retain the 4,5 - double bond needed in thapsigargin. It has been reported that several of these enzymes are CYP450's and therefore oxygen and NADPH are likely to be crucial for its biosynthesis as well as other cofactors such as Mg^{2+} and Mn^{2+}(Drew et al., 2009).

1.6.2 Thapsigargin and ER stress

Tg inhibits SERCA, and blocks the release of Ca^{2+} from the ER lumen and decreases the affinity of GRP78/BiP for Ca^{2+} and its ER chaperone anti-apoptotic effects (Shu et al., 2008, Zhang et al., 2009 and Sasaya et al., 2008). Increases in intracellular Ca^{2+} concentration lead to the accumulation of incompetent proteins and activation of caspase-3-mediated apoptosis (Takadera et al., 2006) by means of the ubiquitin proteasome system (UPS). Tg has the ability not to activate protein kinase C or inhibit protein phosphatase, yet it is a naturally occurring sesquiterpene. Thastrup and co-workers (1990) reported that Tg acts by directly inhibiting ER Ca^{2+}-ATPase resulting in the release of all sequestered Ca^{2+} as well as, Ca^{2+} (ATP-dependent) from

microsomal membrane vesicles (Thastrup et al., 1990). Tg could, therefore, provide a valuable tool against breast cancer by altering the cell's Ca^{2+} levels and inducing cellular arrest.

The aim of this study was to investigate the effects of tunicamycin (Tm), thapsigargin (Tg) and nicotine (Nic) on growth and apoptosis of human breast carcinoma (MCF-7) cells. The objectives included (i) determining the effects of the compounds at selected concentrations on cell viability, ATP concentration, DNA proliferation and early apoptosis at 24, 48 and 72 hrs, (ii) to analyse the combinatorial effects of Nic, Tm and Tg on the above parameters in order to counter the pro-apoptotic properties of nicotine and simultaneously support apoptosis in MCF-7 cells.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

Human breast cancer (MCF-7) cells were donated from the Human Biology Department at The University of Cape Town and placed in cell culture which were maintained in Dulbecco's modified Eagle medium (DMEM)/Nut medium (Mix F–12 with glutamax) (Invitrogen, Germany), supplemented with 10% Foetal bovine serum (FBS) (Invitrogen, Germany) and 1% penicillin, streptomycin (pen-strep) (Sigma-Aldrich, SA), at 37°C, 5% CO_2 in a humidified atmosphere. Before conducting experiments, cells were washed in 1X phosphate buffered saline (PBS) (Invitrogen, Germany). Adherent cells were brought into suspension by trypsinating with 0.25% trypsin/EDTA (Invitrogen, Germany).

Suspended cells were used to conduct the following experimental procedures: Cell viability was determined by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (CAS: 298-93-1; Sigma, SA). ATP production was determined using Promega CellTiter Glo® Luminescent Cell viability assay (Cat# G7571) (Promega, SA). DNA proliferation was assessed by means of BrdU Cell proliferation ELISA Chemiluminescence assays (Cat# 11669915001) (Roche, Germany). Early apoptosis was investigated using, Annexin V:FITC (cat# AN300FITC) (AbDserotec; Europe). MCF–7 cells were exposed to various concentrations of tunicamycin (CAS: 11089-65-9) (Sigma, SA), thapsigargin (CAS: 67526-95-8) (Sigma, SA) and nicotine (CAS: 54-11-5) (Merck SA).

All assays, except early apoptotic detection were conducted in flat-bottomed 96-well microtiter plates (LASEC and Biosmart, SA) and absorbance and luminescence were read using the Glomax multidetection system. (Serial No: 51187002). Early apoptosis was analysed by means of flow cytometry using the Becton Dickinson FACSCalibur (serial no: E2023) flow-cytometer with a 488 nm Coherent laser. The software used for the acquisitioning of the data, was Cellquest Pro version 5.2.1.

2.2 Methods

2.2.1 Cell Viability Assay using 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)

MTT is a colourimetric assay used to detect cell viability. It uses a tetrazolium salt, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, which is reduced to purple formazan crystals (1-[4,5-Dimethylthiazol-2-yl]- 3,5-diphenylformazan) in living cells. The tetrazolium salt is cleaved in the mitochondria of living/viable cells where the redox reaction transforms the salts into formazan via the 'succinate-tetrazolium reductase' system. The formazan crystals produced by the cells are a direct indication of the quantity of metabolically active and viable cells (Mosmann, 1983). The formazan product was solubilised in 99% isopropanol and measured spectrophotometrically at a wavelength of 560 nm.

MCF-7 cell suspension of 5000 cells in 100 µl, was added to each of the experimental wells of a clear 96-well microtiter plate. Cells were allowed to attach by incubating for 24 hours at 37°C in a 5% CO_2 humidified atmosphere. After 24 hours, various concentrations of drugs were prepared on the day in pre-warmed supplemented media. Supernatant from experimental wells were aspirated and cells were treated with 100

µl of the compound dilutions. Controls, ran in parallel to the experimental wells, and contained only media (blanks). The concentrations of the pure compounds (Table 1) and the combinations of these compounds are presented in Tables 2 and 3.

Table 1: Pure compound concentrations (µM) used for cell viability determination (n≥6)

Pure Compounds		
Tunicamycin	**Thapsigargin**	**Nicotine**
0.0001	0.0001	0.0001
0.001	0.001	0.001
0.0039	0.01	0.01
0.0078	0.0125	0.1
0.01	0.025	1
0.0156	0.05	10
0.03125	0.1	100
0.0625	0.2	1000
0.1	0.3	
0.125	0.4	
0.25	0.8	
0.5	1	
1		

Table 2: Selected concentrations (μM) of nicotine in combination with tunicamycin and thapsigargin (1:1) ratio used for cell viability determination (n≥6)

Nicotine Combinations	
Tm : Nic	**Tg : Nic**
0.0001	0.0001
0.001	0.001
0.01	0.01
0.1	0.1
1	1
10	5
100	50
200	
300	
400	
500	
1000	

Tm = Tunicamycin, Tg = Thapsigargin, and Nic = Nicotine

Table 3: Selected combinatorial concentrations of tunicamycin and thapsigargin (μM), using concentrations below IC_{50} for cell viability determination (n≥6)

Tm : Tg combination	
0.105 Tm : 0.03 Tg	0.06 Tg : 0.0525 Tm
0.105 Tm : 0.06 Tg	0.06 Tg : 0.105 Tm
0.105 Tm : 0.12 Tg	0.06 Tg : 0.21 Tm

Fixed concentration of 0.105 μM Tm was combined with various Tg concentrations. Fixed concentration of 0.06 μM Tg was combined with the various Tm concentrations.

Cells were treated (with the various compounds mentioned in Tables 1, 2 and 3) and incubated for 24, 48 and 72 hours. Subsequently, 10 μl of MTT (5 mg/ml) was added to each well, including controls and media-only wells (blanks) in minimal amount of light, and incubated at 37°C, in 5% CO_2 for 2 hours. After the 2 hr incubation, supernatants were removed and 200 μl of 99 % isopropanol added to each well to dissolve the formazan crystals. The microtiter plate was placed on an orbital shaker with a microtiter plate adaptor for 25 min. Absorbance was read at 560 nm using the Glomax multidetection system.

$$\%\, Viability = \frac{Experiment\ -\ Blank}{Control\ -\ Blank} \times 100$$

2.2.2 Cell Titer Glo® Luminescent Cell Viability Assay

This assay is used for high throughput screening, proliferation and cytotoxicity determinations. It is accurate and robust, in which the phenol red containing medium does not interfere with luminescent output. Temperature, however, greatly influences the rate of enzymatic reaction and, in turn, affects light intensity output. The assay determines cell viability via the detection of ATP activity and relies on the thermostable luciferase reaction. This single step assay involves adding a single reagent, causing cell lysis and generation of the luminescent signal, in the presence of ATP, Mg^{2+}, and molecular oxygen (Fig. 8).

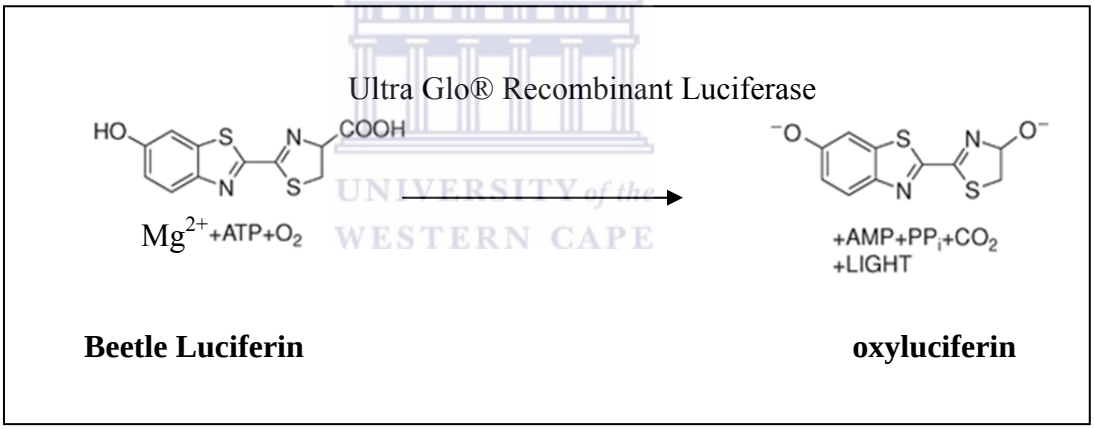

Fig. 8. The enzymatic reaction of luciferin in the presence of Mg^{2+}.

The selected drug concentrations used for the ATP assay were based on the IC_{50} concentrations calculated from the viability assay results. Concentrations selected for the pure compounds and the combinations of these compounds were as follows:

Table 4: Pure compound concentrations (μM) used for the determination of ATP and DNA proliferation (n≥6)

Pure Compounds		
Tunicamycin	**Thapsigargin**	**Nicotine**
0.25	0.05	0.0001
0.5	0.1	0.001
1	0.2	0.01
1.5	0.3	0.1
2	0.4	1

Table 5: Nicotine in combination with tunicamycin and thapsigargin (1:1 ratio) concentrations (μM) used for the determination of cellular ATP and DNA proliferation (n≥6)

Nicotine Combinations	
Nic : Tm	**Nic : Tg**
0.01	0.01
0.1	1
1	5

Tm = Tunicamycin, Tg = Thapsigargin, and Nic = Nicotine

Table 6: Selected combinatorial concentrations of tunicamycin and thapsigargin (μM), using concentrations below IC_{50} for the determination of cellular ATP and DNA proliferation (n≥6)

Tm : Tg combination	
0.105 Tm : 0.03 Tg	0.06 Tg : 0.0525 Tm
0.105 Tm : 0.06 Tg	0.06 Tg : 0.105 Tm
0.105 Tm : 0.12 Tg	0.06 Tg : 0.21 Tm

Fixed concentration of 0.105 μM Tm was combined with various Tg concentrations. Fixed concentration of 0.06 μM Tg was combined with the various Tm concentrations.

The experiment was conducted by seeding 5000 cells in 100 μl, into each well of white, flat-bottom 96-well microtiter plates. Following the addition of the selected drug concentrations, the cells were incubated at 37°C, in 5% CO_2 for 24, 48 and 72h. The thawed Cell Titer Glo® buffer was transferred into the amber substrate bottle in order to reconstitute the lyophilized enzymes to obtain a homogenous solution as per the instructions of manufacturer. Prior to the addition of the reconstituted substrate, it was allowed to equilibrate to room temperature (RT) (±22°C).

The microtiter plate containing the compound-exposed cells was allowed to equilibrate for 30 min at RT, non-sterile in minimal light exposure. 100 μl of reconstituted substrate was added to each of the wells, including the controls and the blanks. The microtiter plate was incubated for a further 2 min at RT with minimal light exposure before placing it on an orbital shaker for an additional 3 min. Luminescence was detected using the Glomax multidetection system.

2.2.3 DNA Proliferation Detection using 5-bromo-2`-deoxyuridine (BrdU) Chemiluminescent Assay

DNA replication is a pivotal part of cellular survival. It identifies the proliferation activity of the compound induced cell. Screening for DNA production over time in the presence of cytotoxic or genotoxic compounds, would not only signify proliferation rates, but also allow for the examination of DNA synthesis and regulation. [^{3}H]-thymidine conventionally binds to DNA of proliferative cells. In this assay, the nonradioactive analogue 5-bromo-2'-deoxyuridine (BrdU) is used for the purpose of detecting DNA proliferation. This assay integrates the pyrimidine analogue, BrdU, rather than thymidine into the DNA of cells within the S phase of the cell cycle. The BrdU isotope is detected using ELISA techniques.

MCF-7 cells were cultured into sterile flat bottom, black 96-well microtiter plates with a cell density of 5000 cells per well and incubated for 24, 48 and 72 hours at 37°C, 5% CO_2. After which, compound concentrations and combinations (as described in Tables 4, 5 and 6) were freshly prepared and 100 µl added to their designated wells, while control cells only received supplemented media. Reagents were reconstituted as instructed by the manufacturer.

Preparation of Working Solutions (as per manufacturer's instructions)

BrdU labelling solution	BrdU labelling reagent was diluted 1:100 with sterile culture medium (final concentration: 100 µM BrdU). (For each well, 10% labelling solution was added to existing well content).

Anti-BrdU-POD stock solution	Anti-BrdU-POD was dissolved in 1.1 ml of double distilled water.
Anti-BrdU-POD working solution	Anti-BrdU-POD stock solution was diluted 1:100 with antibody dilution solution. (100 µl of anti-BrdU-POD working solution was added to each well).
Washing solution	washing buffer concentrate (10X PBS) was diluted 1:10 with double distilled water. (Each well was washed thrice with 200 µl of washing solution).
Substrate solution	Tetramethylbenzidine (TMB) was reconstituted at a ratio of 1:100. The mixture was allowed to equilibrate at RT for at least 15 min before adding 100 µl of the substrate to each well.

After the required incubation period, 10% labelling solution was added to all wells including controls and blanks (media-only wells). The microtiter plate was incubated for 2 hrs at 37°C, 5% CO_2. After 2 hrs, the media containing labelling solution was aspirated and 100 µl of Fix Denat was added and incubated for a further 30 min at RT. Subsequently the Fix Denat was aspirated and anti-BrdU-POD added, the microtiter plate was incubated for an additional 90 min at RT. After the incubation period, the anti BrdU-POD was aspirated and cells were washed thrice with 200 µl of washing buffer per well. Concluding the assay, a 100 µl of reconstituted substrate was added to each well and the microtiter plate was placed on an orbital shaker for

approximately 3 min, after which luminescence was detected using the Glomax multidetection system.

$$\%\ DNA\ proliferation\ = \frac{Experiment\ -\ Blank}{Control\ -\ Blank} \times 100$$

2.2.4 Detection of Early Apoptosis using Flow Cytometry and Annexin V:FITC

Annexin V:FITC (fluorescein isothiocyanate) is a fluorescent quantitative method of detecting apoptotic cells in a population. Annexin V is an anticoagulant protein which binds to negatively charged phospholipids. During early apoptosis, phospholipids are disrupted exposing phosphatidylserine (PS) extracellularly. The FITC conjugated to the annexin binds to and labels the extracellularly exposed PS. Propidium iodide (PI) included in the kit, binds to cellular DNA of necrotic cells. Combining these stains, allows for the detection of percentage of live (negative) cells, cells undergoing early apoptosis, cells deadas a result of apoptosis as well as dead cells (PI positive) of one cell population (Sigma-Aldrich).

Cells $3x10^5$, were seeded in 25 cm^2 tissue culture treated flasks and allowed to attach for 24 hrs. Subsequently, the supernatant was aspirated and cells were treated with compound concentrations (single and combination) as described in the previous section (Tables 4, 5 and 6). After incubation of 24, 48 and 72 hrs, the supernatants were aspirated and transferred to labelled centrifuge tubes while attached cells were trypsinated. Once cells had detached, the trypsinated cell suspension was also added to the labelled centrifuge tube. The tubes were centrifuged at 2500 rpm for 2 min in order to pellet cells. Subsequently, the supernatant was aspirated and cells were resuspended in 0.5 ml binding buffer (100 mM HEPES/ NaOH, pH7.5 containing 1.4

M NaCl and 25 mM $CaCl_2$), and centrifuged at 2500 rpm for an additional 2 min. Cells were then resuspended in 195 µl of binding buffer and 5 µl of Annexin V (50 µg/ml), and allowed to incubate for 10 min at RT.

After the 10 min incubation period, the cells were centrifuged at 2500 rpm for 2 min at RT and the supernatant aspirated. The cells were then resuspended in 190 µl of binding buffer and 10 µl of PI (100 µg/ml) and placed on ice with minimal light exposure. The samples were analysed with the Becton Dickinson FACSCalibur (serial no: E2023) flow-cytometer with a 488 nm Coherent laser. The Cellquest Pro version 5.2.1 software was used for the acquisitioning of the data. The negative control contained unstained cells. The positive control contained 1 µM staurosporine, which when incubated for 3 hours, induced apoptosis, and the sample was prepared to contain only the Annexin V stain. Other controls included PI only stained cells, in which the Annexin V was omitted from the sample preparation.

2.3 Statistical Analysis

Results were analysed using GraphPad Prism 5 and MedCalc 12.2.1.0. The Mann-Whitney test was used for non-normally distributions of unpaired samples and $p<0.05$ denoted a significant event compared to controls.

CHAPTER 3

3. RESULTS

3.1 Effect of Nicotine on MCF-7 cells

3.1.1 Effect on cell viability

Table 7: The effect of selected nicotine concentrations on MCF-7 cell viability (Mean ± SEM)

Concentration (µM)	24 Hours	48 Hours	72 Hours
0.0001	90.261 ± 3.492	93.367 ± 4.203	114.952 ± 3.062
0.001	89.169 ± 0.361	96.935 ± 8.357	117.902 ± 3.263
0.01	73.459 ± 17.520	98.119 ± 2.975	111.397 ± 4.275
0.1	77.193 ± 0.528	75.480 ± 9.365	93.440 ± 2.326
1	114.305 ± 3.812	73.264 ± 4.701	105.418 ± 3.115
10	101.359 ± 3.254	95.320 ± 3.706	93.904 ± 4.283
100	98.890 ± 9.301	89.138 ± 2.941	102.528 ± 1.849
1000	95.005 ± 4.482	115.257 ± 5.926	120.859 ± 3.200

Table 7 displays the arithmetic mean and standard error of mean, of the % viability of cells treated with nicotine for 24, 48 and 72 hrs. MCF-7 cell viability remained above 73% for all time intervals tested. Comparison between individual time intervals showed that significant differences were only observed at 0.1 nM between 48 and 72 hrs (p=0.0105), and 24 and 72 hrs (p=0.0105). At 1 mM concentration of nicotine, a marked

difference was also observed between 24 and 48 hrs (p=0.0163) and 24 and 72 hrs (p=0.0039).

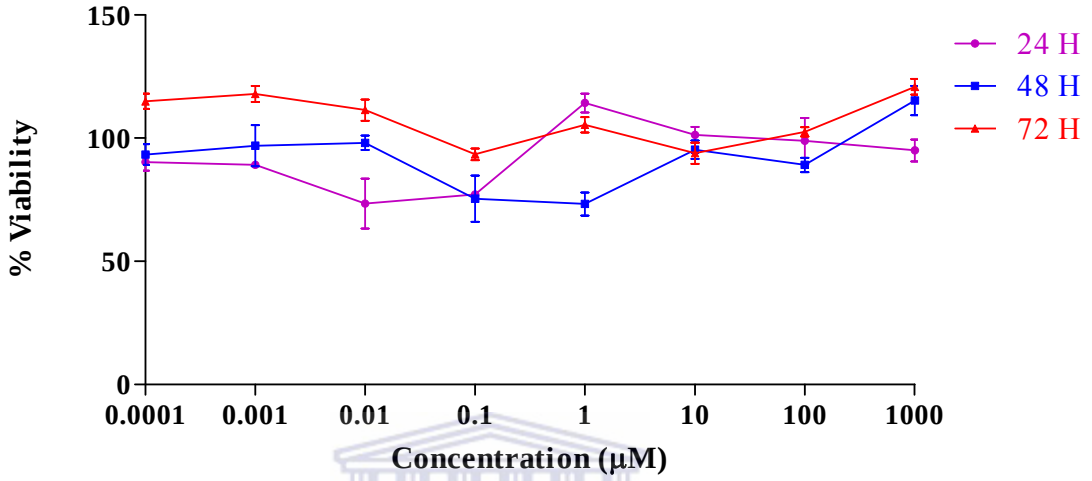

Fig. 9. Effects of various nicotine concentrations on MCF-7 cell viability.

Fig. 9 illustrates the effects of Nicotine on MCF-7 cells between 24, 48 and 72 hr incubation periods. Nicotine did not inhibit cell viability at all time intervals across all concentrations treated, except at 0.1 nM between 24 and 72 hrs (p=0.0105), and 48 and 72 hrs (p=0.0105), and at 1 mM between 24 and 48 hrs (p=0.0163) and between 24 and 72 hrs (p=0.0039).

3.1.2 Effect on ATP production

Table 8: The effect of selected nicotine concentrations on ATP production in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hours	48 Hours	72 Hours
0.0001	104.173 ± 1.627	107.884 ± 1.486	113.827 ± 2.542
0.001	105.093 ± 2.261	121.261 ± 0.744	116.158 ± 1.492
0.01	98.707 ± 2.838	117.916 ± 1.001	120.544 ± 1.760
0.1	105.865 ± 1.968	115.039 ± 1.314	118.939 ± 1.378
1	106.197 ± 1.576	113.216 ± 1.012	117.108 ± 0.722

Table 8 shows the effects of Nicotine on the ATP levels of MCF-7 cells. No difference was, observed between 0.1 nM and 1 µM at 24 hrs, 48 and 72 hr exposure. There was, however, a significant increase in ATP production between 24 and 72 hrs at all concentrations of Nicotine ($p \leq 0.0143$).

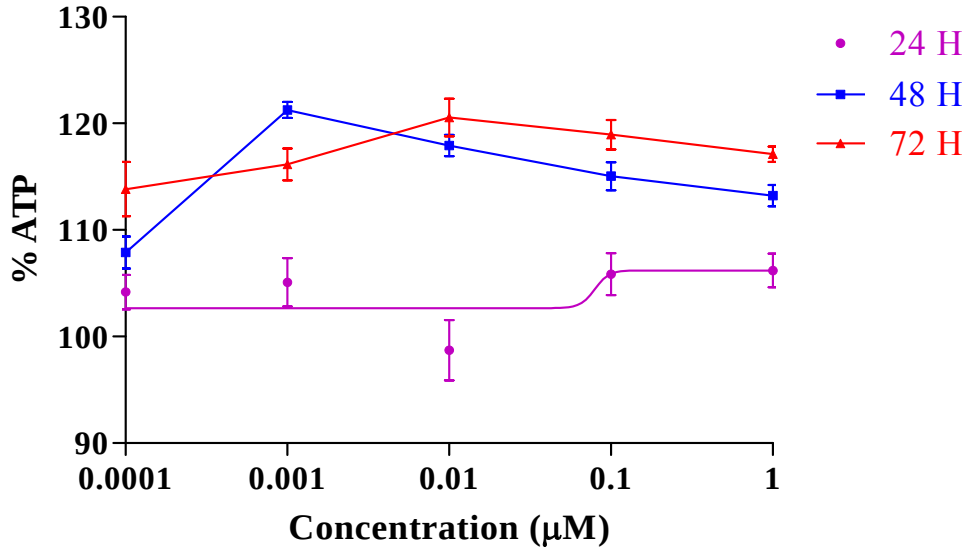

Fig. 10. The effects of selected nicotine concentrations on ATP production.

Though a significant increase in ATP production was noted between 0.1 nM and 1 nM (p=0.0079) at 48 hrs, ATP levels were similar and displayed similar trends at 24, 48, and 72 hrs at all other concentrations. % ATP production at 24 hrs was, however, markedly lower than those observed at 72 hrs.

3.1.3 Effect on DNA proliferation

Table 9: The effect of selected nicotine concentrations on DNA proliferation in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hours	48 Hours	72 Hours
0.0001	129.174 ± 7.276	113.552 ± 8.242	105.934 ± 26.652
0.001	169.801 ± 1.586	126.747 ± 6.511	143.693 ± 15.249
0.01	172.140 ± 3.026	143.712 ± 2.490	160.523 ± 13.366
0.1	159.631 ± 4.187	138.565 ± 6.145	162.259 ± 20.163
1	149.370 ± 1.819	129.408 ± 2.060	155.907 ± 15.587

Table 9 displays DNA proliferation by MCF-7 cells when exposed to various concentrations of Nicotine. A significant increase was only observed between 0.1 nM and 1 µM at 24 hrs (p<0.029). No differences were observed when comparing 24 and 48 hrs, 48 and 72 hrs and 24 and 72 hrs for all concentrations analysed.

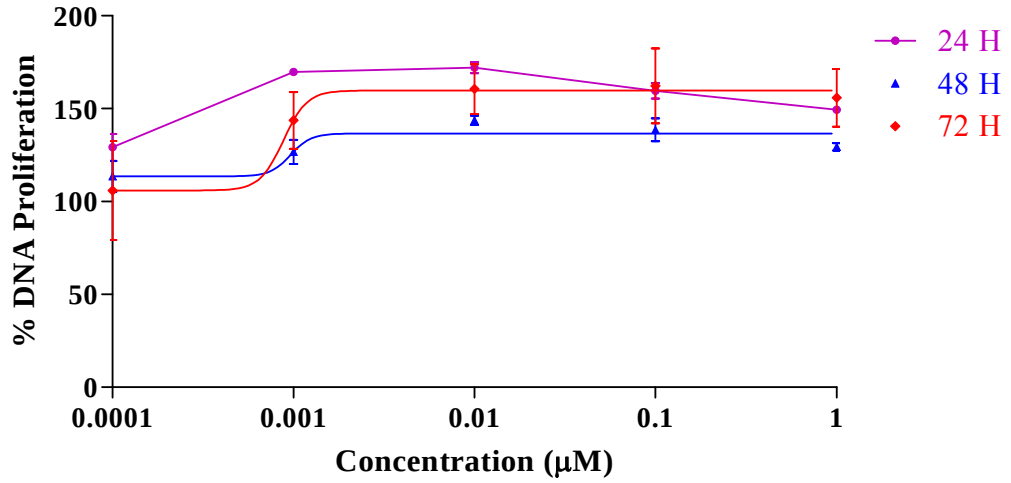

Fig. 11. The effect of selected concentrations of nicotine on DNA proliferation in MCF-7 cells at 24, 48 and 72 hrs.

Fig. 11 shows the effects of Nicotine on DNA proliferation in MCF-7 breast cancer cells. As the concentration increased, proliferation increased, however, proliferation reached a plateau at 0.01 µM at all time periods. Overall, the same trend was observed at 24, 48 and 72 hrs between 0.1 µM and 1mM.

3.1.4 Effect on cell viability, ATP and DNA proliferation

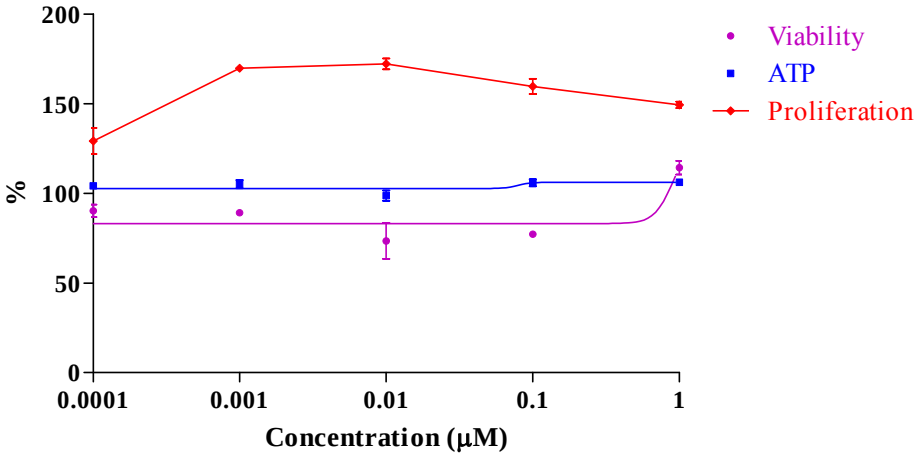

Fig.12. The effects of 24 hr nicotine exposure on cell viability, ATP and DNA proliferation.

Fig. 12 shows that nicotine in MCF-7 breast carcinoma cells at 24 hrs, resulted in unaffected % viability and ATP levels while DNA proliferation was significantly elevated significantly above 100% at all concentrations.

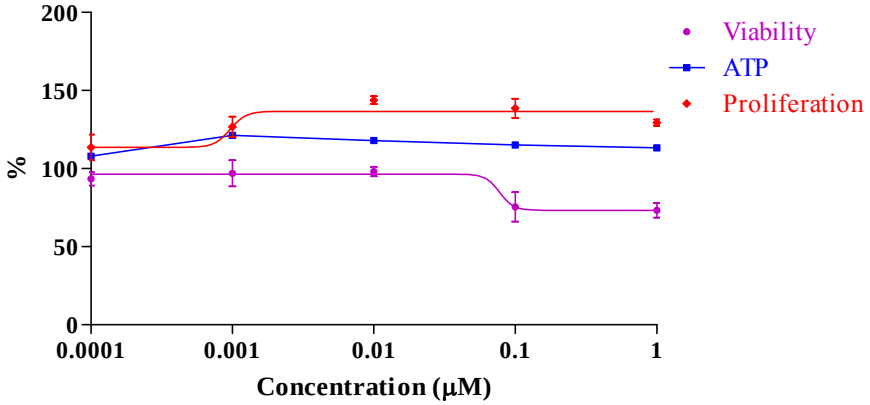

Fig. 13. The effects of 48 hr exposed MCF-7 cells to selected nicotine concentrations.

Fig.13 depicts the effects of nicotine after 48 hrs of exposure on MCF-7 cells. ATP activity and DNA proliferation remained consistently high even though viability had decreased between 0.1 and 1 µM.

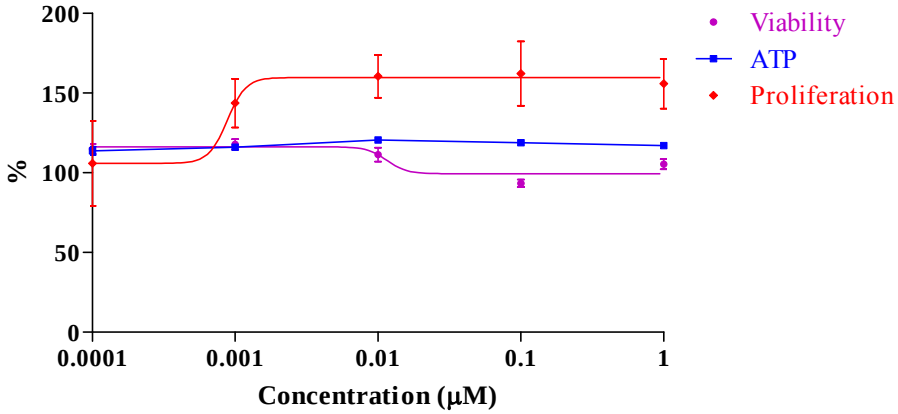

Fig. 14. The effect of nicotine induced MCF-7 cells after 72 hrs.

Fig. 14 indicates that viability and ATP activity remained the same after 72 hrs at all concentrations of nicotine, while, DNA proliferation increased and remained above 140% after 0.001 μM.

3.1.5 Early apoptotic effects on MCF-7 cells

Table 10: The effect of selected nicotine concentrations on early apoptosis in MCF-7 cells after 24 hrs of exposure (20 000 events analysed)

Concentration (μM)	Percentage			
	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.0001	0.09	0.53	98.24	1.15
0.001	0.77	0.77	95.58	2.89
0.01	0.09	0.66	98.31	0.93
0.1	0.1	0.59	98.19	1.12
1	0.07	0.54	98.16	1.23

Table 10 demonstrates that dead cell population, early apoptotic cells and dead apoptotic cells were unaffected. The % live cell populations obtained after 24 hrs, treated with nicotine, were unaffected at all concentrations when compared to controls.

Table 11: The effect of selected nicotine concentrations on early apoptosis in MCF-7 cells after 48 hrs of exposure (20 000 events analysed)

Concentration (µM)	Percentage			
	Dead Cells	**Dead Apoptotic Cells**	**Live Cells**	**Early Apoptotic Cells**
0	0.02	0.01	99.91	0.06
0.0001	0.35	1.6	95.83	2.22
0.001	0.55	1.42	96.48	1.55
0.01	1.09	2.3	94.96	1.65
0.1	0.35	1.81	95.19	2.65
1	0.5	1.63	95.42	2.45

Table 11 shows that nicotine after 48 hrs of exposure, did not result in changes in the number of early apoptotic cells. MCF-7 cells exposed to any nicotine concentration analysed were similar to controls.

Table 12: The effect of selected nicotine concentrations on apoptosis in MCF-7 cells after 72 hrs of exposure (20 000 events analysed)

Concentration (µM)	Percentage			
	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.0001	1.22	4.00	92.27	2.51
0.001	0.94	3.6	92.11	3.35
0.01	1.29	5.92	89.07	3.72
0.1	1.9	4.12	90.87	3.11
1	1.7	2.75	93.19	2.37

Table 12 demonstrated no differences in apoptotic and dead cell numbers at all concentrations. Thus, after 72 hrs, cells remain unaffected relative to control.

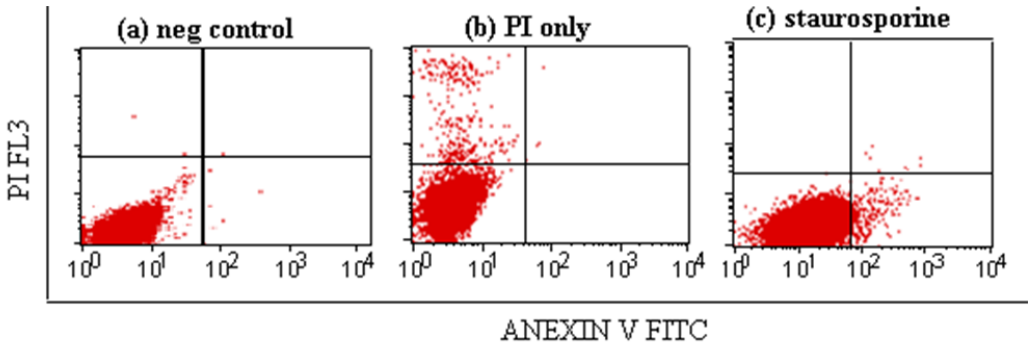

Fig. 15. Scatter plots created from the flow cytometry analysis represent the controls used for comparison of the effects of Nicotine at a) Unstained MCF-7 cells, b) PI only stained MCF-7 cells and c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

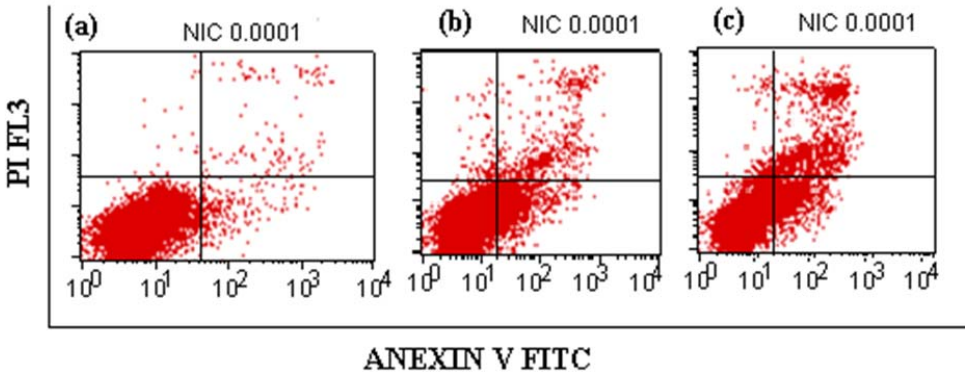

Fig. 16. Scatter plots obtained from flow cytometry analysis depict the effect of 0.1 nM of Nicotine on MCF-7 cells at a) 24, b) 48 and c) 72 hrs.

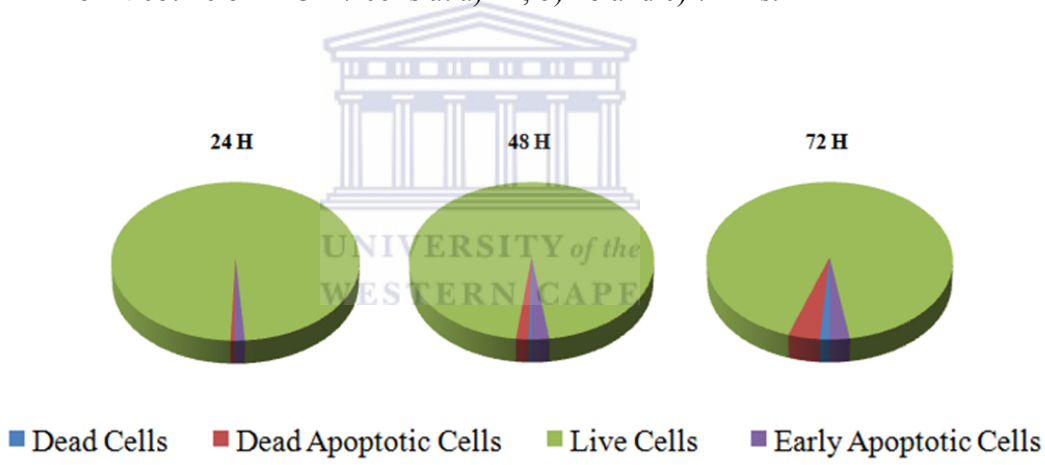

Fig. 17. The effects of 0.1 nM Nicotine on MCF-7 cells (data obtained from flow cytometry scatter plots).

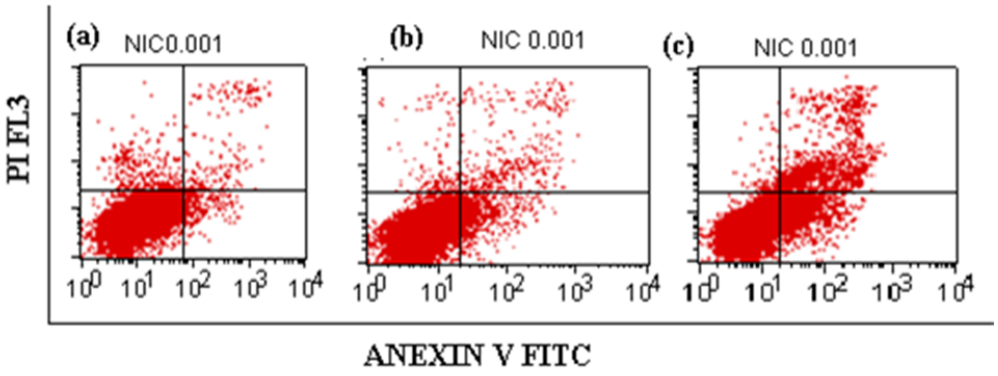

Fig. 18. Scatter plots displaying the effects of 1 nM Nicotine at a) 24 hrs, b) 48 hrs and c) 72 hrs.

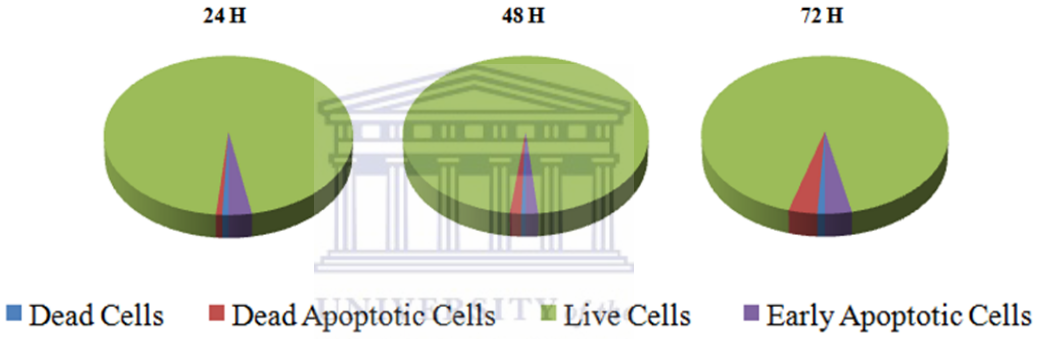

Fig. 19. The effects of 1 nM Nicotine on MCF-7 cells.

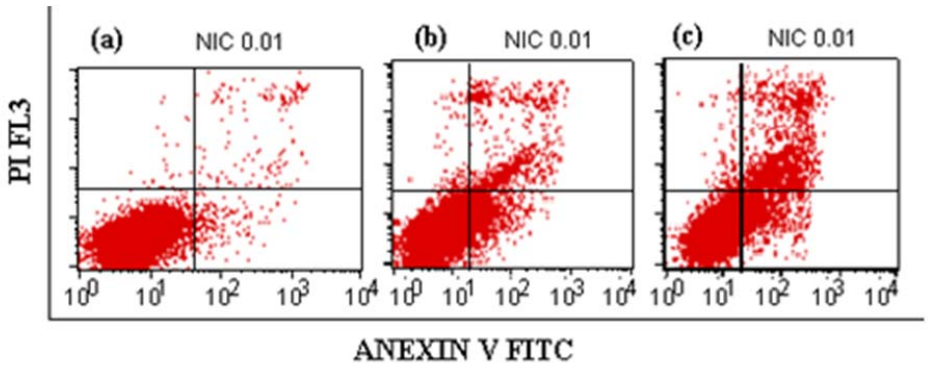

Fig. 20. Scatter plots illustrating the effects of 0.01 µM Nicotine on MCF-7 cell at a) 24 hrs, b) 48 hrs and c) 72 hrs.

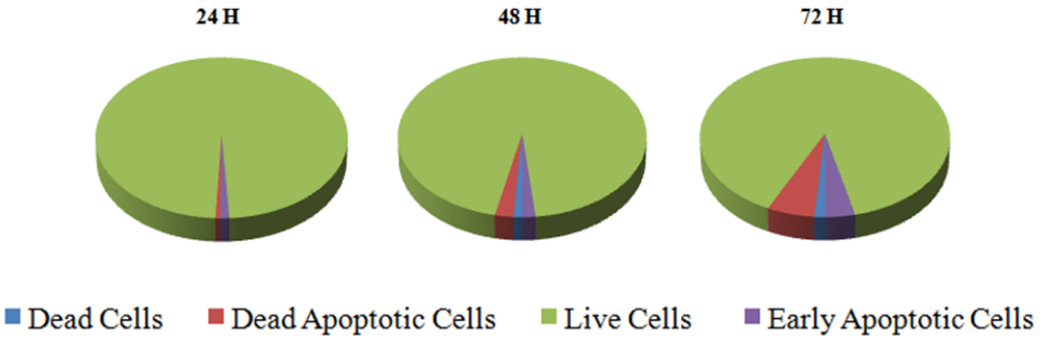

Fig. 21. The effects of 0.01 µM Nicotine on MCF-7 cells.

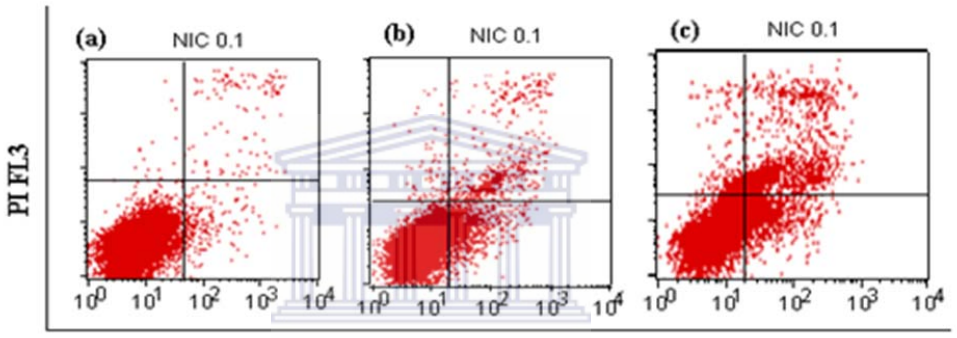

Fig. 22. Scatter plots displaying the effects of 0.1 µM Nicotine on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

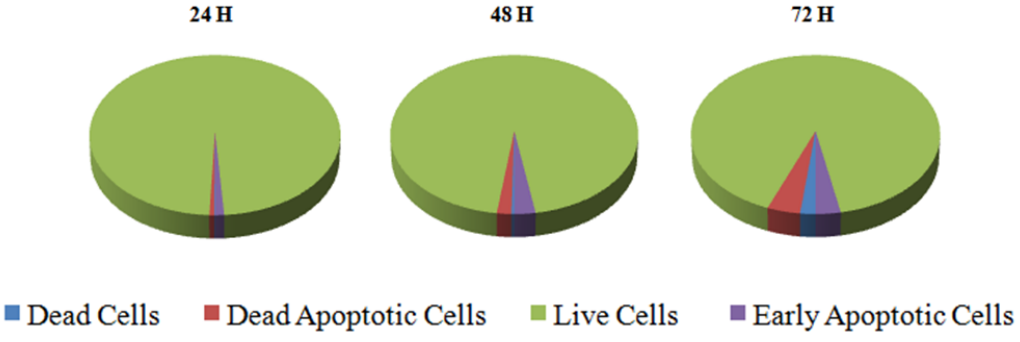

Fig. 23. The effects of 0.1 µM Nicotine on MCF-7 cells.

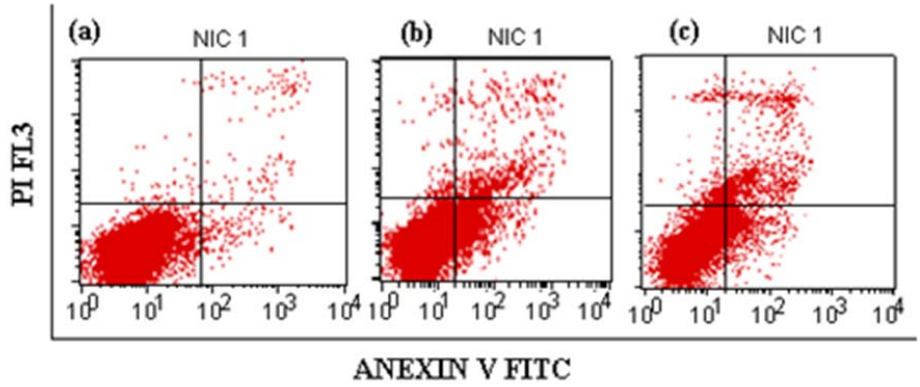

Fig. 24. Scatter plots depicting the effects of 1 µM Nicotine on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

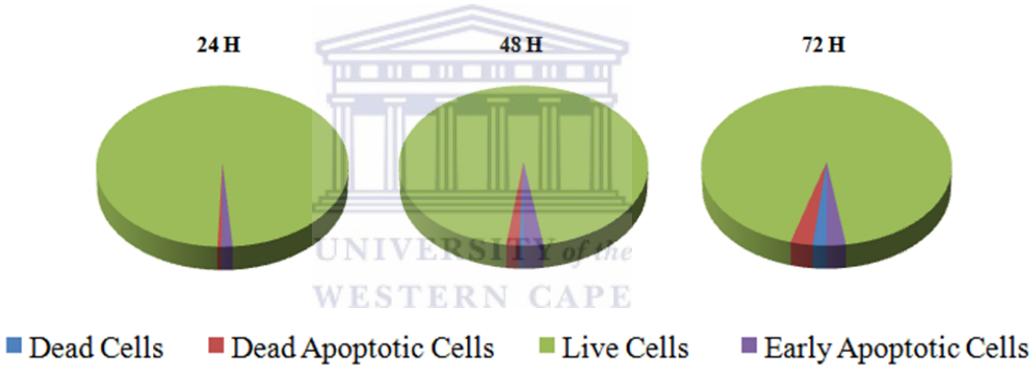

Fig. 25. The effects of 1 µM Nicotine on MCF-7 cells.

Scatter plots and pie charts (Fig. 15 to 25) illustrate the effects of selected concentrations of Nicotine on early apoptosis in MCF-7 cells. Flow cytometry analysis showed that Nicotine did not significantly alter the early apoptotic cell population at any selected concentration or time intervals.

3.2 Effect of Tunicamycin on MCF-7 cells

3.2.1 Effect on cell viability

Table 13: The effect of selected tunicamycin concentrations on MCF-7 cell viability (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.0001	94.788 ± 0.51	91.340 ± 0.712	108.715 ± 3.159
0.001	91.057 ± 1.132	91.924 ± 1.614	103.166 ± 3.137
0.0039	76.382 ± 3.07	76.362 ± 3.070	129.014 ± 2.540
0.0078	67.101 ± 6.257	77.662 ± 9.643	111.508 ± 2.996
0.01	69.884 ± 1.114	69.581 ± 3.743	85.584 ± 1.113
0.0156	60.418 ± 4.621	59.101 ± 5.220	85.693 ± 2.231
0.03125	57.512 ± 2.705	44.356 ± 2.628	56.272 ± 3.324
0.0625	53.117 ± 0.872	43.797 ± 1.731	40.302 ± 2.494
0.1	53.636 ± 1.252	36.489 ± 5.940	10.490 ± 1.758
0.125	55.538 ± 1.590	13.985 ± 1.313	8.258 ± 1.020
0.25	47.841 ± 1.601	13.904 ± 1.012	6.639 ± 1.197
0.5	44.589 ± 0.933	11.959 ± 0.989	6.477 ± 0.999
1	46.180 ± 0.918	10.857 ± 0.765	7.116 ± 0.586

Table 13 shows the effects of Tm on MCF-7 % viability. As Tm concentrations increased for all incubation periods, viability decreased. % viability significantly decreased by 48.61% (p=0.0004) and 80.48% (p=0.0159) and 101.604% (p=0.0011) at 24, 48 and 72 hrs, between 0.1 nM and 1 µM respectively. The viability of MCF-7 cell was unaffected

at the lower concentrations between 0.0001 µM and 0.0078 µM for all incubation times. Cells incubated for 72 hrs with 0.1 µM Tm displayed a significantly reduced viability (p=0.0043) when compared to the viability at 24 hrs. The % viability remained notably higher at 24 hrs for 0.125, 0.25, 0.5 and 1 µM when compared to the 48 and 72 hr viability.

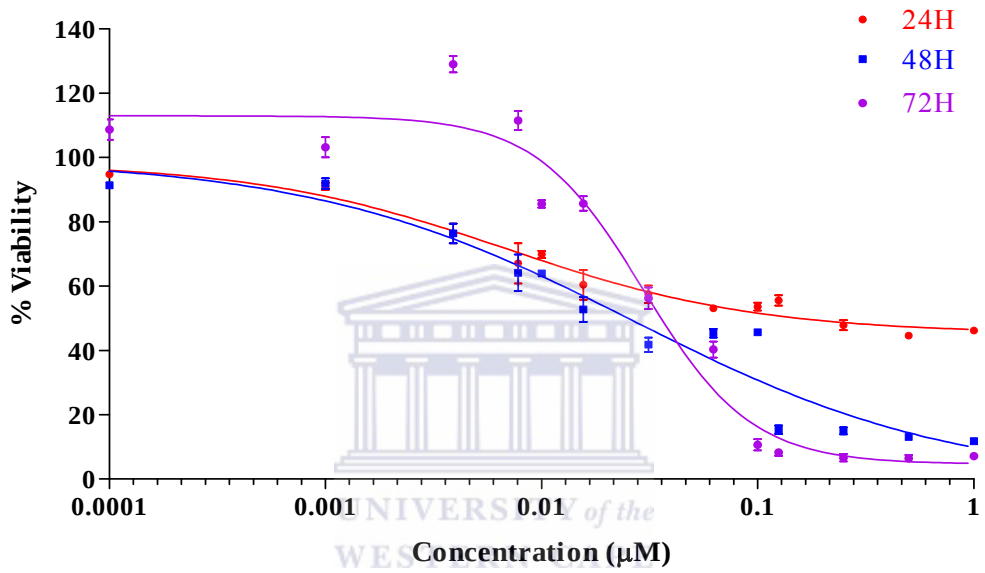

Fig.26. Effects of various tunicamycin concentrations on MCF-7 cells viability.

Fig. 26 illustrates the effects of various Tm concentrations on MCF -7 cell viability at 24, 48 and 72 hrs. Marked differences were observed between highest and lowest concentration of 0.1 nM and 1 µM at, 24 hrs (p=0.0004), 48 hrs (p=0.0159) and 72 hrs (p=0.0011). Significant differences was only observed when comparing 0.0078 µM for 24 and 72 hrs (p=0.0159) and 48 and 72 hrs (p=0.0079). MCF-7 cells displayed a gradual decrease in viability for 24 hrs as the Tm concentration increased. The cells % viability trend at 48 hrs was similar to that of cells incubated for 24 hrs, however at 0.125 µM there was a significant decrease in viability when compared to the 24 hr-incubated cells,

which continued with exposure to the subsequent higher concentrations. Cells incubated for 72 hrs (from 0.0001 µM to 0.0156 µM) also displayed similar trends to 24 and 48 hr-incubated cells, however at higher concentrations of 0.125 µM there was a noticeable decrease in viability, which thereafter continued similarly to that of the viability of cells incubated for 48 hrs. % viability at 24 hr-exposed cells did not go below ±46%.

3.2.2 Effect on ATP production

Table 14: The effect of selected tunicamycin concentrations on ATP production in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.25	53.663 ± 0.725	35.516 ± 0.989	22.015 ± 0.546
0.5	64.748 ± 0.775	38.020 ± 2.347	13.596 ± 0.137
1	66.158 ± 0.920	33.021 ± 0.826	7.471 ± 0.191
1.5	66.511 ± 0.767	32.738 ± 0.879	6.224 ± 0.370
2	68.527 ± 1.071	34.154 ± 1.328	10.831 ± 0.423

Table 14 shows the differences in ATP production observed when cells were treated to selected concentrations at 24, 48 and 72 hrs. Statistical differences were observed between 24 and 48 hrs, between 48 and 72 hrs as well as 24 and 72 hrs at all concentrations of Tm used ($p \leq 0.005$).

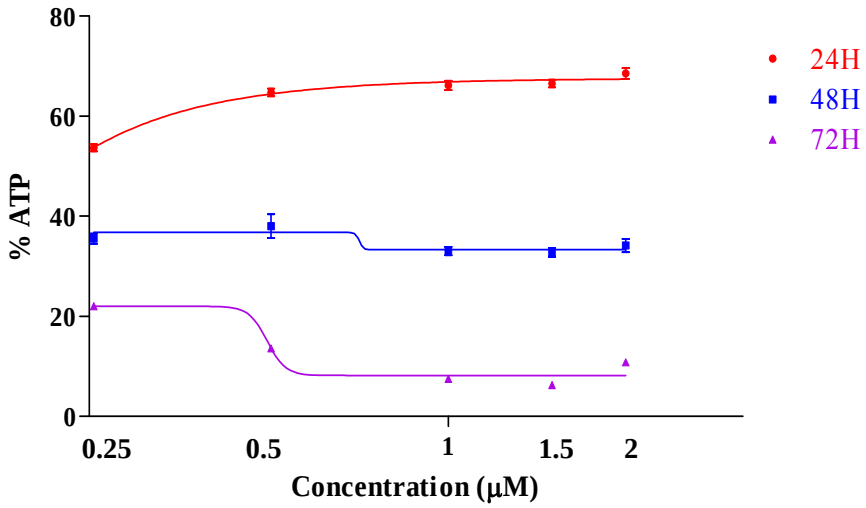

Fig. 27. The effects of selected tunicamycin concentrations on ATP production.

Fig. 27 shows the effects of selected Tm concentrations on the % ATP production of MCF-7 cells at various time periods. ATP production was significantly higher ($p \leq 0.0001$) for all Tm concentrations at 24 hrs when compared to 48 and 72 hrs. The cells displayed similar % ATP production trends for 24, 48 and 72 hrs between 0.25 and 2 µM, although at significantly lower ATP levels, with the lowest ATP levels observed at 72 hrs.

3.2.3 Effect on DNA proliferation

Table 15: The effect of selected tunicamycin concentrations on DNA proliferation in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.25	25.930 ± 1.353	105.435 ± 2.267	241.450 ± 11.5153
0.5	26.345 ± 3.529	121.750 ± 4.268	241.948 ± 6.156
1	27.600 ± 2.628	116.090 ± 13.618	231.028 ± 19.386
1.5	26.513 ± 1.870	129.550 ± 11.113	221.305 ± 4.048
2	28.023 ± 1.441	138.148 ± 2.506	227.065 ± 8.386

The effects of selected Tm concentrations over 24, 48 and 72 hrs are shown in Table 15. For all selected concentrations a significant increase was noted ($p \leq 0.0286$) between 24 and 48 hrs, 48 and 72 hrs and 24 and 72 hrs.

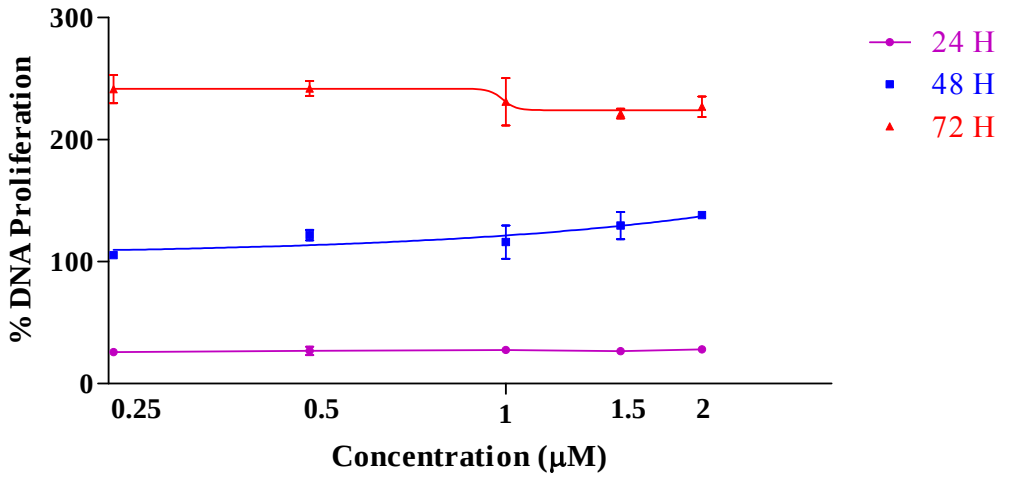

Fig. 28. The effect of selected Tm concentrations on DNA proliferation in MCF-7 cells at 24, 48 and 72 hrs.

Fig. 28 shows the effect of the selected Tm concentrations on DNA proliferation in MCF-7 cells at 24, 48 and 72 hrs. The effect of Tm remained the same at 24 hrs at any of the concentrations used. Similar trends were observed at 48 hr and 72 hrs, although the % of DNA proliferation was markedly higher ($p \leq 0.0286$) when compared to 24 hrs, with the highest proliferation observed at 72 hrs.

3.2.4 Effect on cell viability, ATP and DNA proliferation

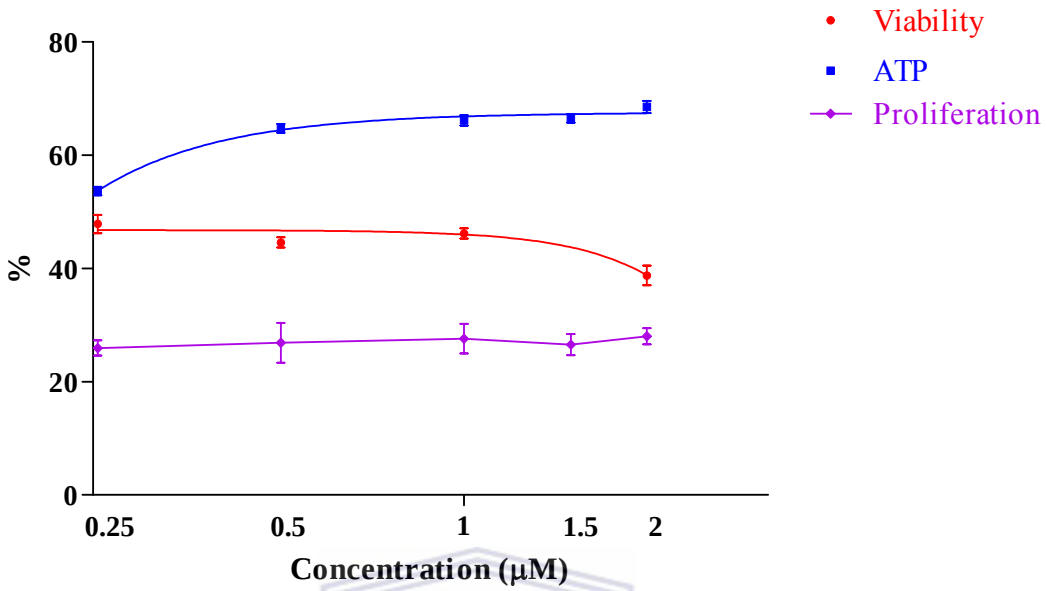

Fig. 29. The effects of 24 hr tunicamycin exposure on cell viability, ATP activity and DNA proliferation.

Cell viability decreased gradually between 1 nM and 1 µM at 24 hrs (Fig. 29). ATP production was greater in % than cell viability, and DNA proliferation was suppressed at these concentrations at 24 hrs.

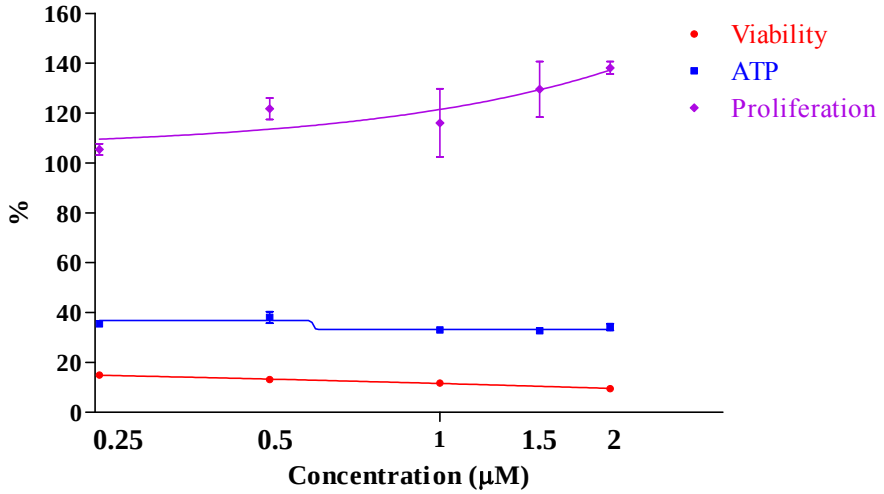

Fig. 30. The effectof tunicamycin treated MCF-7 cells after 48 hr.

Fig. 30 exhibits the effect of Tm concentrations after 48 hrs on viability, ATP and DNA proliferation. The graph shows that viability and ATP productions were considerably suppressed. However, DNA proliferation had exceeded 100% and augmented as concentrations of Tm increased.

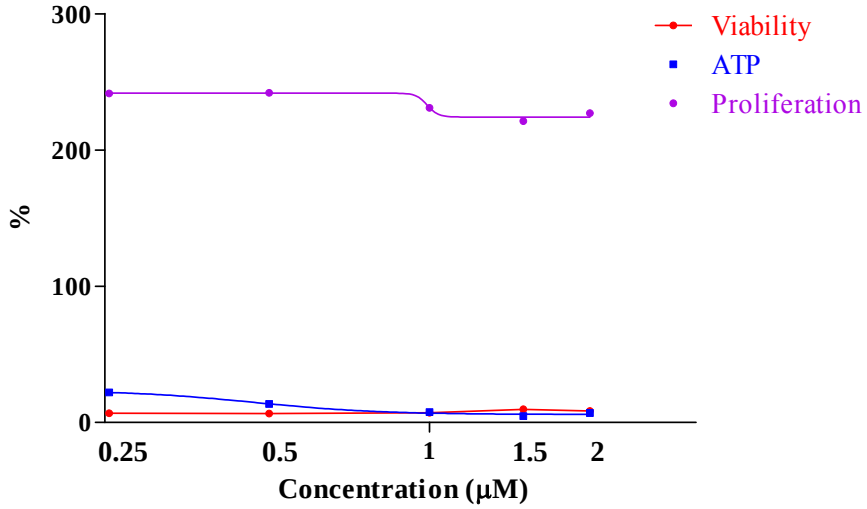

Fig. 31. The effect of tunicamycin induced MCF-7 cells after 72 hrs.

Fig. 31 demonstrates the effects of Tm on MCF-7 cells after 72 hrs. Viability and ATP productions were below 20%. A dramatic average of 230% in DNA proliferation was observed after 72 hrs of exposure for all concentrations.

3.2.5 Early apoptotic effects on MCF-7 cells

Table 16: The effect of selected tunicamycin concentrations on early apoptosis in MCF-7 cells after 24 hrs of exposure (20 000 events analysed)

Concentration (µM)	Percentage			
	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.25	0.59	10.19	72.51	16.7
0.5	0.79	13.74	69.02	16.46
1	0.51	11.42	71.47	16.6
1.5	0.58	12.29	68.99	18.15
2	0.05	12.70	70.80	16.50

Table 16 shows the early apoptotic effects of Tm at selected concentrations on MCF-7 cells after 24 hrs. All concentrations of Tm exposure resulted in, early apoptotic cells that were below 18.15%. The population of live cells were above 68.99% with less than 0.8% dead cell population.

Table 17: The effect of selected tunicamycin concentrations on early apoptosis in MCF-7 cells after 48 hrs of exposure (20 000 events analysed)

Concentration (µM)	Percentage			
	Dead Cells	**Dead Apoptotic Cells**	**Live Cells**	**Early Apoptotic Cells**
0	0.02	0.01	99.91	0.06
0.25	22.91	61.58	5.83	9.69
0.5	12.98	61.82	7.93	17.27
1	17.49	66.8	4.31	11.4
1.5	13.6	62.03	5.23	19.14
2	8.9	72.65	2.92	15.53

Table 17 shows the apoptotic phases of MCF-7 cells induced with various Tm concentrations after 48 hrs. More than 61% of the population at all concentrations were dead due to apoptosis after 48 hrs. The % of live cells for all concentrations, were below 10%.

Table 18: The effect of selected tunicamycin concentrations on early apoptosis in MCF-7 cells after 72 hrs of exposure (20 000 events analysed)

Concentration (µM)	Percentage			
	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.25	16.33	44.02	4.7	34.95
0.5	11.8	35.27	8.36	44.57
1	15.04	46.91	2.68	35.38
1.5	16.17	48.7	2.19	32.94
2	11.5	41.06	3.72	43.72

Table 18 shows the early apoptotic effects after 72 hrs when MCF-7 cells were exposed to selected Tm concentrations. Populations representing dead apoptotic cells were similar to those of the early apoptotic phase.

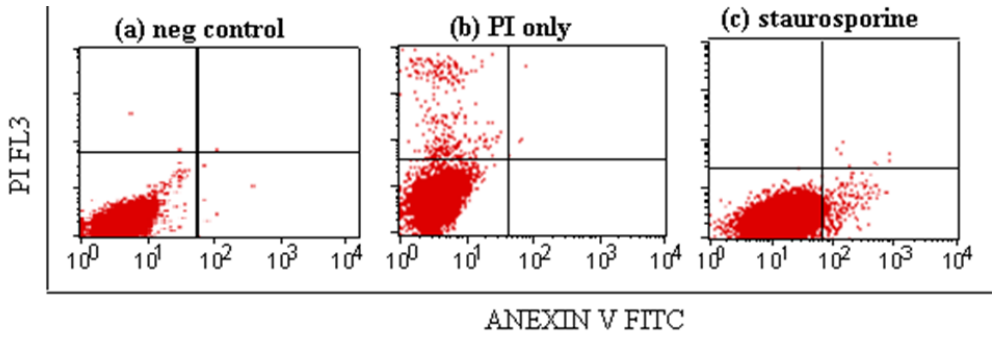

Fig. 32. The scatter plots created from the flow cytometry analysis represent the controls used for comparison of the effects of tunicamycin, a) Unstained MCF-7 cells, b)

PI only stained MCF-7 cells and c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

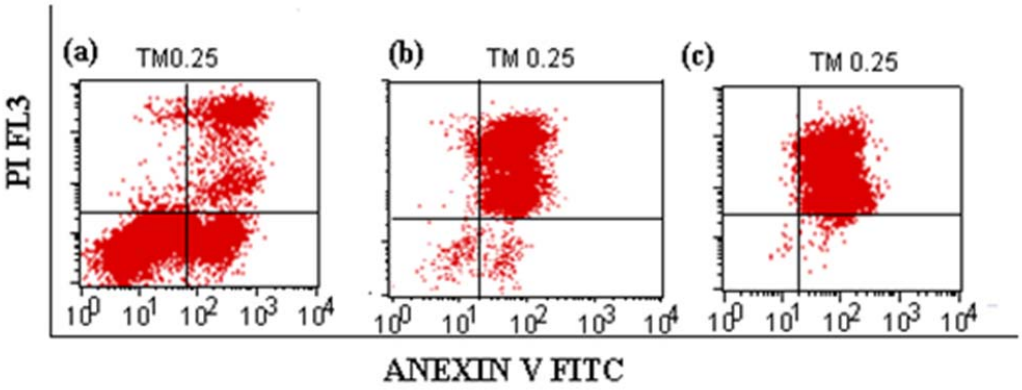

Fig. 33. Scatter plots represent the effects of 0.25 μM Tm on early apoptosis in MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

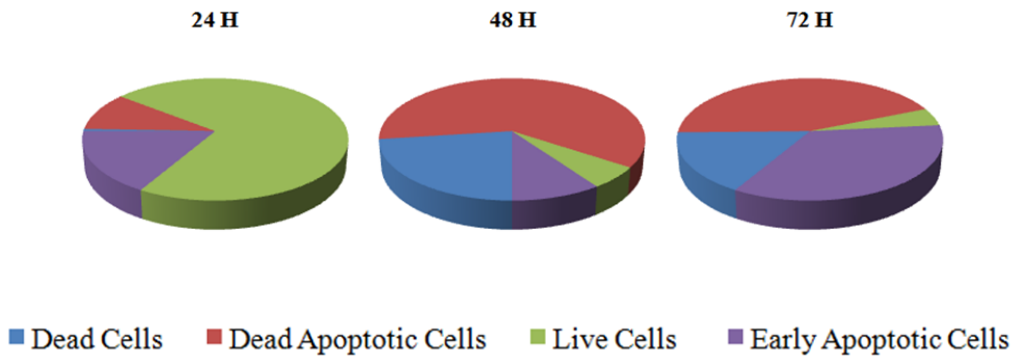

Fig. 34. The effects of 0.25 µM Tm on early apoptosis in MCF-7 cells.

Fig. 33 and 34 shows that 0.25 µM Tm at 24 hrs, resulted in 72.51% of live cells and 16.7% undergoing early apoptosis. However, after 48 hrs, only 5.83% of the cells were alive and 61.58% of the cell population were dead due to apoptosis. After 72 hrs, 34.95% of the population was in early apoptosis compared to 9.69% after 48 hrs of incubation.

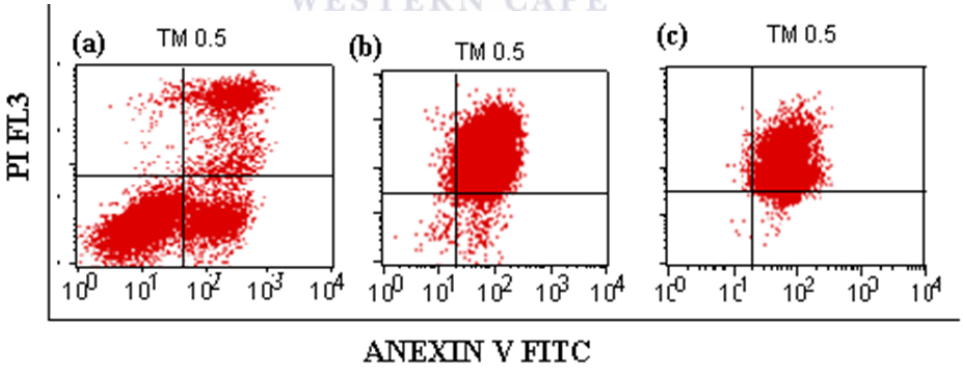

Fig. 35. Scatter plots displaying effects of 0.5 µM Tm on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

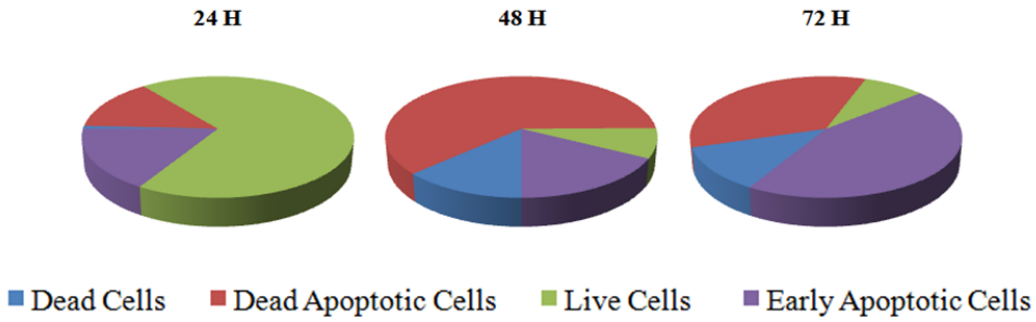

Fig. 36. The effects of 0.5 µM Tm on early apoptosis in MCF-7 cells.

Fig. 35 and 36 illustrates the effects of 0.5 µM of Tm on MCF-7 cells at the selected time intervals. At 24 hrs, 69% of the cell population was alive, and decreased to 12.98 % and 11.8% at 48 and 72 hrs respectively. Cells undergoing early apoptosis were similar at 24 hrs (16.46%) when compared to 48 hrs (17.27%). At 72 hrs early apoptotic cells had increased to 44.57%.

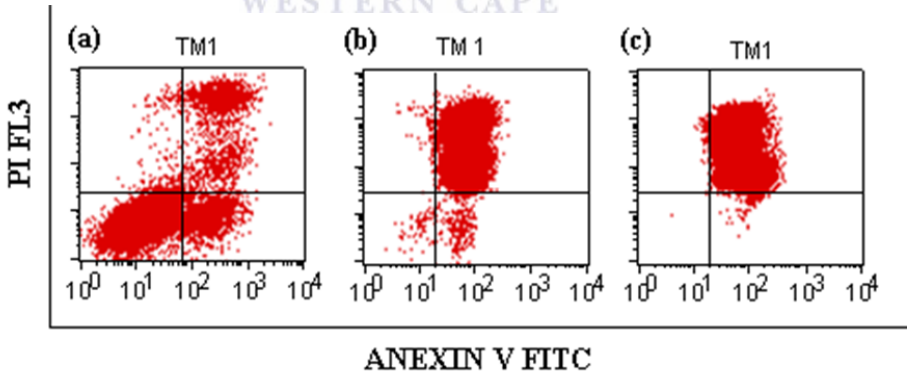

Fig. 37. The apoptotic effects of 1 µM Tm on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

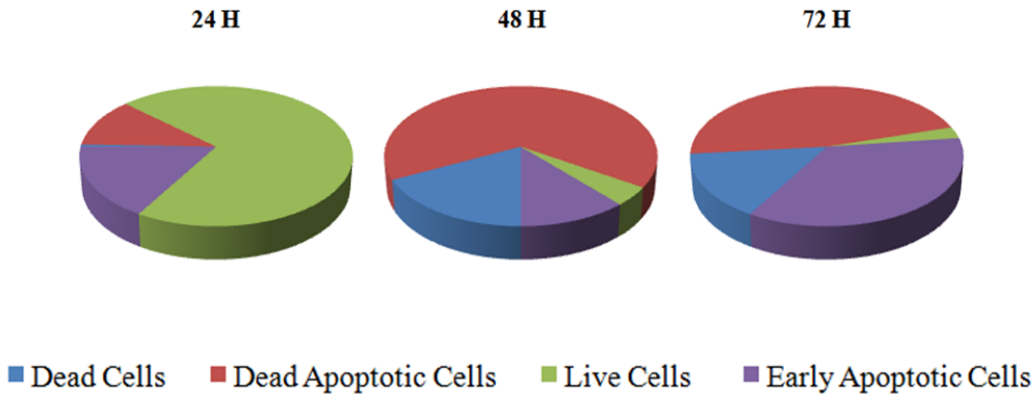

Fig. 38. The effects of 1 µM Tm on MCF-7 cells.

Fig. 37 and 38 displays a decline of 65.16% in live cell population between 24 and 48 hrs. There were similar early apoptotic populations with a 55.38% increase in dead apoptotic cells. An increase of 24% was observed between 48 and 72 hrs for early apoptotic cells, while dead apoptotic cell population decreased by 19.9%

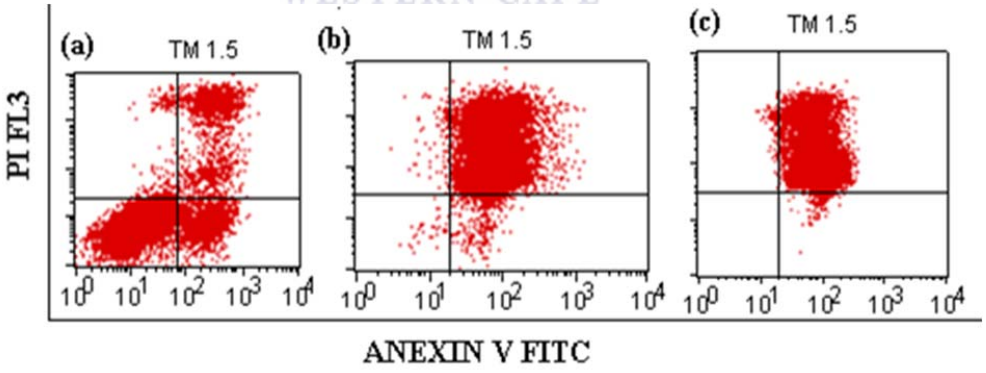

Fig. 39. Scatter plots of the apoptotic effects of 1.5 µM Tm on MCF-7 cells at a)24 hrs, b) 48 hrs and c) 72 hrs.

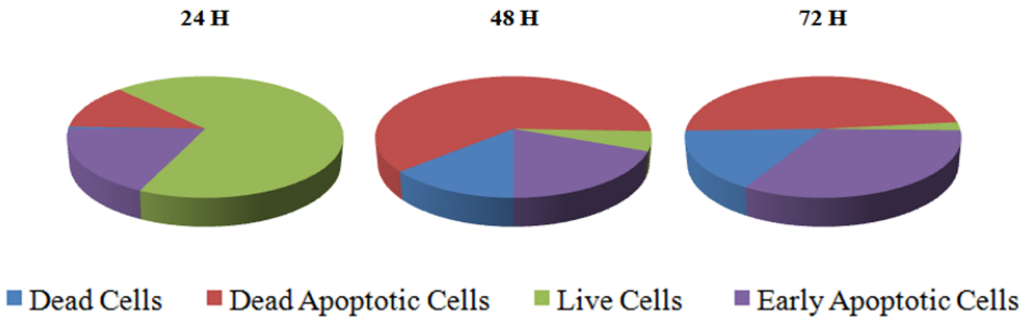

Fig. 40. The effects of 1.5 µM Tm on MCF-7 cells.

1.5 µM Tm resulted in a 63.76% decrease in live cell population between 24 and 48 hrs, while the dead apoptotic cell population increased by 50.74% (Fig. 39 and 40). The population of early apoptotic cells increased from 19.14 to 32.94% between 48 and 72 hrs and the dead apoptotic cell population decreased from 62.03% to 48.7%.

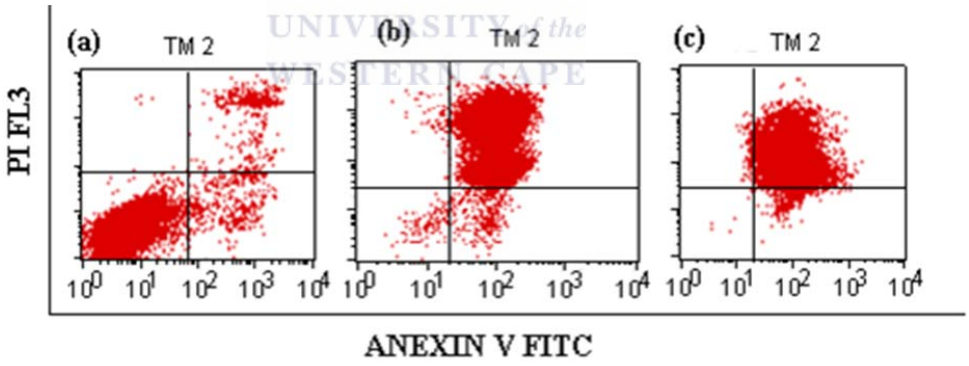

Fig. 41. Scatter plots of the effects of 2 µM Tm on MCF-7 cells a) 24 hrs, b) 48 hrs and c) 72 hrs.

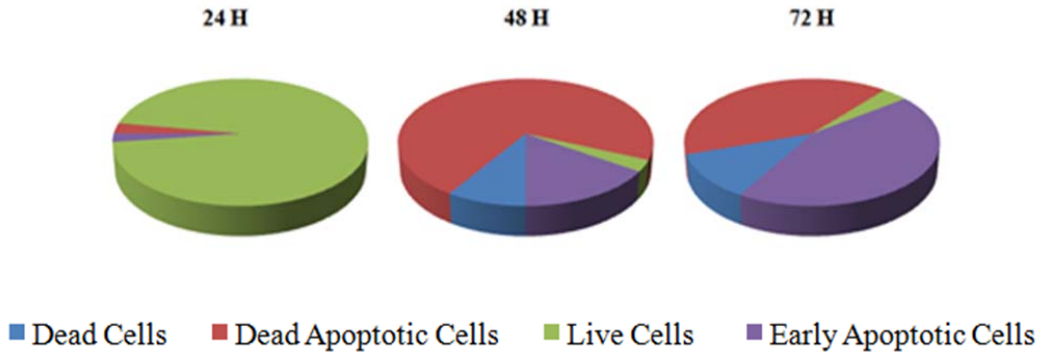

Fig. 42. The effects of 2 µM Tm on MCF-7 cells.

Fig. 41 and 42 illustrates that 2 µM of Tm resulted in a 91.43% decrease in live cells between 24 and 72 hrs. Early apoptotic cell population increased by 41.62% and dead apoptotic cells increased from 2.7% to 41.06%.

Overall (Fig. 33 to Fig. 42) data showed that 24 hr exposure to Tm resulted in live cell populations above 69%. At 72 hrs this population decreased to a maximum of 48.7%. Dead apoptotic cells increased between 24 and 48 hrs and decreased between 48 and 72 hrs. At 24 hrs the dead apoptotic cells never exceeded 13.74% and the early apoptotic cells never exceeded 18.15%. However, at 48 hrs the dead apoptotic cells ranged between 61.58 and 72.62% compared to the 9.69 and 19.14% range for early apoptotic cells. At 72 hrs the dead apoptotic cells (35.27 to 48.7%) were similar to the early apoptotic cells (32.94 to 44.57%).

3.3 The effect of Thapsigargin on MCF-7 cells

3.3.1 Effect on cell viability

Table 19: The effect of selected thapsigargin concentrations on MCF-7 cell viability (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.0001	124.121 ± 3.552	122.066 ± 1.988	125.192 ± 5.787
0.001	115.036 ± 6.058	116.588 ± 4.860	113.563 ± 3.418
0.01	60.383 ± 2.315	109.692 ± 3.203	116.827 ± 4.663
0.0125	81.798 ± 2.339	77.540 ± 3.340	124.725 ± 7.442
0.025	50.440 ± 3.721	61.790 ± 1.418	87.414 ± 2.461
0.05	46.814 ± 2.285	27.707 ± 2.818	45.737 ± 1.752
0.1	41.340 ± 2.793	19.007 ± 0.555	10.154 ± 0.997
0.2	40.266 ± 2.005	17.404 ± 0.574	5.093 ± 1.266
0.3	40.044 ± 2.167	20.008 ± 1.446	4.109 ± 2.729
0.4	36.198 ± 1.512	16.266 ± 1.348	2.079 ± 1.194
0.8	31.3677 ± 1.842	10.216 ± 1.654	2.999 ± 1.878
1	44.586 ± 4.057	23.554 ± 2.359	3.145 ± 0.386

Results obtained (Table 19) shows that the lowest concentration of Tg (0.1 nM and 0.001 µM) resulted in similar effects on % viability at the selected time intervals. Overall, Tg exposure resulted in a significant decrease ($p \leq 0.001$) in % viability when comparing the effects at the highest Tg concentration of 1 µM to the lowest of 0.1 nM at 24, 48 and 72 hrs ($p \leq 0.01$). The cells displayed greater viability at 72 hrs between

0.1 nM and 0.025 µM when compared to the 24 hr-exposed cells. However, the MCF-7 cells displayed noticeably (p<0.05) lower viability at 48 and 72 hrs between 0.1 µM and 1 µM when compared to the 24 hr incubation time.

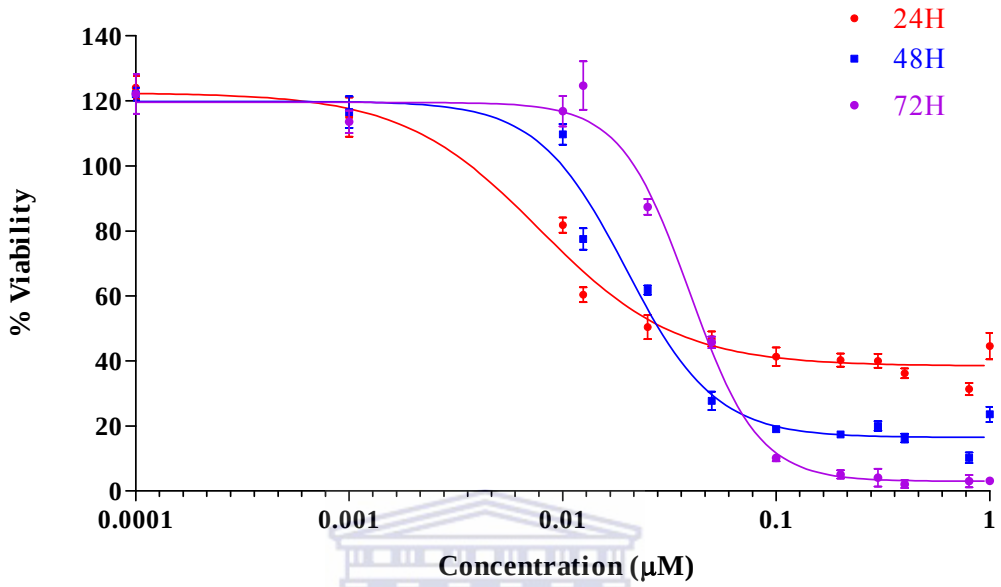

Fig. 43. The effect of various concentrations of thapsigargin on MCF-7 cell viability.

Fig. 43 shows the effect of various concentrations of Tg on MCF-7 viability. A statistical difference was observed between 0.1 nM and 1 µM for all time intervals. The trends were similar when comparing the incubation times. % viability was similar at 0.1 nM for all time periods; however it was evident that viability was significantly different between 0.1 nM (p=0.0044) and 1 µM (p=0.0044) for each time period. Cell viability were significant at 1 µM between 24 and 48 hrs (p=0.0022), 48 and 72 hrs (p=0.0055) and between 24 and 72 (p=0.0021).

3.3.2 Effect on ATP production

Table 20: The effect of selected thapsigargin concentrations on ATP production in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.05	61.891 ± 1.280	61.178 ± 0.796	71.289 ± 2.789
0.1	52.417 ± 2.947	34.778 ± 0.887	17.243 ± 0.923
0.2	56.634 ± 1.456	23.186 ± 0.646	8.757 ± 0.696
0.3	56.704 ± 1.033	20.294 ± 0.503	4.925 ± 0.182
0.4	59.343 ± 1.344	20.658 ± 1.066	6.869 ± 0.611

Table 20 shows that ATP concentrations at 0.05 µM, was similar at all incubation times. No difference was observed between 0.05 and 0.4 µM at 24 hrs. However, at 48 and 72 hrs (between 0.05 µM to 0.4 µM) difference ($p \leq 0.009$) was observed. A decrease of 40.52% in ATP production was evident from 0.1 µM to 0.4 µM at 48 hrs and a decline of 64.42% at 72 hrs.

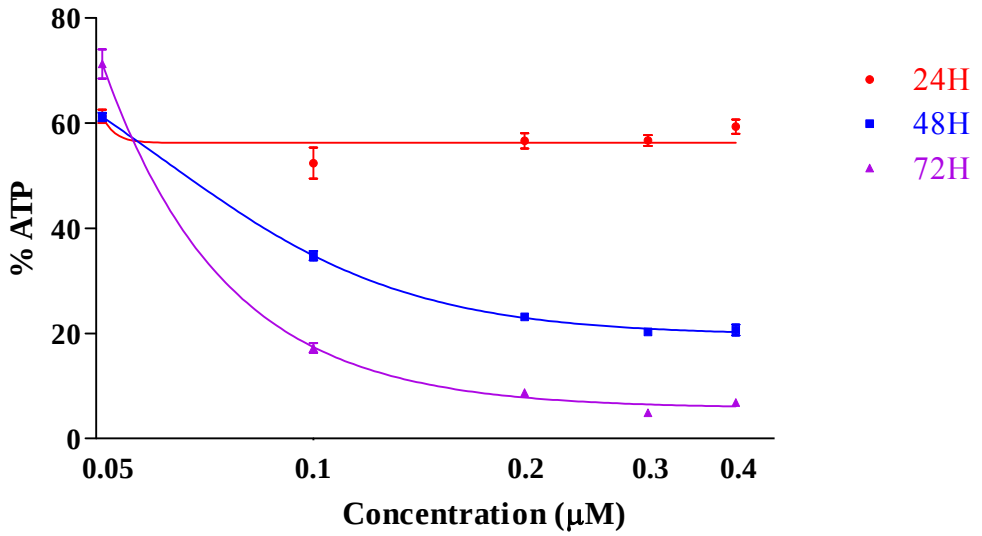

Fig. 44. The effect of selected Tg concentrations on ATP production of MCF -7 cells.

Fig. 44 illustrates that as time increases, ATP production decreased at all concentrations of Tg analysed. While 24 hrs ATP concentrations was similar, both 48 and 72 hr levels displayed the same declining trend with the latter displaying lower concentrations than the former ($p \leq 0.009$)

3.3.3 Effect on DNA proliferation

Table 21: The effect of various concentrations of thapsigargin on DNA proliferation in MCF -7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.05	19.568 ± 3.284	130.577 ± 13.215	208.320 ± 9.9338
0.1	17.208 ± 1.547	139.925 ± 5.695	210.860 ± 7.8548
0.2	15.858 ± 1.393	143.355 ± 7.087	180.470 ± 13.910
0.3	15.160 ± 1.012	161.933 ± 5.061	204.213 ± 6.249
0.4	13.428 ± 2.093	150.298 ± 6.615	204.880 ± 7.173

Table 21, shows that DNA proliferation did not exceed 19.57% at 24 hrs, however after 48 hrs, proliferation exceeded 130.56% and at 72 hrs, it reached 210.86% at 0.1 µM. No differences were observed between 0.05 µM and 0.4 µM at 24, 48 and 72 hrs.

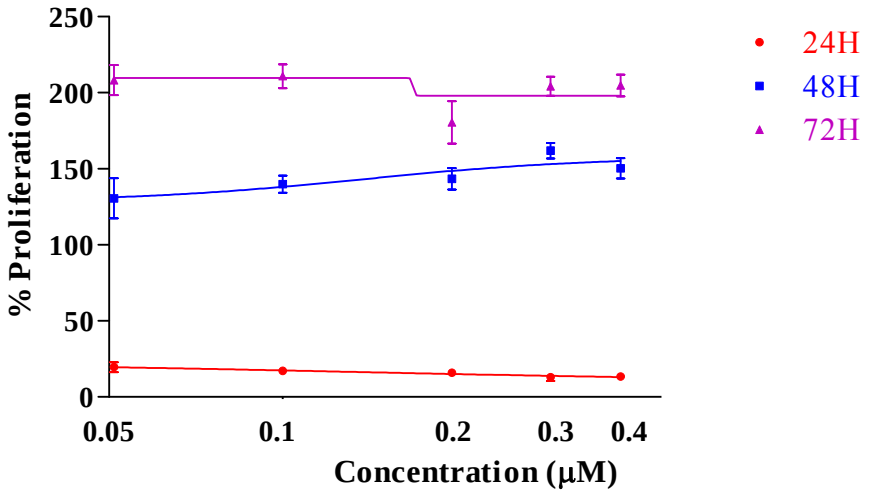

Fig. 45. The effect of selected Tg concentrations on DNA proliferation of MCF-7 cells.

Fig. 45 demonstrates that DNA proliferation, after 72 hrs was above 180%, significantly higher than at 24 or 48 hrs ($p \leq 0.0286$) for all concentrations. The proliferation at 48 hrs was markedly higher that at 24 hrs at all concentrations of Tg.

3.3.4 Effect on cell viability, ATP and DNA proliferation

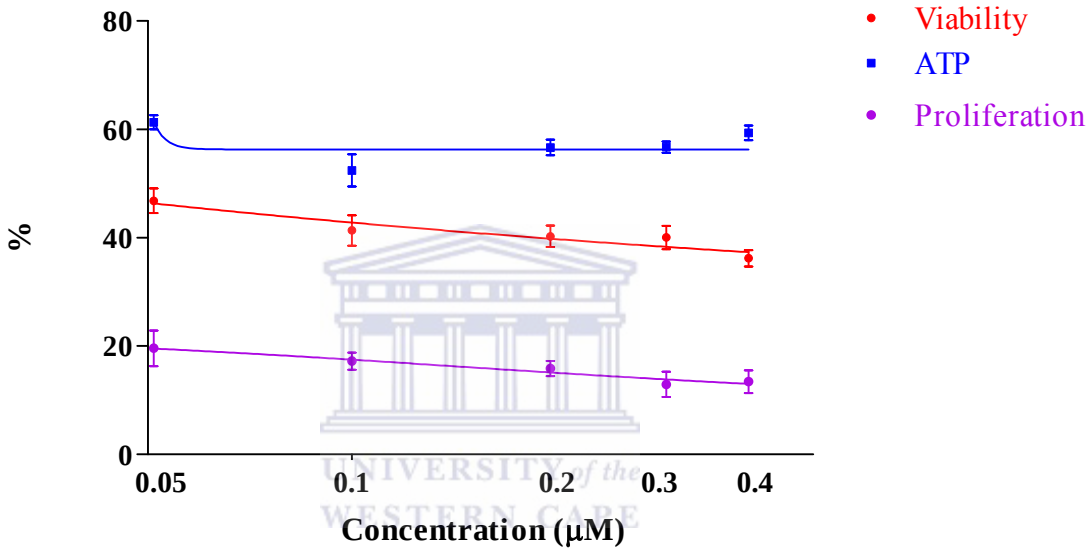

Fig. 46. The effect of Tg after 24 hrs of exposure on viability, ATP and DNA proliferation.

At 24 hrs, a range of 52.42 to 61.89% ATP was observed, with viability levels lower than 46.81% and DNA proliferation not exceeding 19.57%.

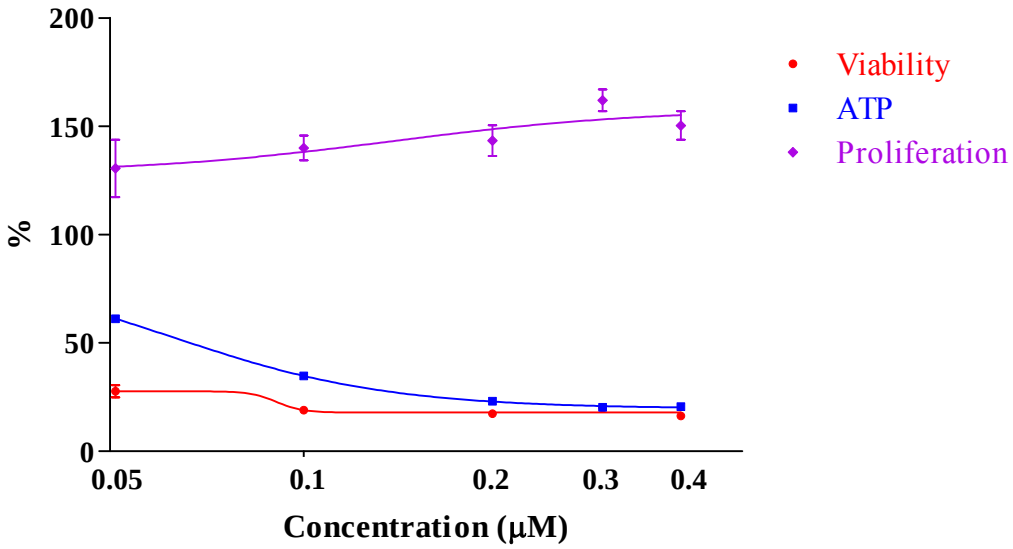

Fig. 47. The effect of Tg after 48 hrs of exposure on viability, ATP and DNA proliferation.

Fig. 47 indicated that viability did not exceed 27.7% at 48 hrs. MCF-7 cells displayed an ATP production of 61.18% at 0.05 µM. A significant decrease in ATP was observed at 0.4 µM and remained the same for the lower Tg concentrations. DNA proliferation was significantly high ranging between 130.58% and 161.9%.

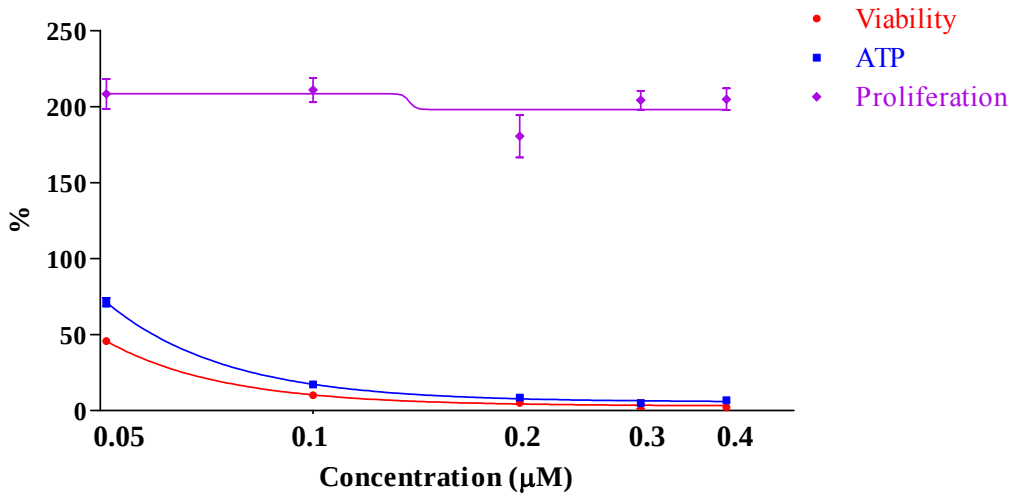

Fig. 48. The effect of select Tg concentrations after 72 hrs of exposure on viability, ATP activity and DNA proliferation.

Fig. 48 shows that levels of viability did not exceed 45.7% after 72 hrs. The cells displayed an ATP production of 71.28% at 0.05 µM. A significant decrease in ATP was observed at 0.1 µM with increasing Tg concentrations. DNA proliferation at 72 hrs ranged between was 180.47% and 210.8%.

3.3.5 Early apoptotic effects on MCF-7 cells

Table 22: The apoptotic effects of selected thapsigargin concentrations at 24 hr exposure in MCF-7 cells (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.05	0.59	8.21	81.88	9.32
0.1	0.88	7.58	83.17	8.38
0.2	0.08	3.74	92.97	3.21
0.3	1.07	10.03	79.49	9.42
0.4	2.27	34.73	24.21	38.79

Table 22 shows that an increase in dead apoptotic cells between 0.05 and 0.4 µM. Similarly, an increase in early apoptotic cells were noted and a decline in live cells compared to the subsequent lower concentrations. Overall, MCF-7 cells were unaffected between 0.05 µM and 0.3 µM.

Table 23: The apoptotic effects of selected thapsigargin concentrations at 48 hr exposure on MCF-7 cells (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.05	8.19	51.26	24.34	16.21
0.1	35.94	36.54	19.23	8.29
0.2	11.18	67.41	8.69	12.72
0.3	9.57	68.66	9.76	12.01
0.4	10.66	67.74	8.07	13.54

Table 23 illustrates that as concentrations increased live cell numbers decreased. Similarly, a trend between concentrations and their effect on dead apoptotic cells was also observed except at 0.1 µM where an increase in dead cells was observed. Generally, more dead cells as a result of apoptosis was observed at 48 hrs, when compared to early apoptotic cells.

Table 24: The apoptotic effects of selected thapsigargin concentrations at 72 hrs exposure in MCF-7 cells (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.05	9.37	36.38	31.02	23.23
0.1	16.11	41.34	3.82	38.73
0.2	17.91	45.2	1.35	35.54
0.3	11.41	46.48	1.01	41.1
0.4	15.82	45.34	2.16	36.69

Table 24 shows that at 72 hrs, dead apoptotic cells exposed to 0.05 µM to 0.4 µM Tg was 36.38% to 45.34% respectively. The early apoptotic cell population ranged between 23.23% and 41.1% for the same concentration range. The dead cell population did not exceed 17.91%.

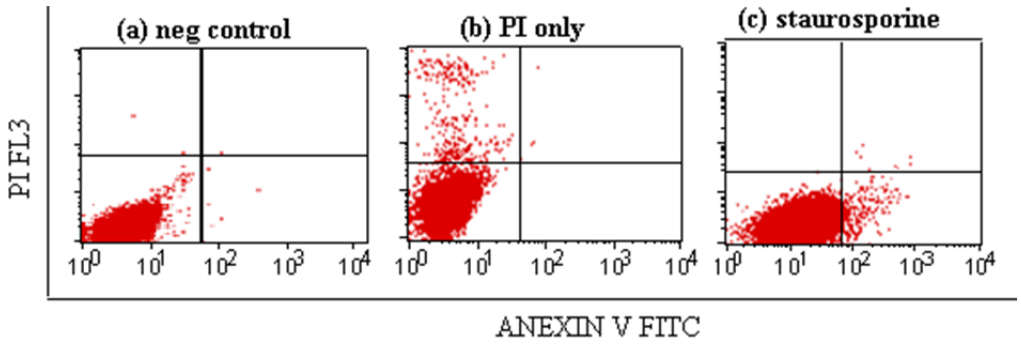

Fig. 49. The scatter plots created from the flow cytometry analysis represent the controls used for comparison of the effects of Thapsigargin. a) Unstained MCF-7 cells b) PI only stained MCF-7 cells and c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

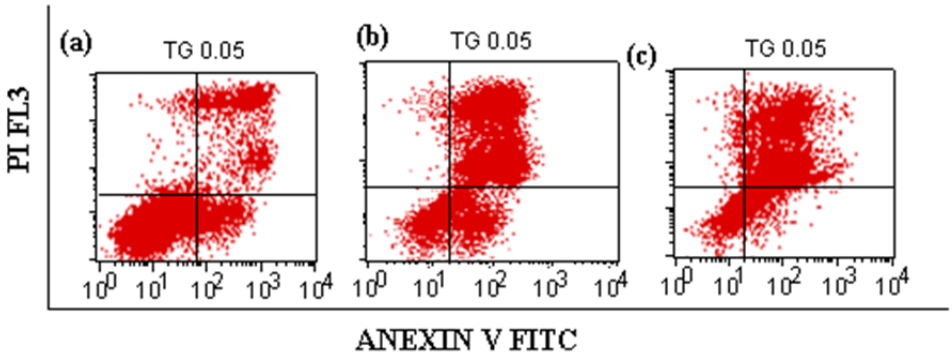

Fig. 50. Scatter plots representing the effect of 0.05 µM Tg at a) 24 hrs, b) 48 hrs and c) 72 hrs.

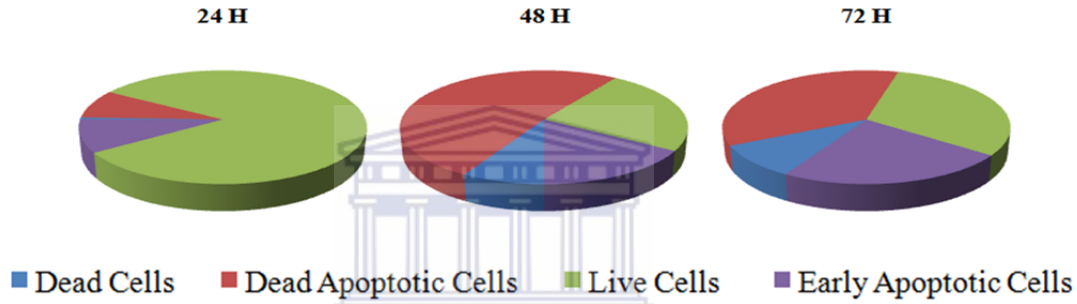

Fig. 51. pie charts indicating the effect of 0.05 µM Tg at various time intervals.

Early apoptotic population increased from 9.32% to 23.23% from 24 to 72 hrs when exposed to 0.05 µM Tg (Fig.50 and 51). At 24 hrs, live cell population was 81% and at 48 and 72 hrs the population did not exceed 31.02%. Between 24 and 48 hrs, there was an increase of 43.05% in cells dead due to apoptosis which declined by 14.88% after 72 hrs.

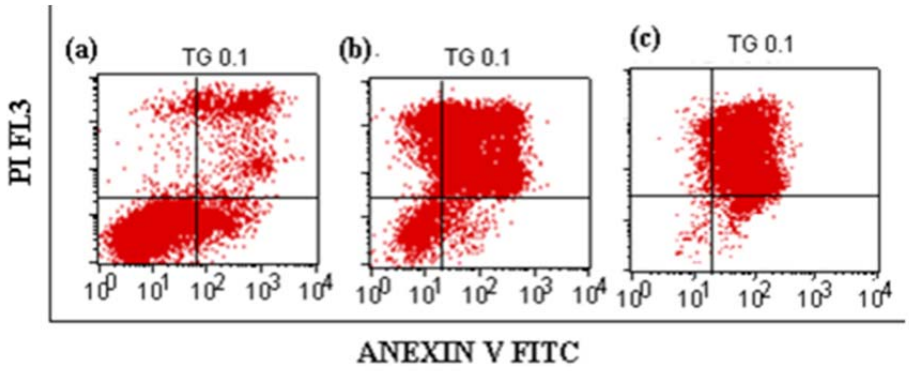

Fig. 52. Scatter plots illustrated apoptotic effects of 0.1 µM Tg at a) 24 hrs, b) 48 hrs and c) 72 hrs.

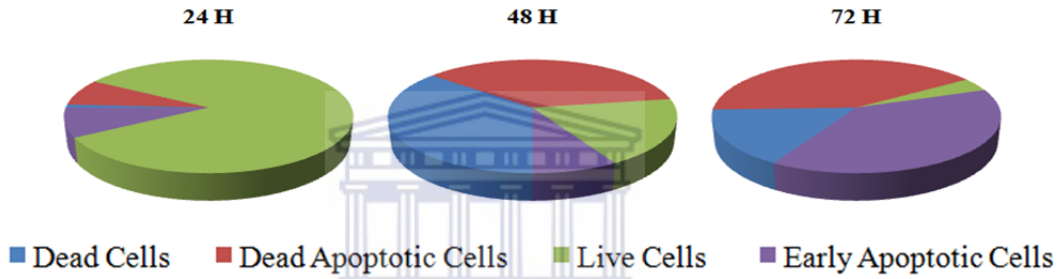

Fig. 53. The apoptotic effects of 0.1 µM Tg.

Fig. 52 and 53 show an increase of 30.35% in early apoptotic cells, and 33.76% in cells dead due to apoptosis between 24 and 72 hrs. Live cells exposed to 0.1 µM Tg decreased by 79.35% for the same time interval.

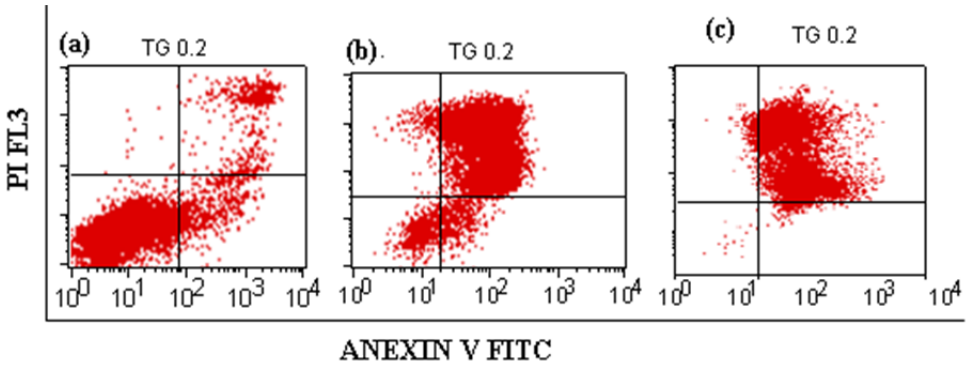

Fig. 54. Scatter plots displaying the effects of 0.2 µM Tg on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

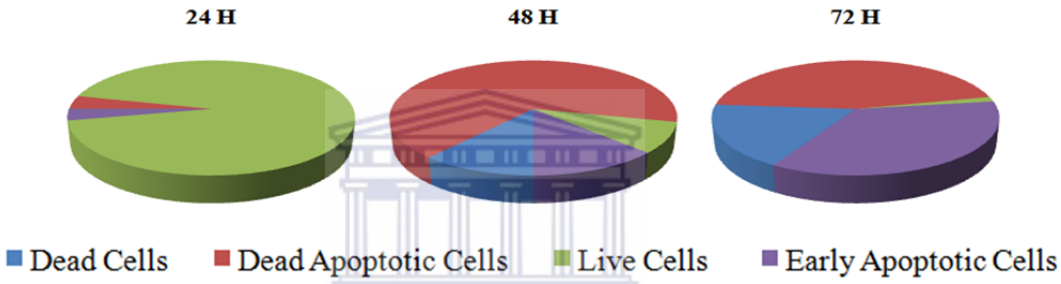

Fig. 55. The apoptotic effects of 0.2 µM Tg.

Fig. 54 and 55 demonstrates that 92.97% of cells after 24 hrs were still alive and significantly decreased by 91.62% after 72 hrs. Furthermore, as time increased early apoptotic population increased by 32.33% and the amount of dead apoptotic cells increased by 41.46%.

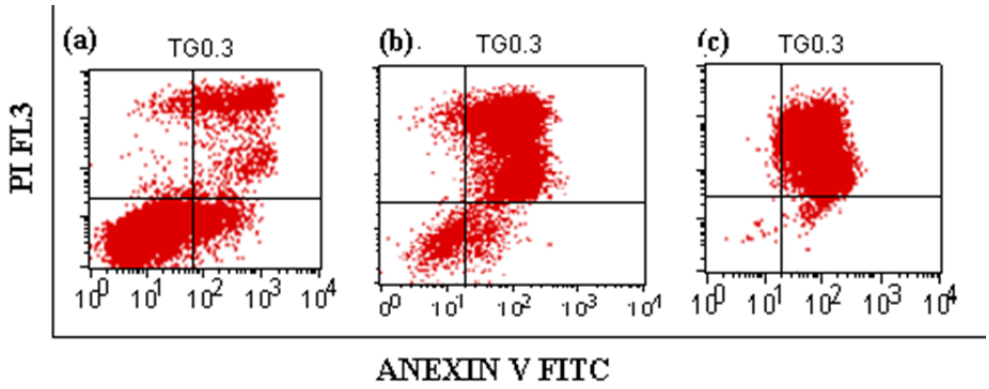

Fig. 56. Scatter plots showing the effects of 0.3 µM Tg on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

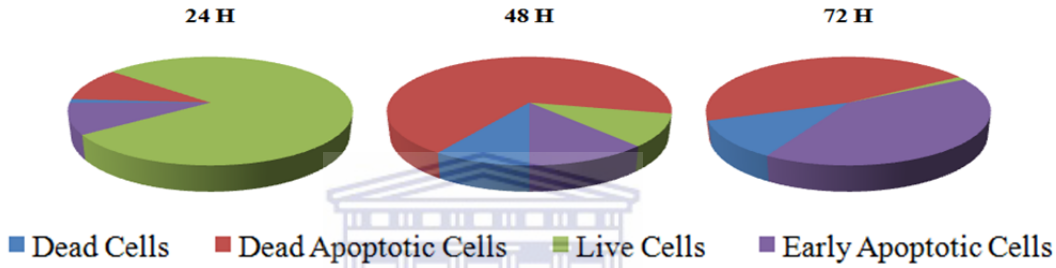

Fig. 57. The apoptotic effects of 0.3 µM Tg.

Fig. 56 and 57 show that, dead apoptotic cells increased by 58.63%, between 24 and 48 hrs, while all other populations were similar. The dead apoptotic cell population decreased by 22.18% between 48 and 72 hrs, with the early apoptotic cells displaying similar levels.

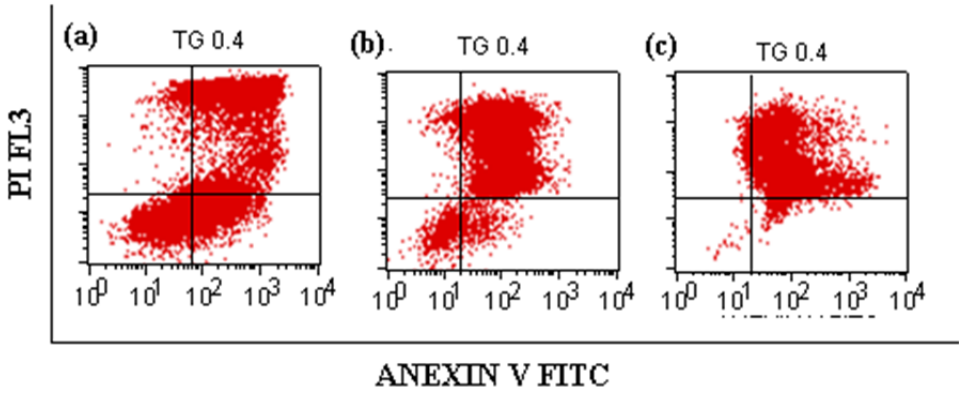

Fig. 58. Scatter plots representing the effect of 0.4 µM Tg on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

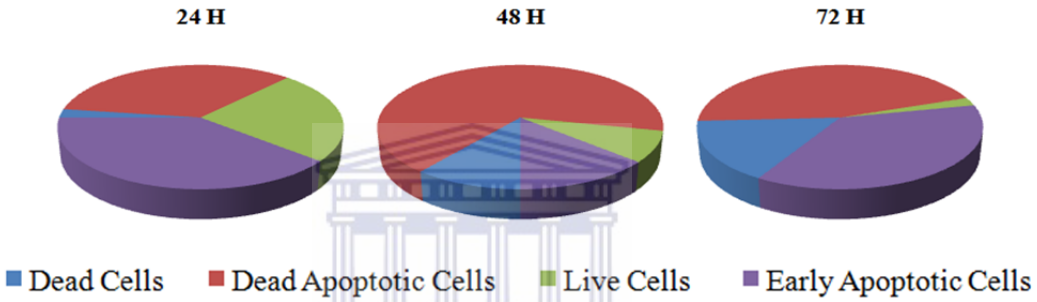

Fig. 59. The apoptotic effects of 0.4 µM Tg.

Fig. 58 and 59 showed an increase in dead apoptotic cells observed between 24 and 48 hrs, followed by a decrease at 72 hrs. While early apoptotic populations decreased at 48 hrs, followed by an increase at 72 hrs.

3.4 The combinatorial effect of Tunicamycin and Nicotine on MCF-7 cells

3.4.1 Effect on cell viability

Table 25: The effect of combinations (ratio 1:1) of selected tunicamycin and nicotine concentrations on MCF-7 cell viability (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.0001	73.040 ± 6.439	64.234 ± 2.823	69.963 ± 2.645
0.001	66.921 ± 2.663	48.683 ± 1.370	53.154 ± 1.286
0.01	66.016 ± 4.035	45.901 ± 0.576	51.199 ± 1.658
0.1	56.707 ± 1.912	32.945 ± 4.209	37.902 ± 2.967
1	56.696 ± 3.099	27.561 ± 1.959	27.563 ± 2.421
10	56.63 ± 2.546	26.878 ± 2.002	28.251 ± 3.306
100	43.865 ± 2.358	22.133 ± 3.317	22.223 ± 1.318
200	12.485 ± 1.252	9.835 ± 0.384	8.580 ± 1.148
300	7.956 ± 0.862	11.794 ± 0.779	1.217 ± 0.991

Table 25 shows that % viability significantly decreased between 0.1 nM and 300 µM at 24, 48 and 72 hrs (p<0.05). At 0.1, 1, 10, and 100 µM of 1:1 ratio of Tm:Nic, viability was noticeably lower at 48 and 72 hrs when compared to 24 hrs.

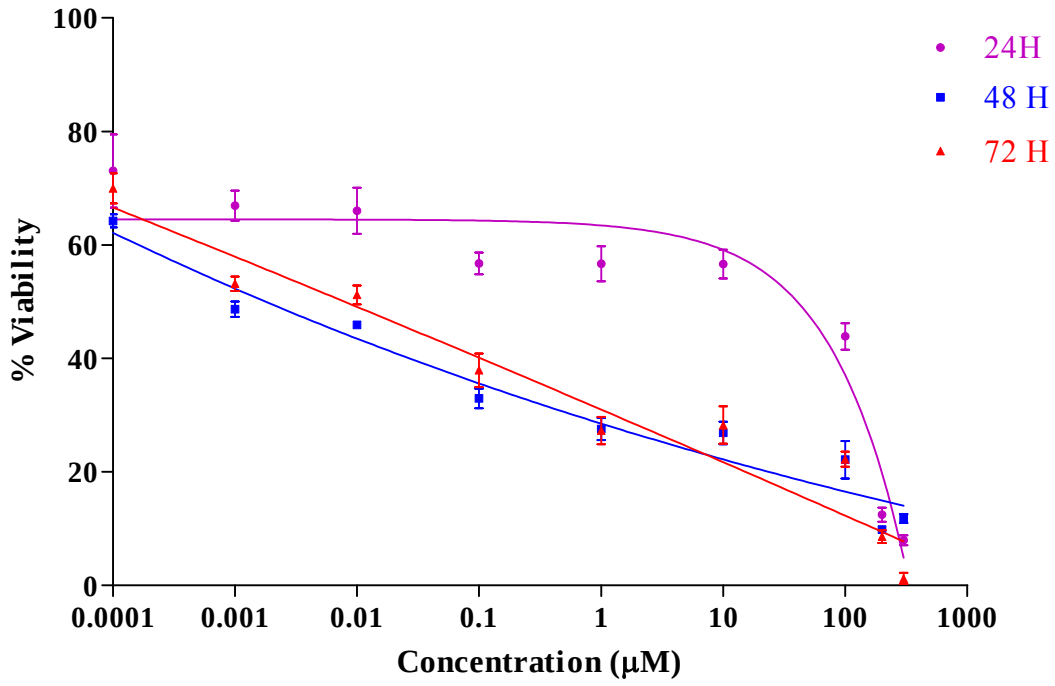

Fig. 60. Effects of various Tm:Nic combinations on MCF-7 cells viability.

% viability was similar between 0.1 nM and 10 µM at 24 hrs. Fig. 60 shows that, at the subsequent concentrations, viability decreased significantly between 10 µM and 300 µM ($p<0.0043$). The trend observed at 48 and 72 hrs, were similar between 0.1 nM and 300 µM, decreasing ($p<0.0043$) by 20.451% and 36.685%, respectively.

3.4.2 Effect on ATP production

Table 26: The effect of combinations (ratio 1:1) of selected tunicamycin and nicotine concentrations on ATP production in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.01	100.707 ± 1.9	95.545 ± 1.055	89.443 ± 0.770
0.1	77.35 ± 1.969	20.36 ± 0.435	19.368 ± 1.326
1	66.137 ± 1.196	9.119 ± 0.199	4.495 ± 0.147

Table 26 displays the effect of Tm:Nic combinations on ATP production at the selected concentrations and time intervals. As concentrations increased from 0.01 to 1 µM, % ATP production significantly decreased ($p \leq 0.0286$), by 34.58%, 86.43% and 84.94% respectively.

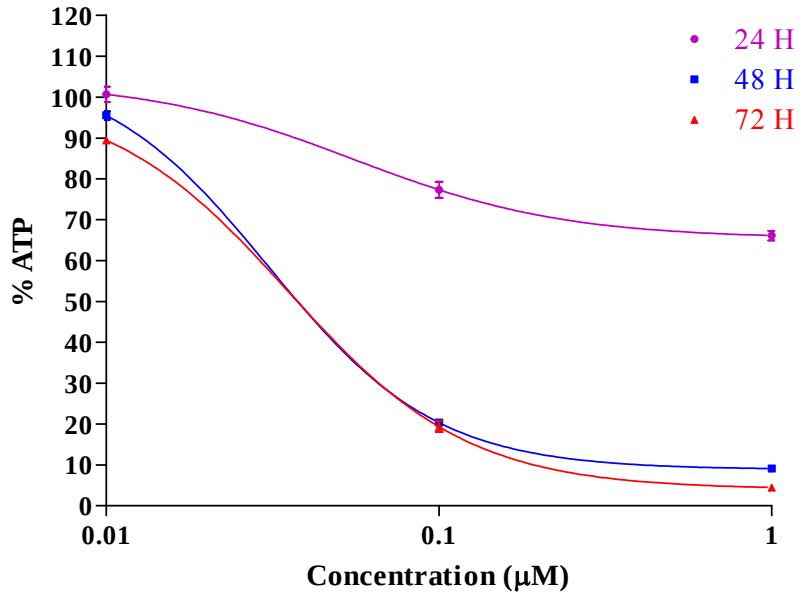

Fig. 61. The effects of selected combinations (ratio 1:1) of Tm:Nic concentrations on ATP production.

Fig. 61 shows that the 1:1 ratio combination of Tm:Nic on ATP production in MCF-7 cells were above 66% at 24 hrs at the highest concentration of 1 µM. Cellular energy levels were greatly inhibited thereafter with ($p \leq 0.0286$) at 48 and 72 hrs. It also illustrated similar trends between 24, 48 and 72 hrs with their steep decline in ATP production.

3.4.3 Effect on DNA proliferation

Table 27: The effect of combinations (ratio 1:1) of selected tunicamycin and nicotine concentrations on DNA proliferation in MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.01	63.133 ± 2.448	49.788 ± 2.327	116.120 ± 9.941
0.1	21.400 ± 1.635	85.595 ± 10.736	218.383 ± 16.686
1	25.105 ± 1.625	98.955 ± 1.989	264.133 ± 7.949

Table 27 shows that, DNA proliferation in MCF-7 cells at 24 hrs significantly ($p<0.05$) decreased between 0.01 and 0.1 µM ratios. However, after 48 hrs DNA proliferation noticeably increased ($p=0.0286$) between 0.01 and 0.1 µM. DNA proliferation decreased, by 38.03% after 24 hrs between 0.01 and 1 µM, in comparison to the increase of 148.01% after 72 hrs.

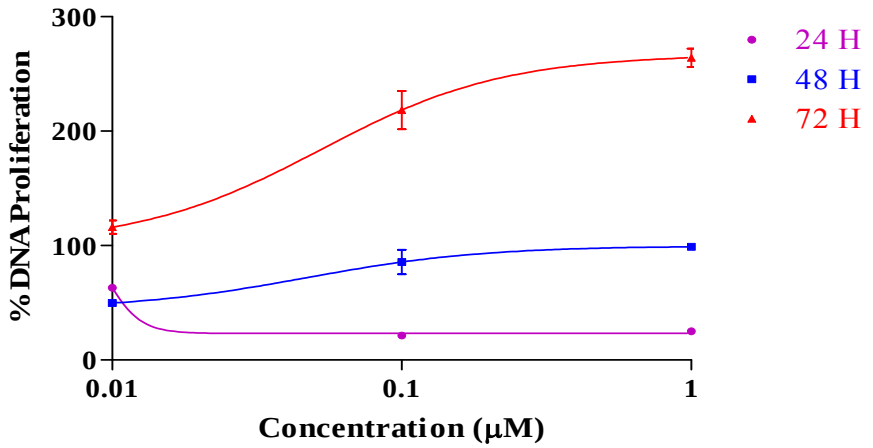

Fig. 62. The effect of selected Tm:Nic (1:1) ratio concentrations on DNA proliferation of MCF-7 cells at 24, 48 and 72 hrs.

MCF-7 cells induced with combinations of the selected Tm:Nic (1:1 ratio) concentrations, (Fig. 62) at 24 hrs, displayed a marked decrease (p=0.0286) in DNA proliferation while at 48 hrs and 72 hrs, % proliferation was elevated. The trend observed at 72 hrs was at significantly higher levels when compared to 48 or 24 hrs.

3.4.4 Effect on cell viability, ATP and DNA proliferation

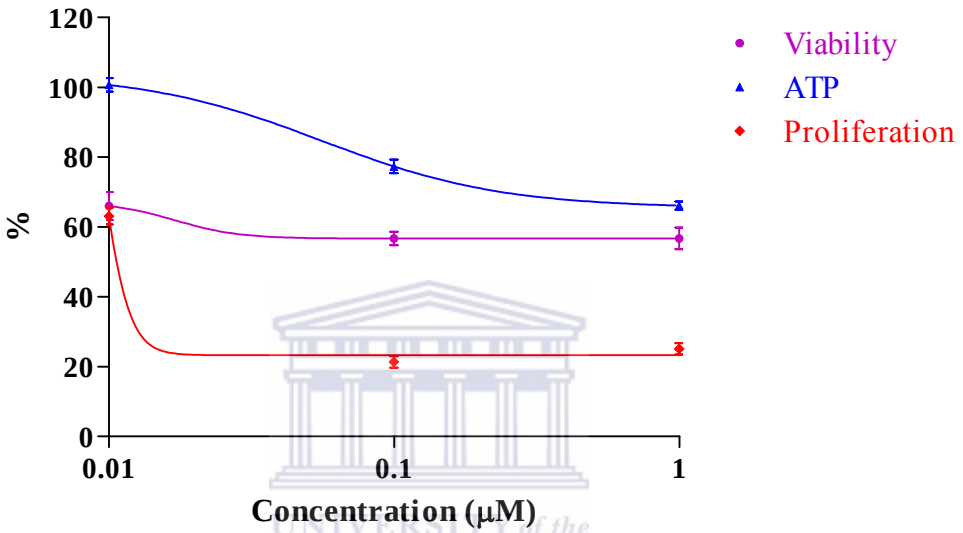

Fig. 63. The 24 hr effects of the combination Tm:Nic (1:1) ratio on viability, ATP and DNA proliferation.

Fig. 63 illustrates that viability remained similar at the concentrations analysed. ATP levels significantly decreased and reached 66.137% at 1 µM with % viability at 56.7% for the same concentration. DNA proliferation at 0.01 µM was similar to % viability, but was significantly decreased at 0.1 and 1 µM.

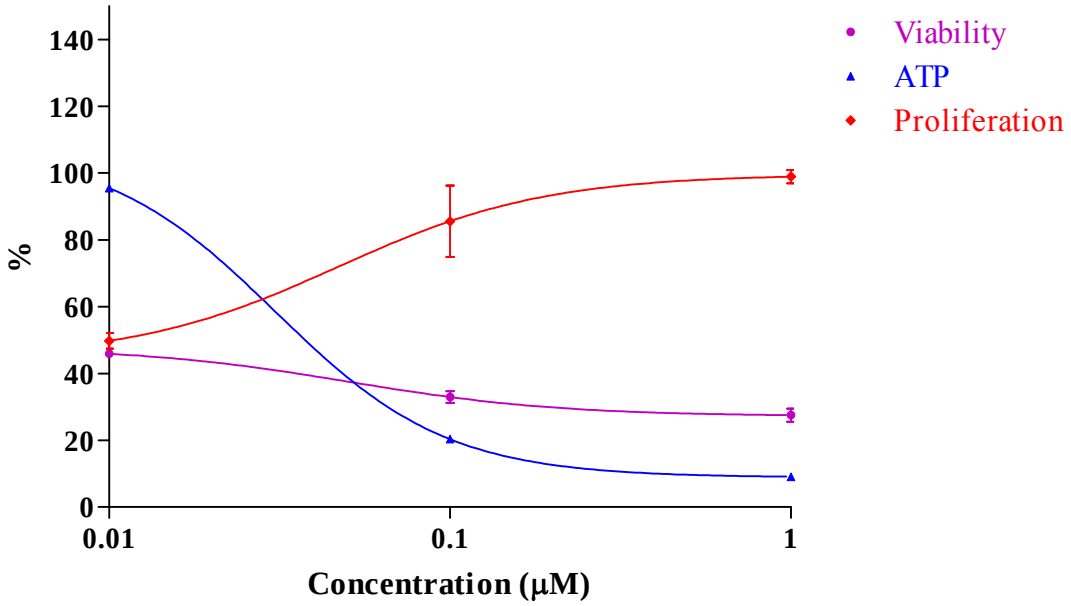

Fig. 64. The effects 48 hrs exposed MCF-7 cells to selected Tm:Nic (1:1) ratio concentrations.

At 48 hrs of Tm:Nic combinations, as depicted in Fig. 64, viability decreased from 45.901% to 27.56%. ATP production markedly declined as DNA synthesis significantly increased as the concentration of the combinations increased.

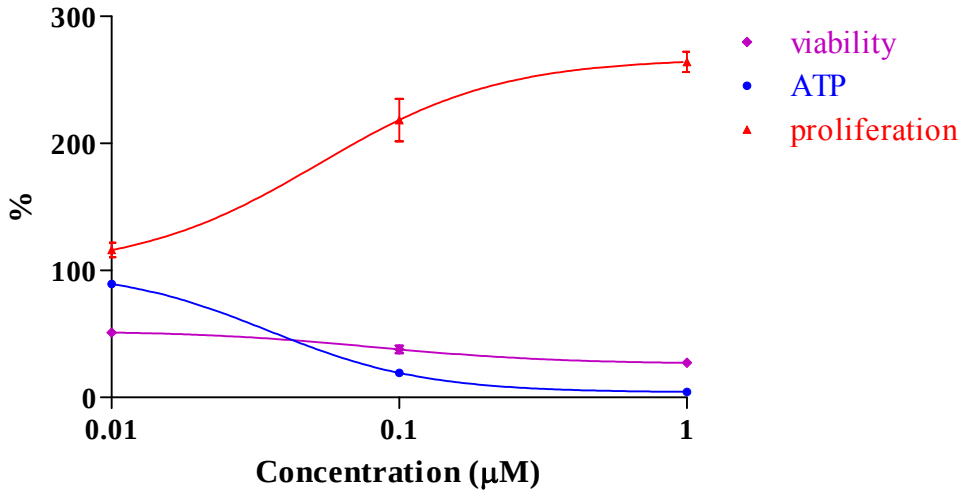

Fig. 65. The effect of Tm:Nic (1:1) ratio induced MCF-7 cells after 72 hrs.

Fig. 65 displays the effects of the 1:1 combination after 72 hrs. % viability and ATP production steadily decreased with the increase in concentration. DNA synthesis rose significantly between 0.01 and1 μM 1:1 ratio.

3.4.5 Early apoptotic effects on MCF-7 cells

Table 28: The effect of combination (1:1 ratio) of selected tunicamycin and nicotine concentrations on early apoptosis in MCF-7 cells after 24 hrs of exposure (20 000 events analysed)

Concentration (μM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.01	0.08	2.65	93.03	4.23
0.1	0.45	9.8	75.54	14.22
1	1.35	12.49	66.08	20.07

Table 28 shows the effect on early apoptosis of the 1:1 ratio of Tm:Nic combination after 24 hrs. Early apoptotic cell population increased by 20.07% at 1 μM when compared to control. The live cell population decreased by 16.95% between 0.01 and 1 μM

Table 29: The effect of combinations (1:1 ratio) of selected tunicamycin and nicotine concentrations on early apoptosis in MCF-7 cells after 48 hrs of exposure (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.01	0.95	3.74	94.63	0.69
0.1	4.25	90.22	0.66	4.87
1	2.15	90.27	0.44	7.13

Table 29 indicates that at 0.1 µM and 1 µM, 90.22% and 90.27% of the respective populations were dead due to apoptosis while early apoptotic cells remained low for both concentrations, when compared to control. 0.01 µM had no effect on early apoptosis.

Table 30: The effect of combinations (1:1 ratio) of selected tunicamycin and nicotine concentrations on early apoptosis in MCF-7 cells after 72 hrs of exposure (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.01	0.93	4.9	90.54	3.62
0.1	16.42	42.35	3.82	37.42
1	14	43.55	3.98	38.47

Table 30 shows that after 72 hrs at 0.1 and 1 µM of the combinations; 42.35% and 43.55% cells entered early apoptotic phase, respectively. For both concentrations, the live cell populations was below 4% and early apoptotic populations were at 37.42 and 38.47%, respectively compared to control. 0.01 µM had a live cell population of 90.54%.

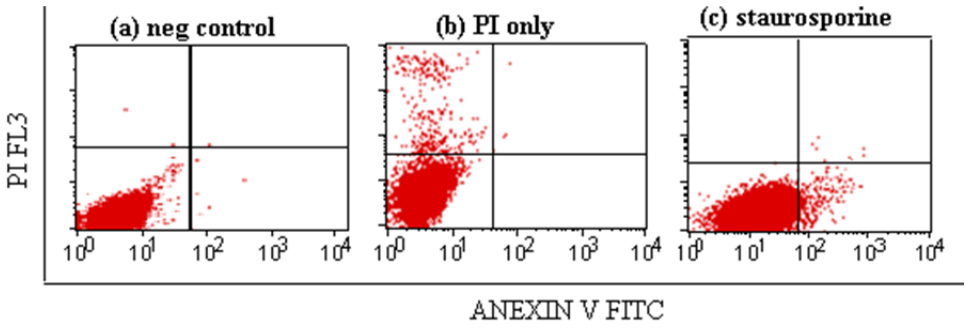

Fig. 66. The scatter plots created from flow cytometry analysis represent the controls used for comparison of the effects of the Tm:Nic (1:1) combination. a) Unstained MCF-7 cells, b) PI only stained MCF-7 cells and c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

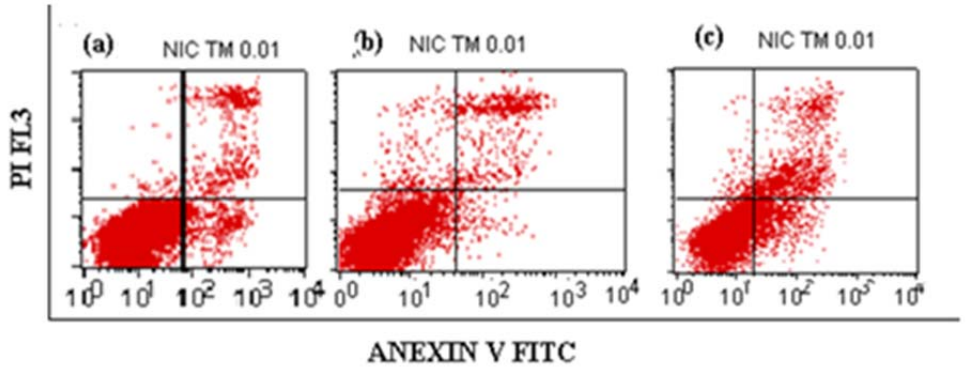

Fig. 67. Scatter plots depicting the effect of 0.01 µM of tunicamycin and nicotine combinations on MCF-7 cells at a) 24 hrs, b) 48 hrs and c) 72 hrs.

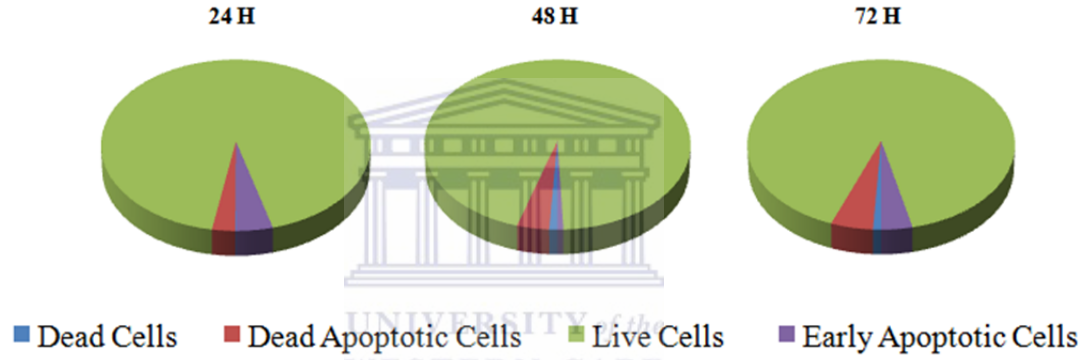

Fig. 68. The effects of 0.01 µM Tm:Nic on MCF-7 cells.

Fig. 67 and 68 displays the effect of 0.01 µM of the (1:1) combination of Tm:Nic over time. At 24 hrs, early apoptotic cells were similar to 48 hrs and 72 hrs of exposure. Dead apoptotic cells were also similar when compared at 24, 48 and 72 hrs.

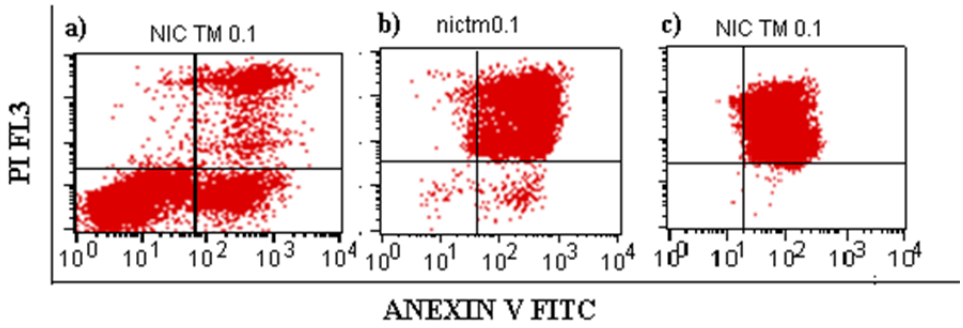

Fig. 69. Scatter plots depicting the effect of 0.1 µM of Tm:Nic combinations at a) 24 hrs, b) 48 hrs and c) 72 hrs.

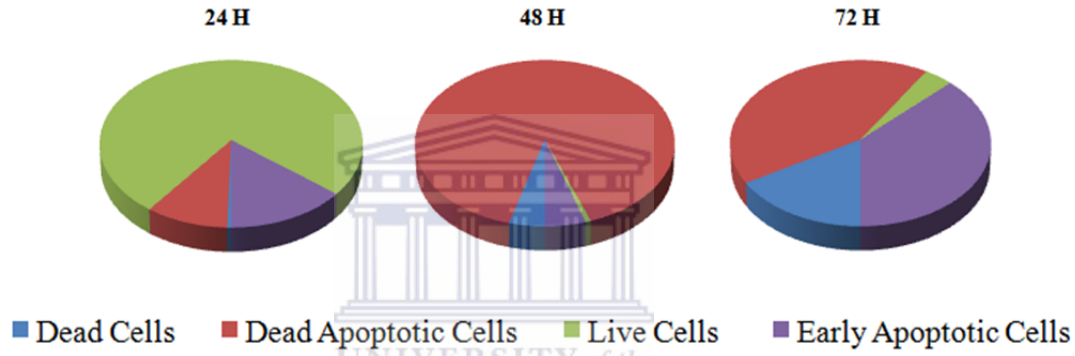

Fig.70. The effects of 0.1 µM Tm:Nic on MCF-7 cells.

Fig. 69 and 70, illustrates the change in population when MCF-7 cells were exposed to 0.1 µM of the Nic and Tm combination. Early apoptotic cell populations decreased from 14.22% at 24 hrs to 4.87% after 48 hrs. At 72 hrs, an increase of 37.42% in early apoptotic population was observed while cells dead due to apoptosis was at 42.35%

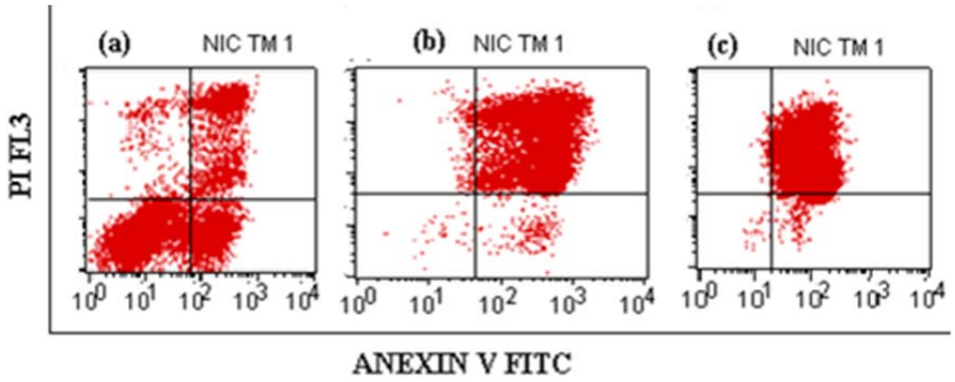

Fig. 71. Scatter plots depicting the effect of 1 µM of Tm:Nic at a) 24 hrs, b) 48 hrs and c) 72 hrs.

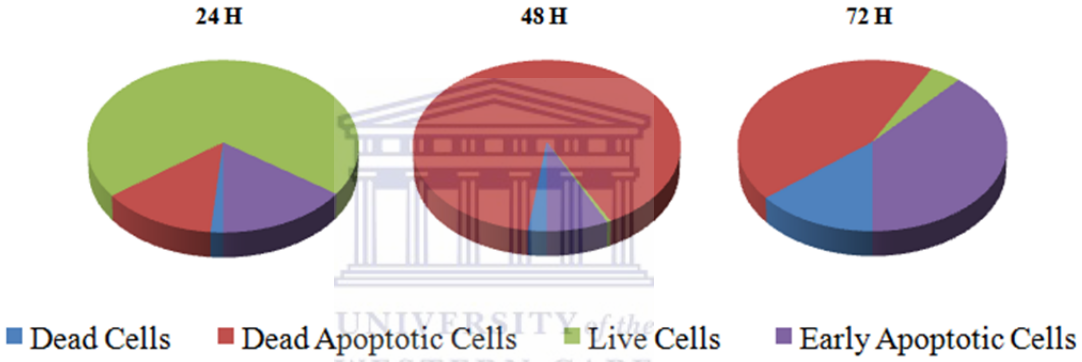

Fig. 72. The effects of 1 µM Tm:Nic on MCF-7 cells.

At 1 µM of Tm:Nic, 20% of the population was in early apoptosis after 24 hrs, which declined after 48 hrs to 7.13% and inclined after 72 hrs to 38.47%. Dead apoptotic cells at 24 hrs were at 12.49%, 48 hrs were at 90.27% and at 72 hrs were 43.55%, as shown in Fig. 71 and 72.

3.5 The combinatorial effect of Thapsigargin and Nicotine on MCF-7 cells

3.5.1 Effect on cell viability

Table 31: The effect of selected combinations (1:1) of thapsigargin and nicotine concentrations on MCF-7 cell viability (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.0001	110.369 ± 4.177	122.934 ± 5.218	105.015 ± 2.694
0.001	113.056 ± 2.656	115.756 ± 3.322	108.897 ± 4.315
0.01	89.099 ± 1.336	108.340 ± 1.885	105.051 ± 2.277
0.1	65.683 ± 2.756	49.848 ± 1.916	53.762 ± 9.925
1	62.954 ± 2.554	39.599 ± 1.270	29.013 ± 4.456
5	22.962 ± 3.316	15.404 ± 0.817	18.187 ± 1.009
50	-11.935 ± 0.720	-0.497 ± 0.478	4.474 ± 1.120

Table 31 shows the effects of Tg:Nic on MCF-7 % viability. At 0.1 and 1 nM, cell viability exceeded 100% across all time intervals and at 48 and 72 hrs for 10 nM concentration. A significant decline in viability was observed at 24 hrs between 1 µM and 50 µM. For 48 and 72 hrs (p<0.0001) and (p<0.0001) the significant decline was observed from 0.01 µM onwards.

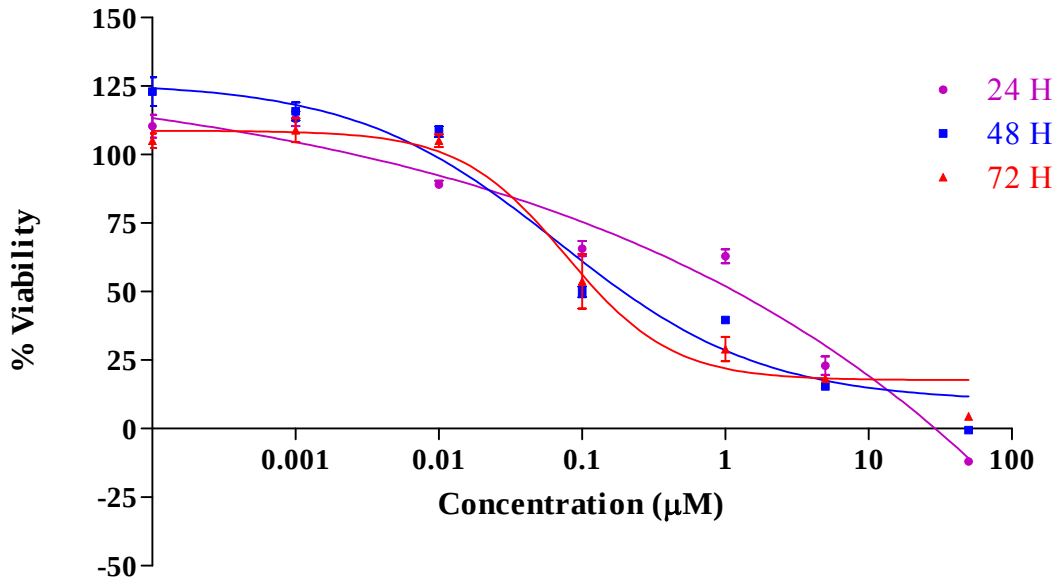

Fig. 73. Effects of various Tg:Nic (1:1) concentrations on MCF-7 cells viability.

Fig. 73 shows the effects of the combination between thapsigargin and nicotine on the MCF-7 cells after 24, 48 and 72 hours. 48 and 72 hrs shared similar trends while cells exposed for 24 hrs displayed delayed inhibitory effects.

3.5.2 Effect on ATP production

Table 32: The effect of selected combinations (1:1) of thapsigargin and nicotine concentrations on ATP production of MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.1	58.920 ± 2.154	16.705 ± 0.128	12.138 ± 0.546
1	57.282 ± 1.135	7.810 ± 0.141	5.340 ± 0.162
5	52.748 ± 1.576	8.125 ± 0.343	5.223 ± 0.106

Table 32 represents the ATP activity in MCF-7 cells when exposed to Tg:Nic combinations. No differences were observed at 24 hrs, between any concentrations tested, nor at 48 or 72 hrs of incubation. However, % ATP was significantly higher ($p \leq 0.0286$) when compared to 48 and 72 hrs.

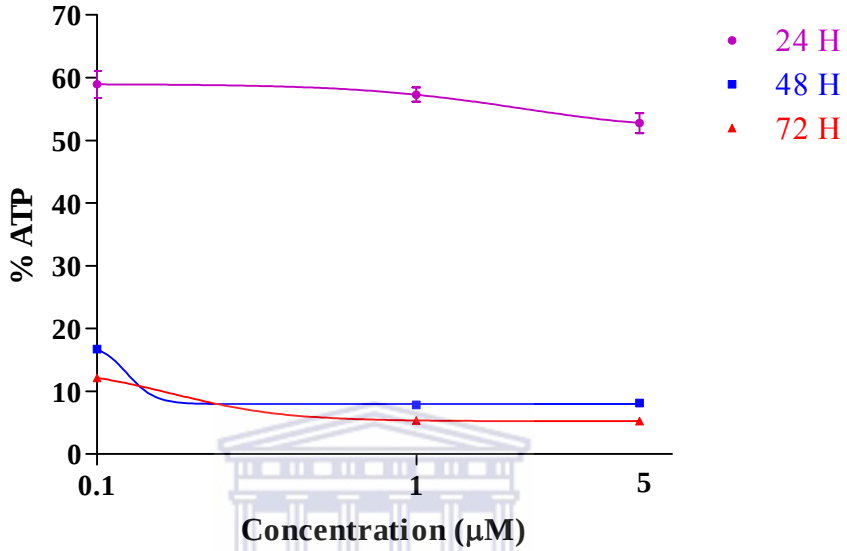

Fig. 74. The effects of selected Tg:Nic (1:1) concentrations on ATP production.

Fig. 74 illustrates the effects of the selected Tg:Nic concentrations on ATP production after 24, 48 and 72 hrs. At 24 hrs, ATP production was between a 50-60% range. However, after 48 and 72 hrs, ATP was noticeably reduced to below 20% and remained suppressed as the concentrations increased.

3.5.3 Effect on DNA proliferation

Table 33: The effect of selected combinations of thapsigargin and nicotine (1:1) concentrations on DNA proliferation of MCF-7 cells (Mean ± SEM)

Concentration (µM)	24 Hour	48 Hour	72 Hour
0.1	16.71± 1.422	190.005 ± 26.615	179.32 ± 8.098
1	16.255 ± 2.372	176.26 ± 23.817	218.383 ± 16.686
5	11.238 ± 1.134	171.203 ± 9.503	247.283 ± 9.111

Table 33 shows the effect Tg:Nic (1:1) had on DNA proliferation at each time interval. DNA levels were significantly lower ($p \leq 0.0286$) at 24 hrs when compared to both 48 and 72 hrs, for all concentrations analysed. Furthermore, % DNA was noticeably higher at 1 and 5 µM ratios at 72 hrs when compared to 48 hrs.

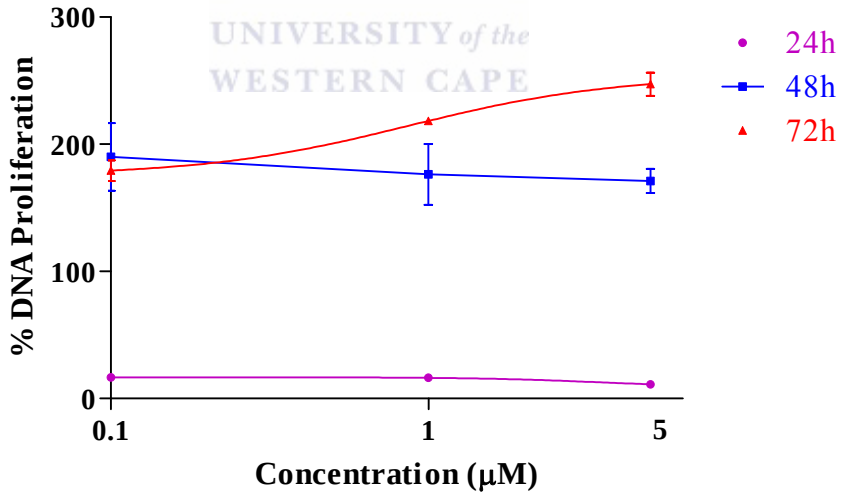

Fig. 75. The effect of selected concentrations of Tg:Nic (1:1) combinations on DNA synthesis in MCF-7 cells at 24, 48 and 72 hrs.

Fig. 75 compares the effects of Tg:Nic on MCF-7 cells' DNA proliferation after 24, 48 and 72 hrs. At 24 hrs a low level of proliferation was observed across concentrations, however, after 48 hrs, proliferation amplified significantly in percentage and decreased with a trend as concentrations increased. At 72 hrs, proliferation of the DNA remained significantly augmented and increased as concentration increased.

3.5.4 Effect on cells viability, ATP and DNA proliferation

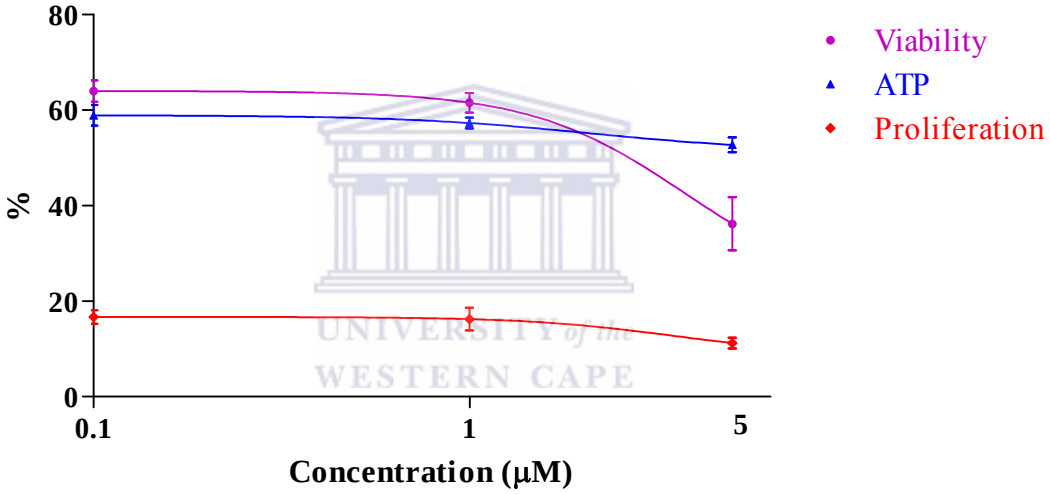

Fig. 76. The combinatorial effects of 24 hr Tg:Nic (1:1) on cell viability, ATP and DNA proliferation.

Fig. 76 represents the effects of thapsigargin and nicotine (1:1) combination of MCF-7 cells after 24 hrs. % viability and ATP were similar at 0.1 and 1 µM, however while ATP levels remained similar at 5 µM, % viability decreased significantly. The % DNA proliferation remained lower for all concentrations.

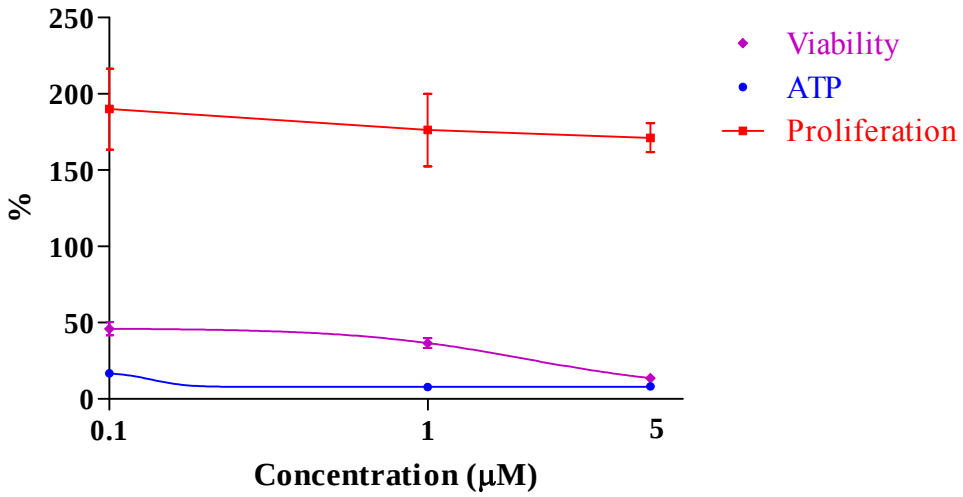

Fig. 77. The effect of 48 hr exposure on MCF-7 cells to selected Tg:Nic (1:1) concentrations.

After 48 hrs of exposure to the combination of thapsigargin and nicotine, Fig. 77 shows that although viability and ATP production was below 50%, DNA proliferation increased to above 150%.

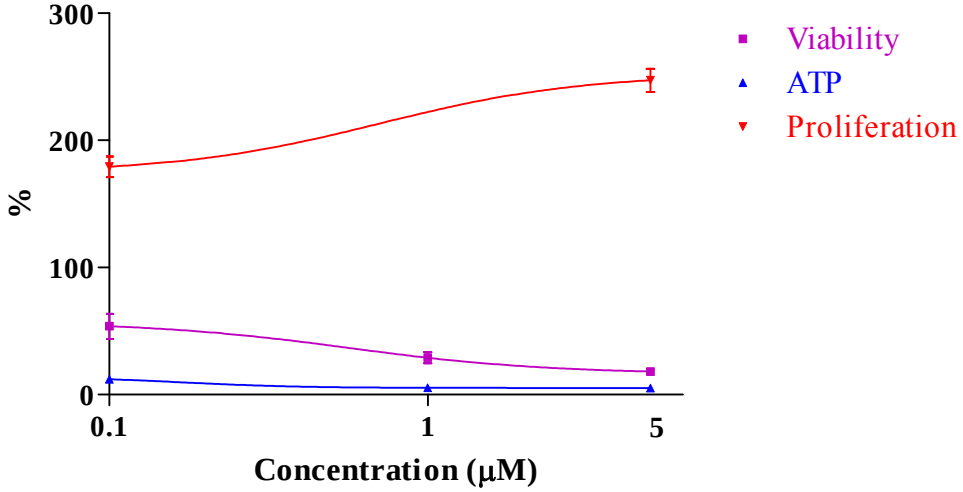

Fig. 78. The effect of Tg:Nic (1:1) induced MCF-7 cells after 72 hrs.

Fig. 78 graphically represents the effects of exposing MCF-7 cells to selected concentration of nicotine and thapsigargin (1:1) combination for 72 hrs. Viability and ATP production were suppressed while proliferation of DNA had exceeded 200%.

3.6.5 Early apoptotic effects on MCF-7 cells

Table 34: The effect of selected combinations of thapsigargin and nicotine (1:1 ratio) concentrations on early apoptosis in MCF-7 cells after 24 hrs of exposure (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.1	0.92	7.38	81.02	10.68
1	3.13	14.34	74.85	7.68
5	1.79	6.67	85	6.53

Table 34 shows that for all concentrations analysed live cell populations were above 74% and early apoptotic cells were less affected (between 6 and 11%). Similar trends were observed for dead apoptotic cells.

Table 35: The effect of selected combinations of thapsigargin and nicotine (1:1 ratio) concentrations on early apoptosis in MCF-7 cells after 48 hrs of exposure (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.1	8.3	75.85	7.49	8.36
1	0.42	88.23	3.42	7.93
5	1.26	83.07	8.87	6.79

Table 35 shows a reduction in live cell population across concentrations and an incline in dead apoptotic cell populations after 48 hrs. Early apoptotic cells remained under 10% for all concentrations investigated.

Table 36: The effect of selected combinations of thapsigargin and nicotine (1:1 ratio) concentrations on early apoptosis in MCF-7 cells after 72 hrs of exposure (20 000 events analysed)

Concentration (µM)	Dead Cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic Cells
0	0.02	0.01	99.91	0.06
0.1	2.07	4.42	91.04	2.48
1	1.11	3.44	91.75	3.71
5	10.63	39.33	5.51	44.53

Table 36 shows an increase in both apoptotic populations as concentrations increased. At 5 µM, 5.51% of the cells were alive and 44.53% were undergoing early apoptosis. At 0.1 µM and 1 µM, similar effects were observed.

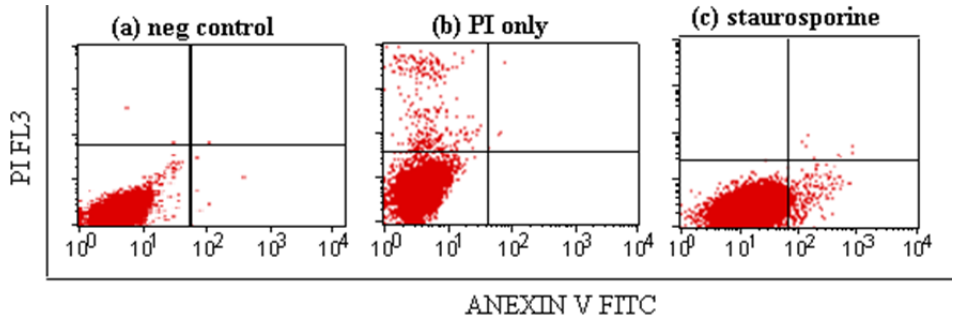

Fig. 79. The scatter plots created from flow cytometry analysis represent the controls used for comparison of the effects of the Tg:Nic combination. a) Unstained MCF-7 cells, b) PI only stained MCF-7 cells and c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

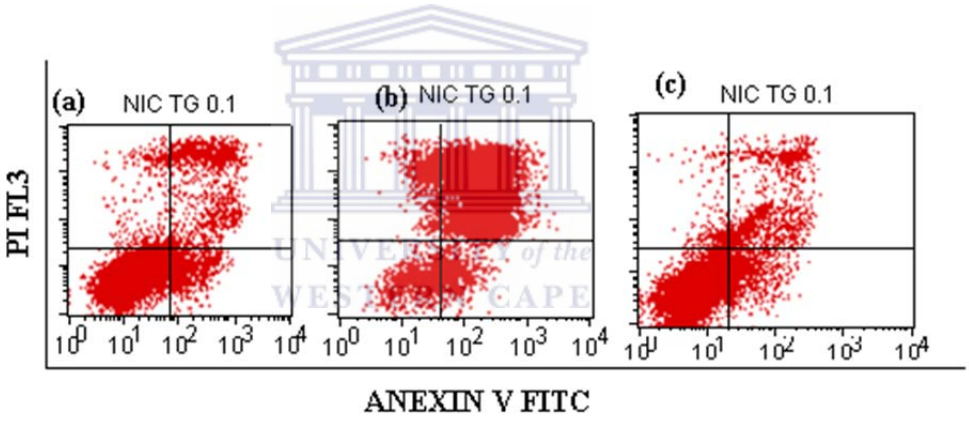

Fig. 80. Displays the scatter plots, a) 24 hr exposure b) 48 hrs and c) 72 hrs of Tg:Nic (1:1) exposure.

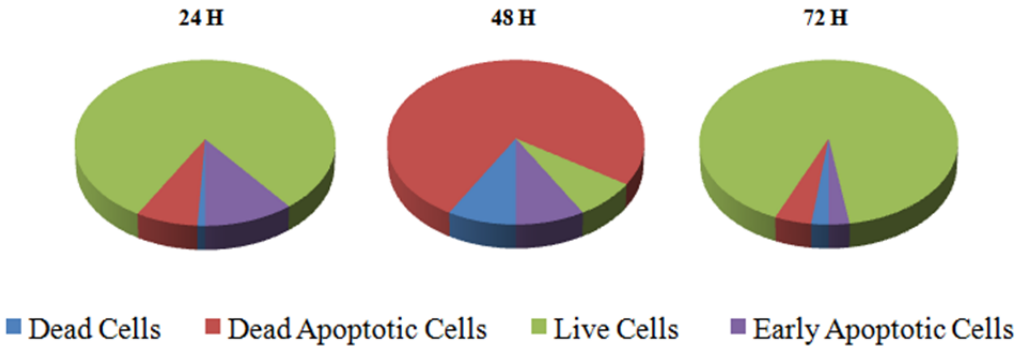

Fig. 81. The effects of 0.1 µM Tg:Nic on MCF-7 cells.

Fig. 80 and 81 illustrates that the combination of Tg:Nic. At 48 hrs, the dead cell population had increased to 75.85% and decreased to 4.48% at 72 hrs.

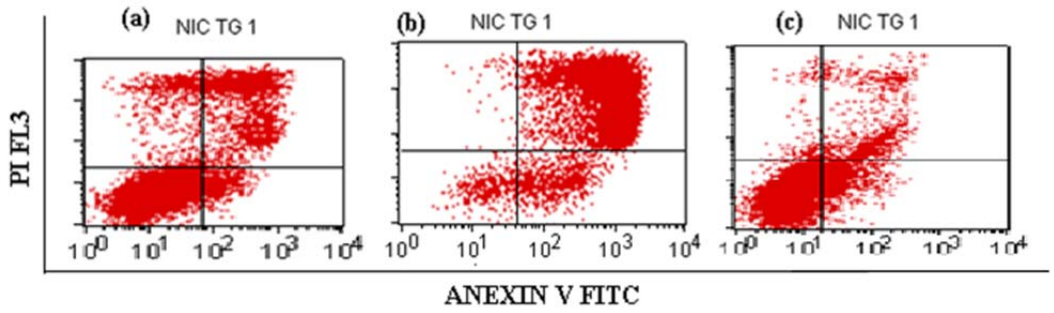

Fig. 82. Scatter plots depicting the effect of 1 µM of thapsigargin and nicotine combinations at a) 24 hrs, b) 48 hrs and c) 72 hrs.

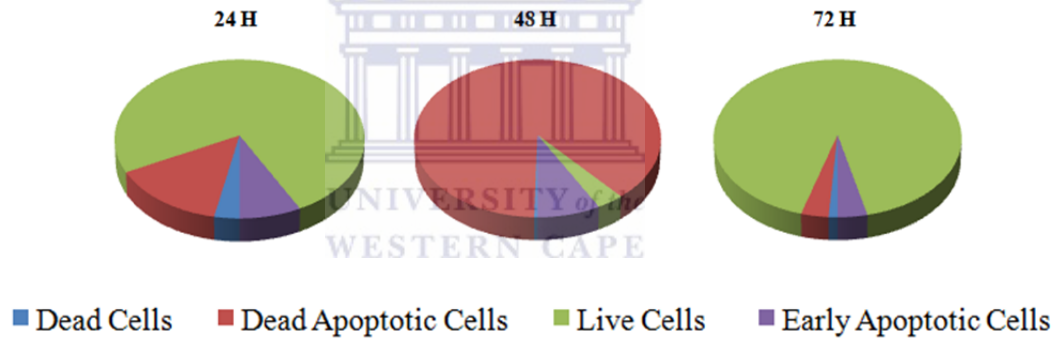

Fig. 83. The effects of 1 µM Tg:Nic on MCF-7 cells.

Fig. 82 and 83 show that, 88.23% were dead due to apoptosis after 48 hr exposure to 1 µM combination, which decreased to 3.44% at 72 hrs. Early apoptotic cells were inversely proportional to time and concentrations.

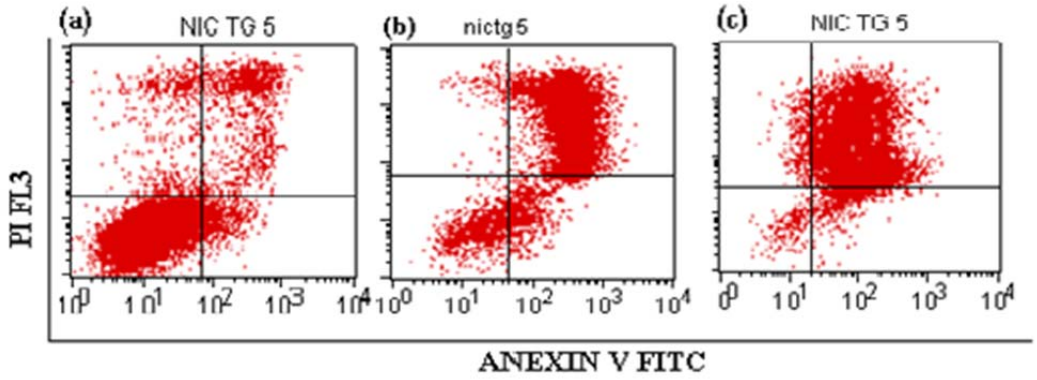

Fig. 84. Scatter plots depicting the effect of 5 µM of thapsigargin and nicotine combinations at a) 24 hrs, b) 48 hrs and c) 72 hrs.

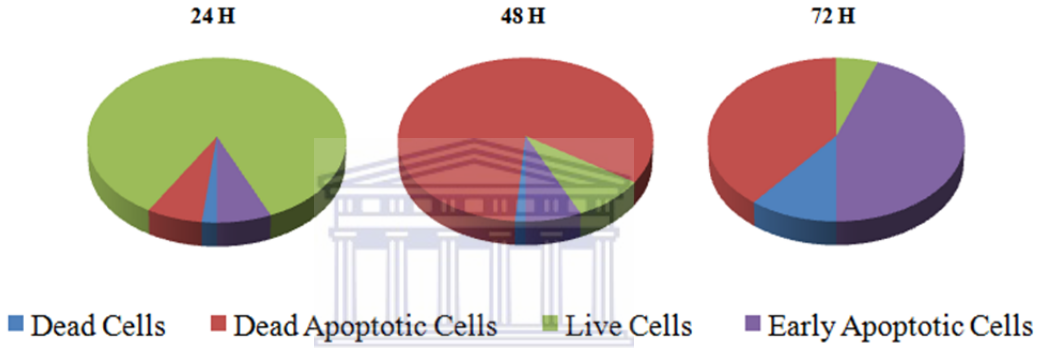

Fig. 85. The effects of 5 µM Tg:Nic on MCF-7 cells.

Fig. 84 and 85 show that between 24 and 72 hrs, early apoptotic cell population increased. Dead apoptotic cells between 48 and 72 hrs decreased from 83.07% to 39.33%, while the early apoptotic population increased to 37.74% .

3.6 The combinatorial effect of Tunicamycin and Thapsigargin on MCF-7 cells

3.6.1 Effect of 0.105 µM Tunicamycin in combination with selected Thapsigargin concentrations

3.6.1.1 Effect on cell viability

Table 37: The effect of selected thapsigargin concentrations in combination with 0.105 µM tunicamycin on MCF-7 cell viability (Mean ± SEM)

Time (Hours)	0.105 Tm		
	0.03 Tg	**0.06 Tg**	**0.12 Tg**
24	43.705 ± 0.648	48.541 ± 1.701	49.415 ± 2.448
48	29.155 ± 2.159	34.477 ± 0.910	11.122 ± 1.877
72	38.368 ± 2.169	22.00 ± 0.550	8.279 ± 1.484

Table 37 shows the effect of 0.105 µM Tm in combination with selected Tg concentrations. Viability was similar at 24 hrs for all concentrations analysed. The % viability did not exceed 49.415 ± 2.448%. At 48 hrs, the lowest and highest Tg combinatorial concentration of 0.03 and 0.12 µM displayed higher viability when compared to Tg 0.06 µM. The viability was significantly higher ($p \leq 0.0022$) at Tg 0.12 combination at 24 hrs when compared to 48 and 72 hrs.

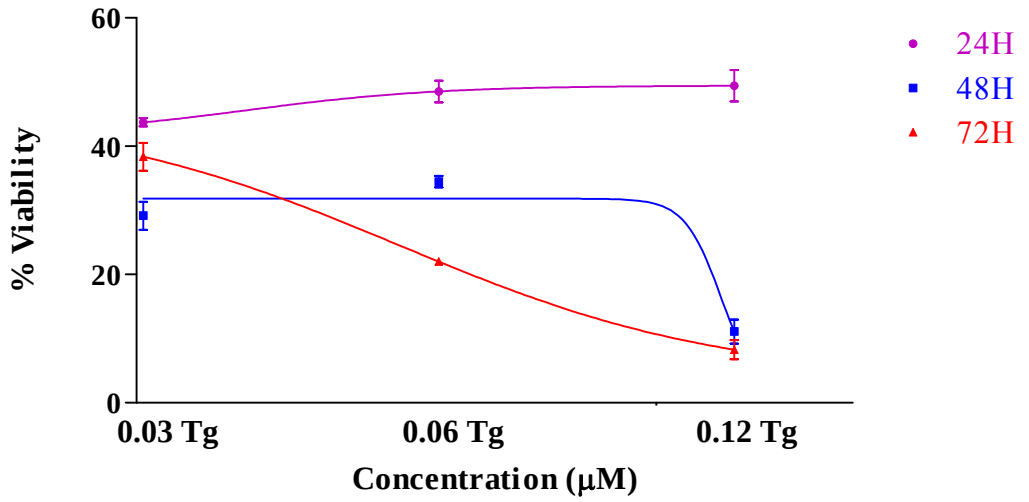

Fig. 86. The effect of selected Tg concentrations in combination with 0.105 µM Tm on the viability of MCF-7 cells.

Fig. 86 shows the effects of various selected Tg concentrations against 0.105 µM of Tm, for the various time intervals. Viability remained similar at 24 hrs while a marked decrease was noted at the higher concentration combinations at 48 hrs. At 72 hrs, a steady decrease in viability occurred as concentrations increased. The intermediate Tg concentration combined with Tm, had the greatest inhibitory effects on viability.

3.6.1.2 Effect on ATP production

Table 38: The effect of selected thapsigargin concentrations in combination with 0.105 μM tunicamycin on ATP production in MCF-7 cells (Mean ± SEM)

Time (Hours)	0.105 Tm		
	0.03 Tg	**0.06 Tg**	**0.12 Tg**
24	62.962 ± 1.464	58.52 ± 1.531	55.076 ± 0.995
48	13.288 ± 0.702	12.345 ± 0.566	11.413 ± 0.473
72	9.251 ± 0.414	7.414 ± 0.115	6.594 ± 0.118

Table 38 displays the effect of thapsigargin and tunicamycin combinations on MCF-7 cells' ATP production. For all concentrations ATP production remained the same for each time interval. Each concentration combination over time resulted in a significant decrease ($p \leq 0.0286$) between 24 and 48 hrs, while no differences were observed between 48 and 72 hrs.

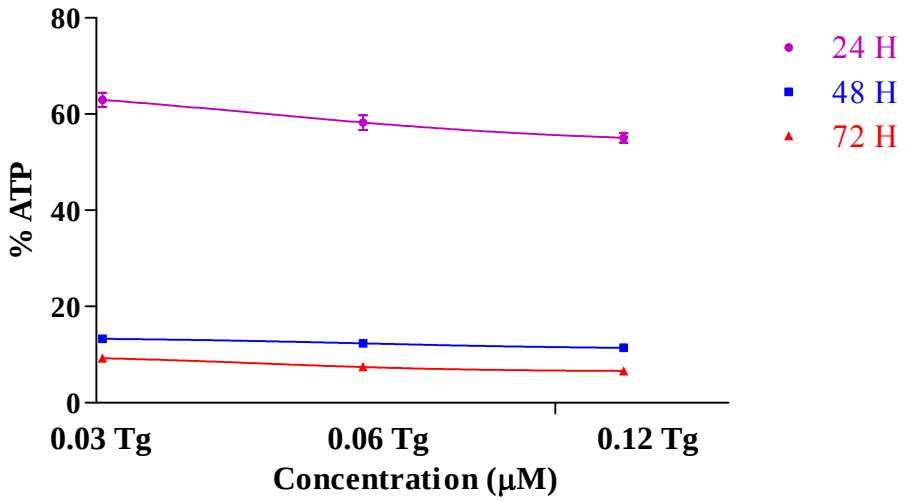

Fig. 87. The effect of selected concentrations of Tg in combination with 0.105 µM Tm on ATP Production in MCF -7 cells.

Fig. 87 displays a statistical inhibition in % ATP after 48 and 72 hrs ($p \leq 0.0286$). At 48 and 72 hrs, ATP levels remained the same at all concentrations analysed.

3.6.1.3 Effect on DNA proliferation

Table 39: The effect of selected thapsigargin concentrations in combination with 0.105 µM tunicamycin on DNA proliferation of MCF-7 cells (Mean ± SEM)

Time (Hours)	0.105 Tm		
	0.03 Tg	**0.06 Tg**	**0.12 Tg**
24	35.894 ± 2.676	33.287 ± 2.524	22.440 ± 6.239
48	88.56 ± 8.362	102.457 ± 9.775	80.503 ± 8.681
72	65.243 ± 8.365	45.935 ± 4.629	48.790 ± 7.170

Table 39 displays the effect of the combined Tm and Tg concentrations on DNA proliferation in MCF-7 cells. DNA proliferation increased between the combination of Tm and Tg 0.03 and 0.12 µM at 24 and 48 hrs were similar. However, at Tg 0.06 (p=0.0238) when comparing 24 to 48 hr proliferation. No differences were observed between 0.03 µM and 0.06 µM Tg combinations at 24 hrs, however the % DNA proliferation increased at 48 hrs. DNA proliferation was similar between 0.03, 0.06 and 0.12 µM Tg combination at 72 hrs. Significant differences were only observed between 24 and 48 hrs at 0.06 µM Tg (p=0.0238) and between 24 and 72 hrs at 0.06 µM Tg (p=0.0381).

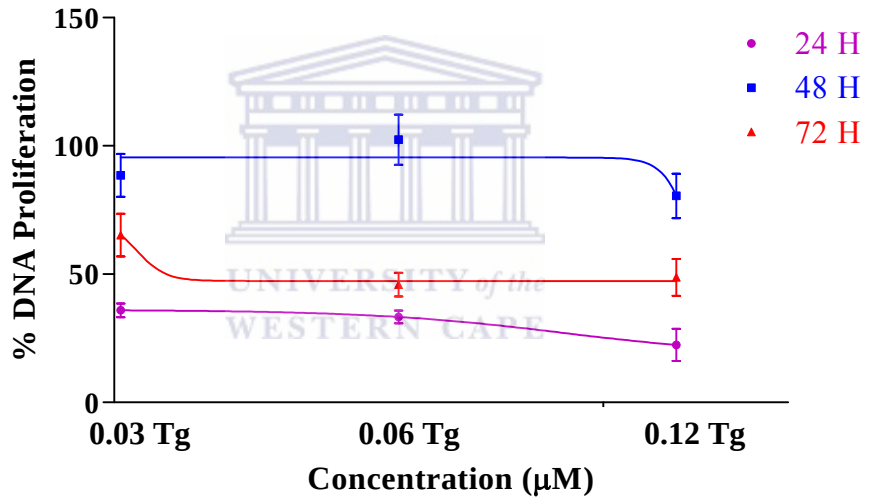

Fig. 88. The effect of selected Tg concentrations in combination with 0.105 µM Tm on DNA proliferation in MCF-7 cells.

DNA proliferation between 24 and 48 hrs saw an increase while between 48 and 72 hrs, % DNA proliferation decreased. The highest % proliferation was observed at 48 hrs.

3.6.1.4 Effect on cell viability, ATP and DNA proliferation

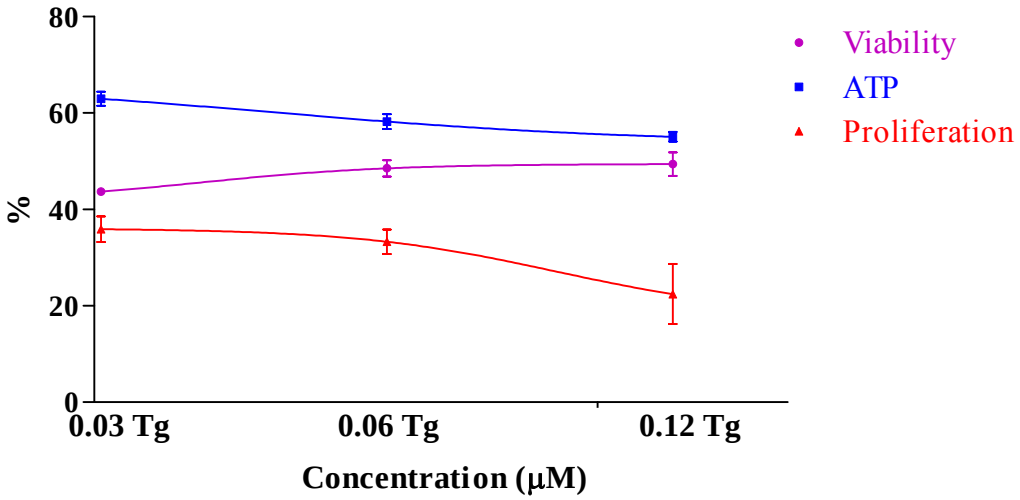

Fig. 89. The effect of 0.105 µM Tm in combination with various Tg concentrations after 24 hrs of exposure.

Fig. 89 illustrates the effect of Tm and Tg combinations on viability, ATP activity and DNA proliferation, at 24 hrs. It was observed that, viability was approximately 50% for all concentrations, and ATP activity was slightly elevated with a significant 20% increase at the Tg 0.03 combination. % DNA proliferation however, decreased as concentrations increased.

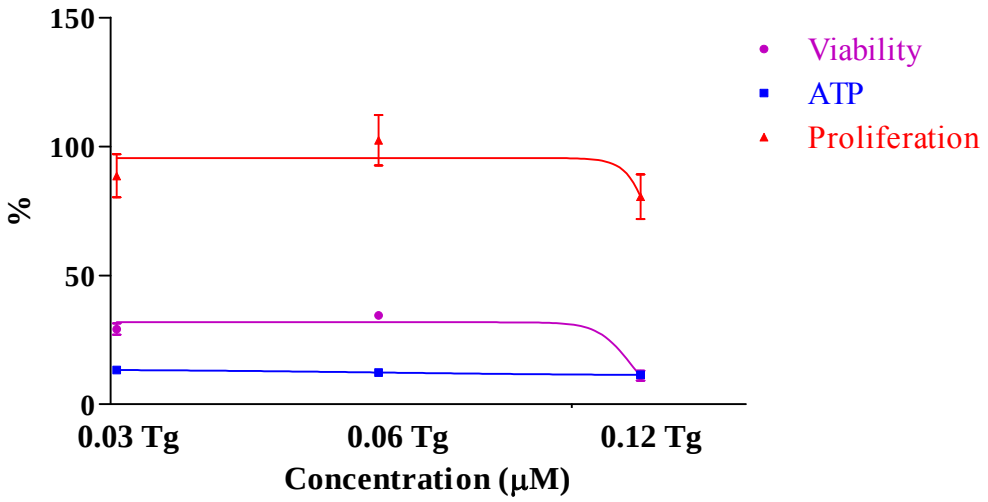

Fig. 90. The effect of Tm 0.105 µM in combination with various Tg concentrations at 48 hrs exposure on % viability, % ATP and % DNA proliferation.

After 48 hrs, both viability and ATP levels are below 40%. However, % DNA proliferation was significantly higher at approximately 80% for all combinations of concentrations.

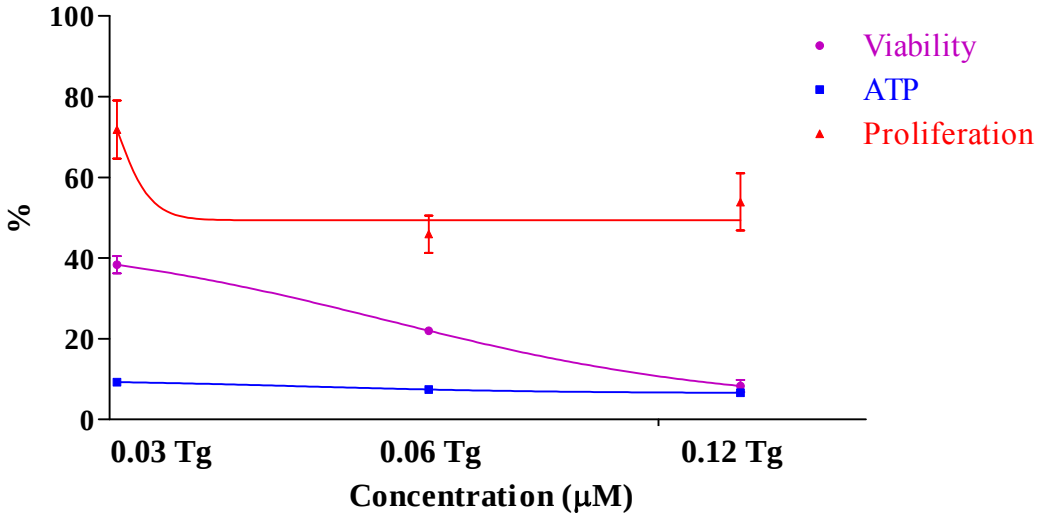

Fig. 91. The graph represents the effect of Tm 0.105 µM in combination with various Tg concentrations at 72 hrs.

After 72 hrs viability decreased as the Tg concentrations increased (Fig. 91). ATP activity remained below 20% for all concentrations analysed. The only statistical significance observed was between combinations of 0.105 μM Tm and 0.03 μM Tg. DNA proliferation when compared to viability and ATP, was elevated after 72 hrs.

3.6.1.5 Early apoptotic effects on MCF-7 cells

Table 40: The effect of selected thapsigargin concentrations in combination with 0.105 μM tunicamycin on early apoptosis in MCF-7 cells after 24 hrs (20 000 events analysed)

Concentration (μM)		**Dead cells**	**Dead Apoptotic Cells**	**Live Cells**	**Early Apoptotic cells**
0.105 Tm	**0.03 Tg**	0.35	9.74	80.56	9.35
	0.06 Tg	0.84	9.87	77.95	11.34
	0.12 Tg	1.56	14.77	70.73	12.91

Table 40 displays that for all combinations, at 24 hrs, the majority of the populations remained unaffected. The cell population in early apoptosis did not exceed 12.91% for all concentration combinations.

Table 41: The effect of various thapsigargin concentrations in combination with 0.105 µM tunicamycin on early apoptosis in MCF-7 cells after 48 hrs (20 000 events analysed)

Concentration (µM)		Dead cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic cells
0.105 Tm	**0.03 Tg**	8.02	85.76	0.51	5.71
	0.06 Tg	2.74	89.69	0.58	6.99
	0.12 Tg	3.11	90.19	1.59	5.11

Table 41 shows that on average 89% of the population, at all combinatorial concentrations, were dead due to apoptosis and approximately 5.5% of the cells were undergoing early apoptosis.

Table 42: The effect of various thapsigargin concentrations in combination with 0.105 µM tunicamycin on early apoptosis in MCF-7 cells after 72 hrs (20 000 events analysed)

Concentration (µM)		Dead cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic cells
0.105 Tm	**0.03 Tg**	16.82	44.49	2.83	35.86
	0.06 Tg	11.61	47.27	2.05	39.07
	0.12 Tg	10.38	57.85	0.76	31.01

Table 42 demonstrates that after 72 hrs of exposure to the various combinations of Tm and Tg, an average of 49% of the population analysed, were dead due to apoptosis and approximately 33% of the population were undergoing early apoptosis.

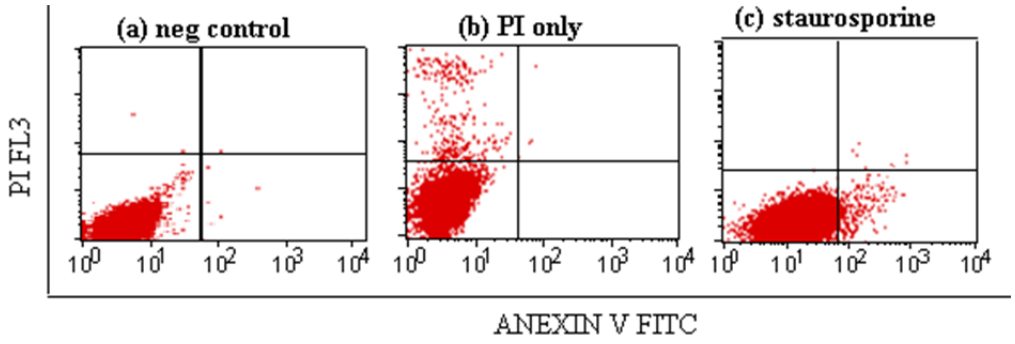

Fig. 92. Flow cytometry controls. a) Unstained MCF-7 cells, b) PI only stained MCF-7 cells and c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

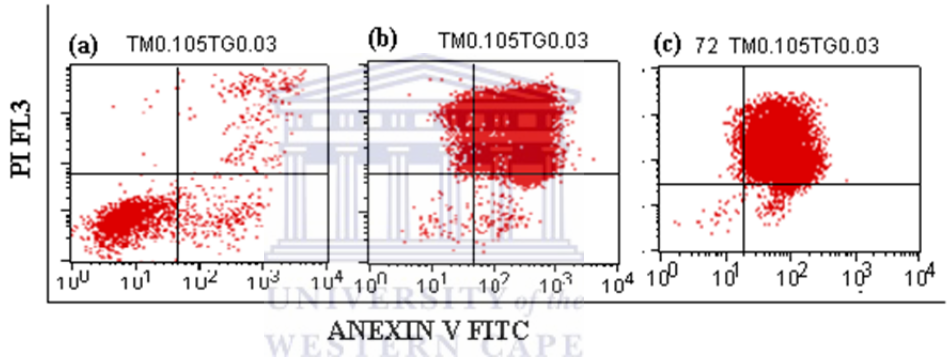

Fig. 93. Scatter plots depicting 0.105 Tm:0.03 µM Tg combination at a) 24 hrs, b) 48 hrs and c) 72hrs.

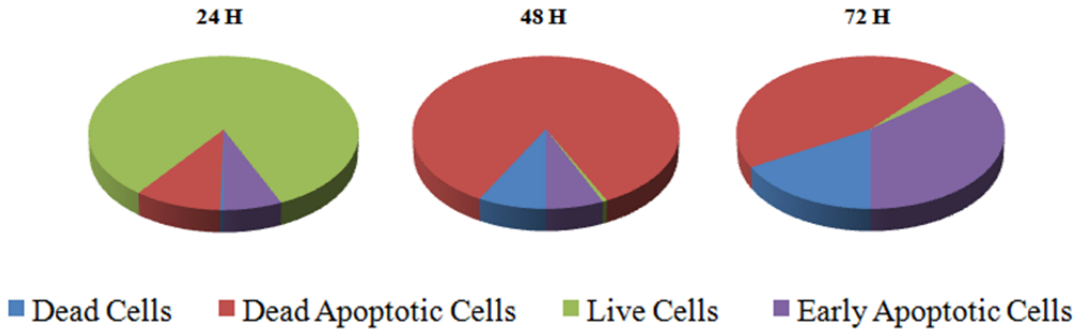

Fig. 94. The effects of 0.105 Tm:0.03 µM Tg on MCF-7 cells.

Fig. 93 and 94 shows the effects of the 0.105 Tm:0.03 µM Tg combination over time. As time increased the amount of dead cells increased. At 48 hrs approximately

85.76% were dead apoptotic cells and, at 72 hrs the population decreased to approximately 45%, with 35.86% of cells in early apoptotic phase.

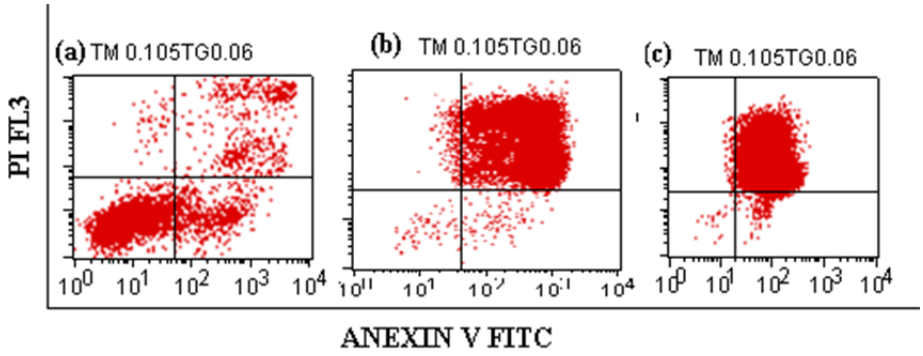

Fig. 95. Scatter plots depicting the effects of 0.105 Tm:0.06 µM Tg combinations at a) 24 hrs, b) 48 hrs and c) 72hrs.

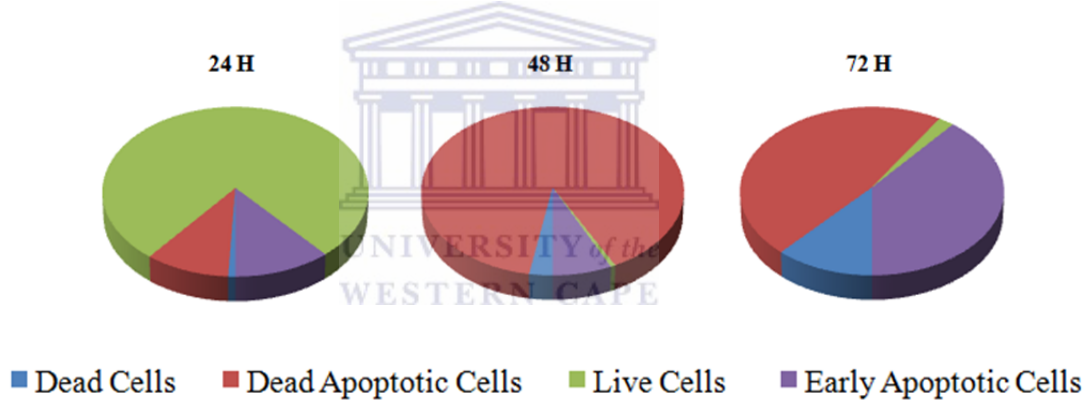

Fig. 96. The effects of 0.105 Tm:0.06 µM Tg on MCF-7 cells.

Early apoptotic cell populations increased as incubation times increased (Fig. 95 and 96). Dead apoptotic cells increased after 48 hrs, but decreased after 72 hrs of exposure to the 0.105 µM Tm and 0.06 µM Tg combinations. Early apoptotic population increased by 28.73% between 24 and 72 hrs.

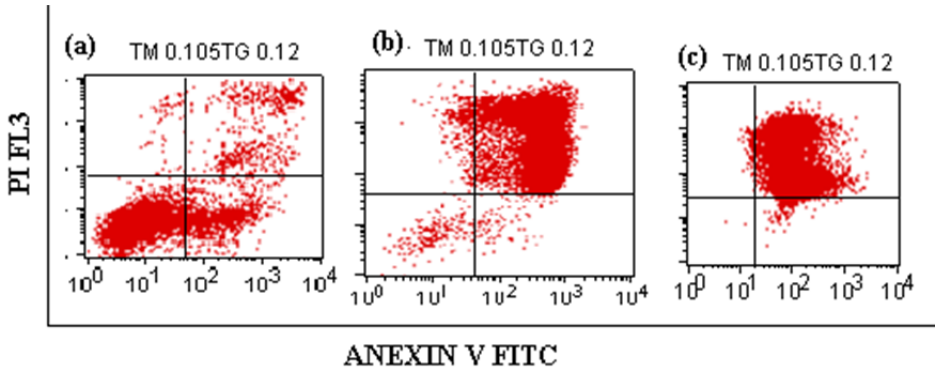

Fig. 97. Scatter plot depicting the effect of 0.105 Tm:0.12 µM Tg combination at a) 24 hrs, b) 48 hrs and c) 72hrs.

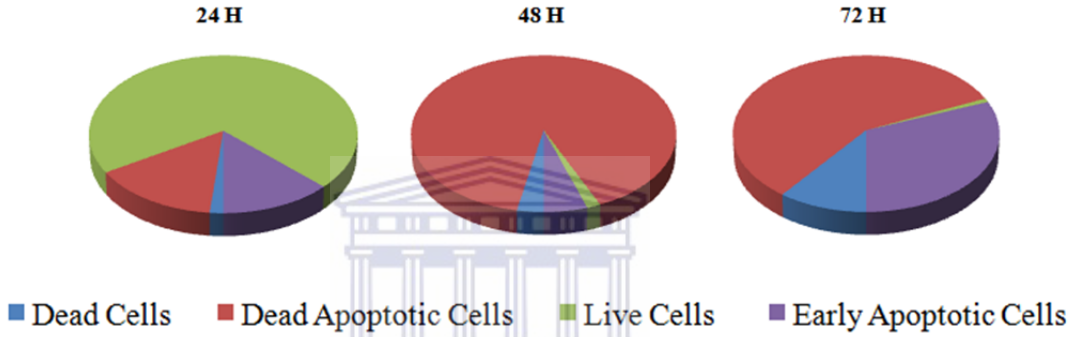

Fig. 98. The effects of 0.105 Tm:0.12 µM Tg on MCF-7 cells.

MCF-7 cells exposed to 0.105 Tm:0.12 µM Tg over time (Fig. 97 and 98) showed an increase in dead apoptotic cells after 48 hrs. At 72 hrs the population of dead apoptotic cells decreased to 57.85%, while early apoptotic cells increased as incubation time doubled. After 48 hrs, live cell populations decreased below 1%.

3.6.2 Effect of 0.06 µM Thapsigargin in combination with selected Tunicamycin concentrations

3.6.2.1 Effect on cell viability

Table 43: The effect of selected tunicamycin concentrations in combination with 0.06 µM thapsigargin on MCF-7 cell viability (Mean ± SEM)

Time (Hours)	0.06 Tg		
	0.0525 Tm	**0.105 Tm**	**0.21 Tm**
24	48.745 ± 0.932	46.816 ± 2.311	50.986 ± 3.486
48	22.108 ± 0.635	34.477 ± 0.910	19.412 ±2 .480
72	19.018 ± 1.448	22.00 ± 0.550	31.862 ± 3.748

Table 43 shows the effect of 0.06 µM Tg in combination with various tunicamycin concentrations on the cell viability of MCF-7 breast carcinoma cells. The combinatorial effect at 24 hrs was similar at all concentrations. At 48 hrs, viability was higher ($p \leq 0.0022$) at 0.105 µM combination when compared to 0.0525 and 0.12 µM Tm. Viability at 72 hrs, showed that the highest Tm concentration combination resulted in the highest viability.

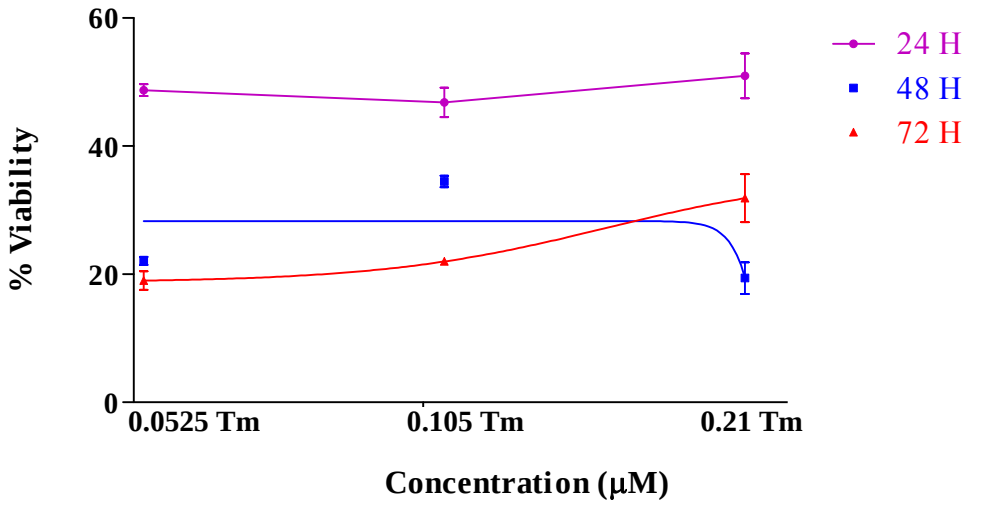

Fig. 99. The combinatorial effect of selected Tm concentrations with 0.06 µM Tg on MCF-7 cell viability.

Fig. 99 illustrates the effect of the various Tm concentrations against 0.06 µM Tg. A significantly elevated ($p \leq 0.0043$) % viability was observed at 24 hrs when compared to 48 and 72 hrs.

3.6.2.2 Effect on ATP production

Table 44: The effect of selected tunicamycin concentrations in combination with 0.06 µM thapsigargin on ATP production in MCF-7 cells (Mean ± SEM)

Time (Hours)	0.06 Tg		
	0.0525 Tm	**0.105 Tm**	**0.21 Tm**
24	61.588 ± 0.898	58.52 ± 1.531	58.798 ± 1.369
48	18.723 ± 1.044	12.345 ± 0.566	10.99 ± 0.637
72	6.398 ± 0.151	7.414 ± 0.115	7.051 ± 0.359

Table 44 illustrates the effects of the Tg:Tm combinations on ATP production in MCF-7 cells. ATP production was the same for all combinations at 24 hrs, but did not exceed 61.588 ± 0.898%. A significant decrease ($p \leq 0.0286$) was noticed between 24 and 48 hrs, and 48 and 72 hrs for all combinatorial concentrations. No differences were observed between 0.105 µM Tm and 0.21 µM Tm, for both 48 and 72 hrs. However, a decrease was noted ($p=0.0286$) between 0.0525 and 0.105 µM Tm at 48 hrs, while no difference in % ATP was observed at 72 hrs.

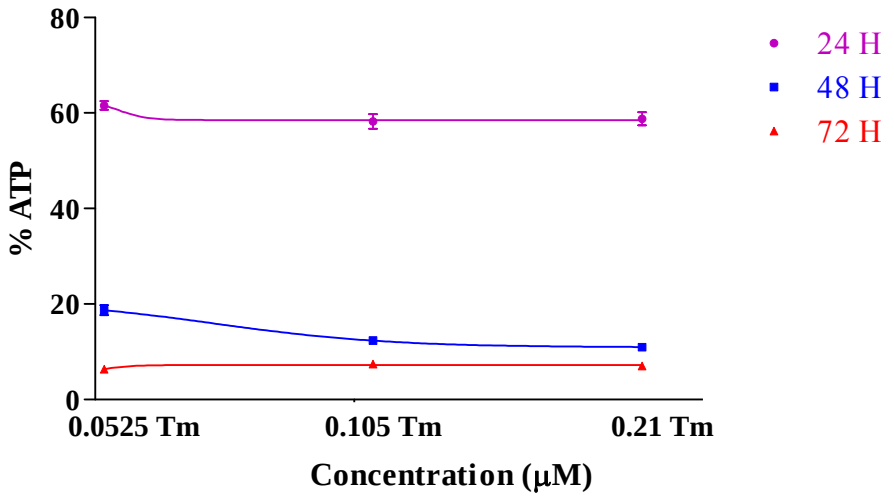

Fig. 100. The effect of various Tm concentrations in the presence of 0.06 µM Tg on % ATP activity in MCF-7 cells.

Fig. 100 indicates that after 48 ($p \leq 0.0286$) and 72 hrs ($p \leq 0.0286$) of exposure a significant inhibition of ATP was observed in comparison with 24 hrs. Furthermore after 48 hrs ATP was below 20% and remained so even after 72 hrs.

3.6.2.3 Effect on DNA proliferation

Table 45: The effect of selected tunicamycin concentrations in combinations with 0.06 μM thapsigargin on DNA proliferation in MCF-7 cell (Mean ± SEM)

Time (Hours)	0.06 Tg		
	0.0525 Tm	**0.105 Tm**	**0.21 Tm**
24	32.00 ± 3.831	33.287 ± 2.524	37.256 ± 1.261
48	89.283 ± 13.019	102.457 ± 9.775	82.36 ± 10.714
72	55.978 ± 3.899	45.935 ± 4.629	54.51 ± 5.4584

No differences were observed between the various combinations for all time intervals. % DNA proliferation increased at 48 hrs, however it was only significant when compared to 24 hrs (p=0.0238) at 0.105 μM Tm. It decreased between 48 and 72 hrs and did not exceed 55.98%.

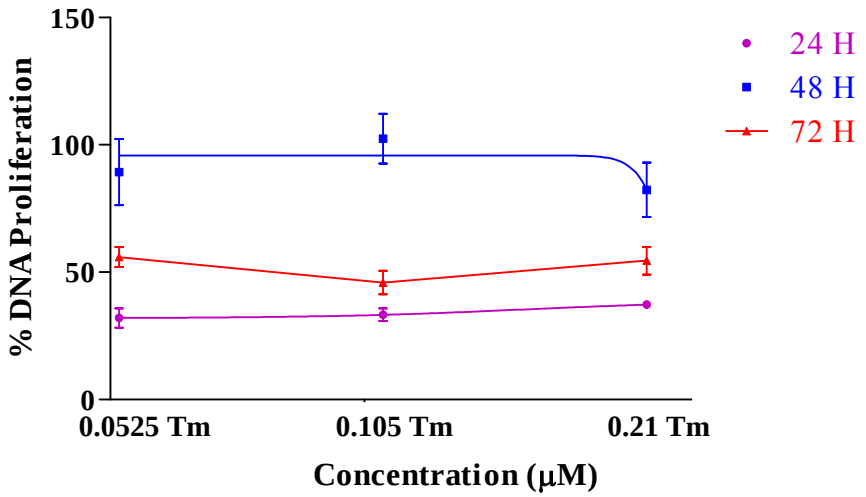

Fig. 101. The effect of 0.06 µM Tg in combination with selected Tm concentrations on DNA proliferation.

After 48 hrs, a significant escalation in proliferation was detected. However after a further 24 hrs an approximate 40% decrease in proliferation was observed.

3.6.2.4 Effect on cell viability, ATP and DNA proliferation

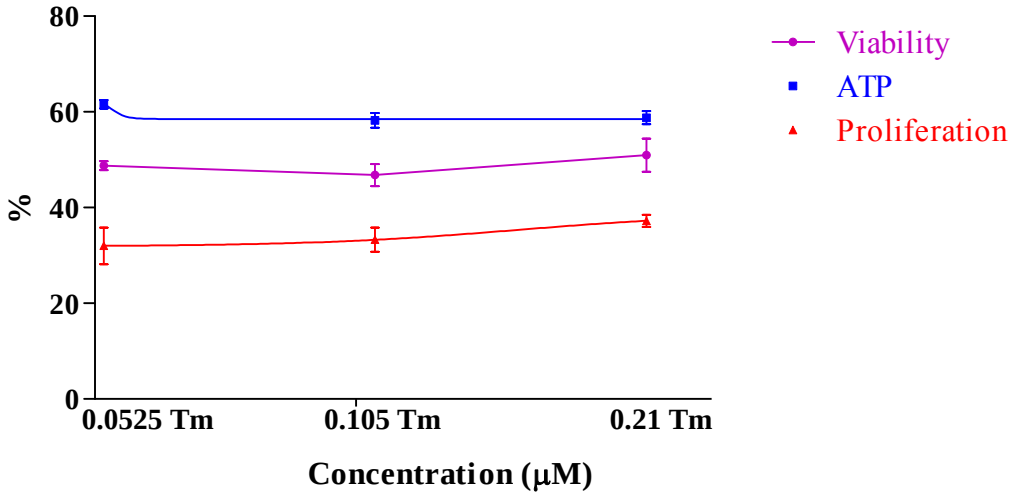

Fig. 102. The effect of 0.06 µM Tg in combination with various Tm concentrations after 24 hrs exposure.

Fig. 102, illustrates the effect of the combinations on viability, ATP activity and DNA proliferation. At 24 hrs it was observed that, ±50% viability was reached for all concentrations, ATP activity was at approximately 60% and proliferation did not exceed 37.3%.

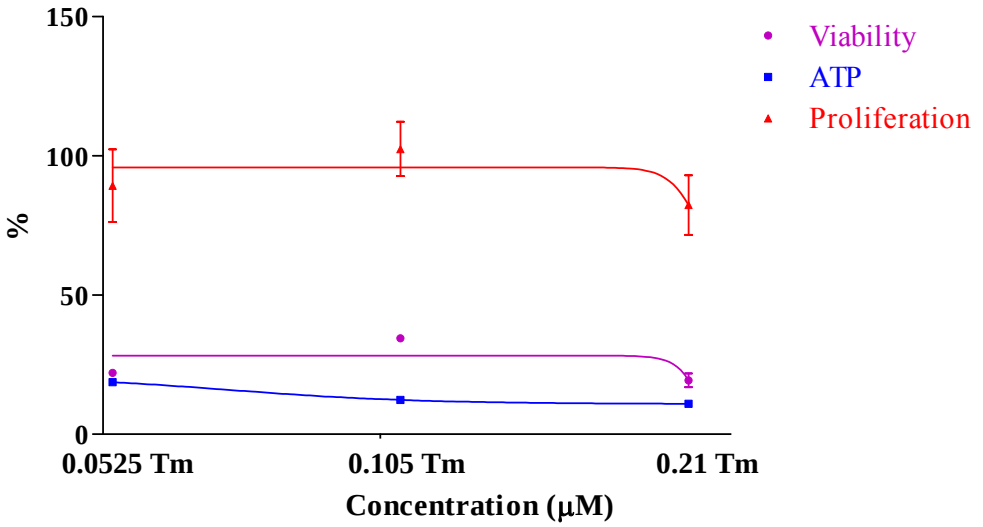

Fig. 103. The effect of 0.06 µM Tg in combination with various Tm concentrations at 48 hrs exposure on % viability, % ATP and % DNA proliferation.

After 48 hrs, both viability and ATP levels were below 40%. However, % DNA proliferation was significantly higher with at least a 50% increase across all combination concentrations.

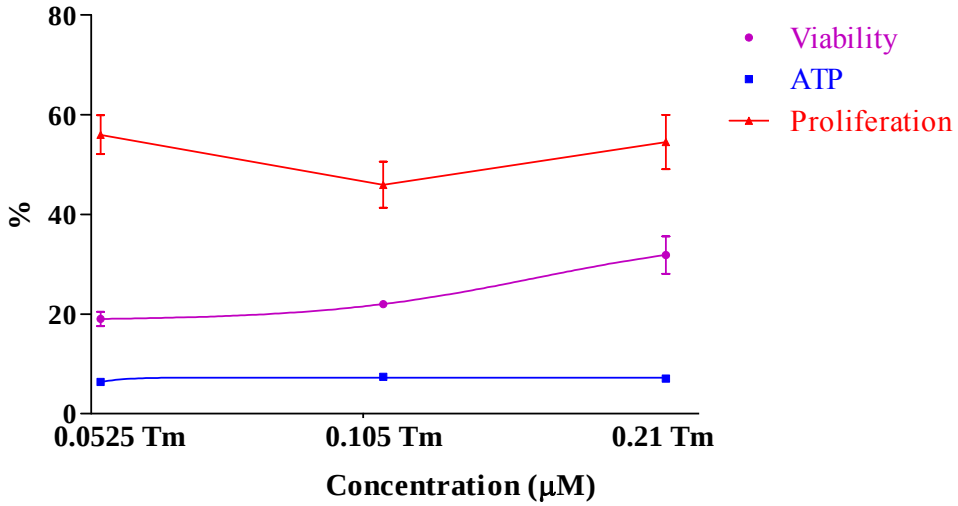

Fig. 104. The graph represents the effect of 0.06 µM Tg in combination with various Tm concentrations at 72 hrs.

As concentrations increased (Fig.104) an increase in viability was observed but did not exceed 31.7%. ATP activity remained below 10% for all concentrations analysed. DNA proliferation did not go below 45.94%.

3.6.2.5 Early apoptotic effects on MCF-7 cells

Table 46: The effect of selected tunicamycin concentrations in combination with 0.06 µM thapsigargin on early apoptosis in MCF-7 cells after 24 hrs (20 000 events analysed)

Concentration (µM)		Dead cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic cells
0.06 Tg	**0.0525 Tm**	1.23	40.17	51.98	6.62
	0.105 Tm	0.84	9.87	77.95	11.34
	0.21 Tm	0.75	7.57	79.17	12.51

Table 46 displays the effect that Tg:Tm combinations had on the early apoptotic phase MCF-7 cells. The combination of 0.0525 µM Tm with 0.06 µM Tg resulted in 51.98% live cells and a 40.17% dead apoptotic cell population, while the other selected combinations resulted in no more than 9.87% as a dead apoptotic population and a minimum of 77.95% were live cells.

Table 47: The effect of selected tunicamycin concentrations in combination with 0.06 µM thapsigargin on early apoptosis in MCF-7 cells after 48 hrs (20 000 events analysed)

Concentration (µM)		Dead cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic cells
0.06 Tg	0.0525 Tm	4.83	89.82	0.78	4.56
	0.105 Tm	2.74	89.69	0.58	6.99
	0.21 Tm	2.67	91.15	0.76	5.41

Table 47 shows that dead apoptotic cell populations were between 89 and 92% for all combinations analysed at 48 hrs.

Table 48: The effect of selected tunicamycin concentrations in combination with 0.06 µM thapsigargin on early apoptosis in MCF-7 cells after 72 hrs (20 000 events analysed)

Concentration (µM)		Dead cells	Dead Apoptotic Cells	Live Cells	Early Apoptotic cells
0.06 Tg	0.0525 Tm	20.49	46.65	2.98	29.87
	0.105 Tm	11.61	47.27	2.05	39.07
	0.21 Tm	15.75	52.37	0.97	30.91

Table 48 displays the effect after 72 hrs on MCF-7 cells induced with various combinations of Tm:Tg. Approximately 30% of cells treated with the combinatorial concentrations, had been inducted into early apoptosis for all concentrations. The dead apoptotic cell population was between 46 and 53% with the dead cell population, not exceeding 21%.

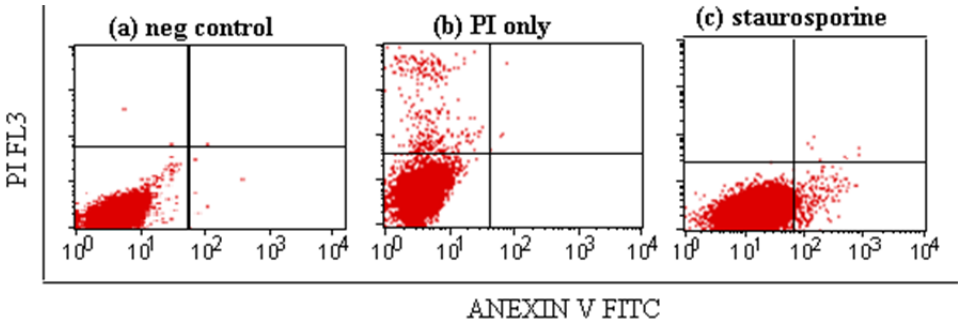

Fig. 105. Flow cytometry controls a) Unstained MCF-7 cells, b) PI only stained MCF-7 cells c) staurosporine treated, annexin V only stained MCF-7 cells (positive control).

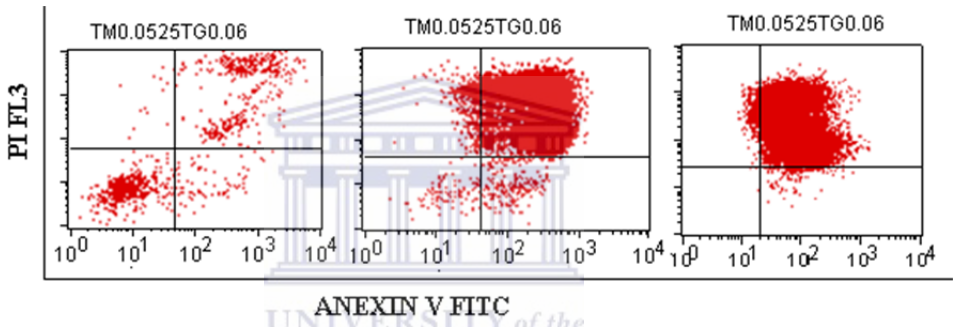

Fig. 106. Scatter plots depicting the apoptotic effect of 0.0525 Tm:0.06 µM Tg combination on MCF-7 at a) 24 hrs, b) 48 hrs and c) 72 hrs.

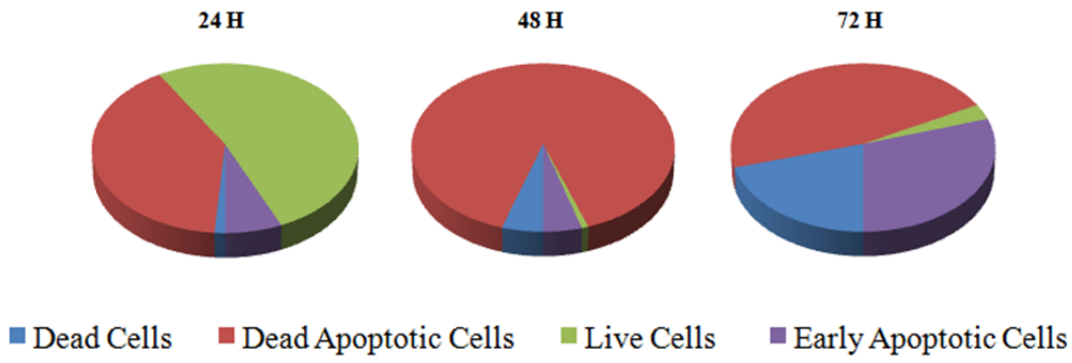

Fig. 107. The effects of 0.0525 Tm:0.06 µM Tg on MCF-7 cells.

Fig. 106 and 107 show that after 24 hrs, dead apoptotic cell population was at 40.17%, a further 24 hr incubation period, resulted in an increase in the apoptotic

population to 89.92%, which decreased after 72 hrs to 46.65%. Early apoptotic cell numbers increased by 23.28% between 24 and 72 hrs.

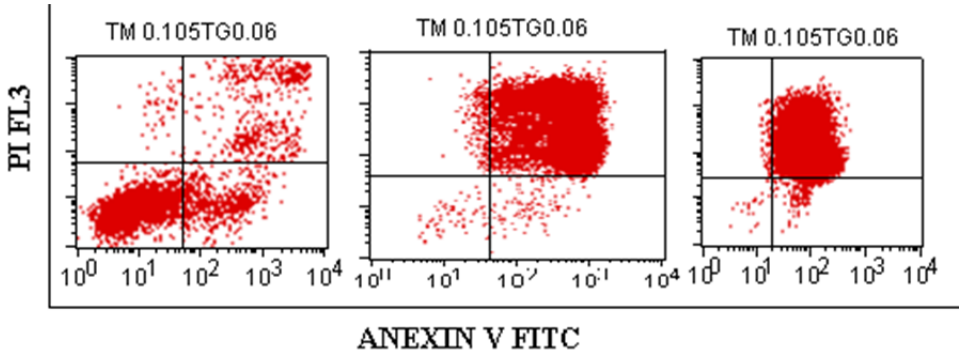

Fig. 108. Scatter plots depicting the apoptotic effect of 0.105 Tm:0.06 µM Tg combination on MCF-7 at a) 24 hrs, b) 48 hrs and c) 72 hrs.

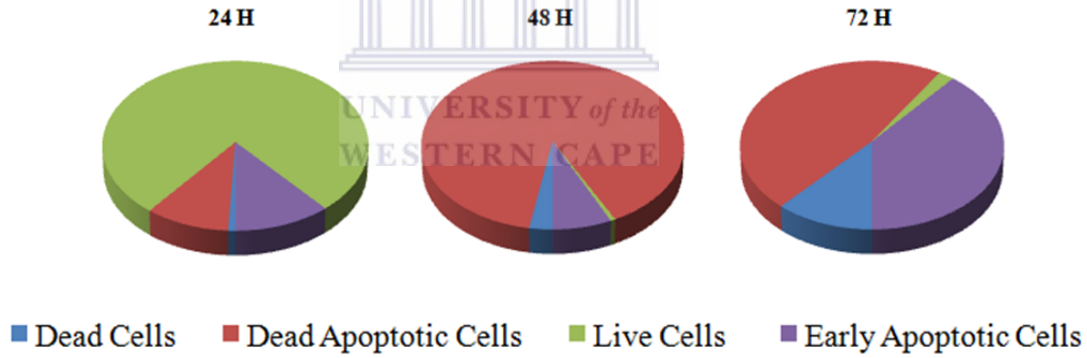

Fig. 109. The effects of 0.105 Tm:0.06 µM Tg on MCF-7 cells.

Scatter plots and pie graphs (Fig. 108 and 109) illustrate that early apoptotic cell populations increased by 27.73% between 24 and 72 hrs. Dead apoptotic cells numbers increased to 89.7% after 48 hrs, but decreased to 47.3% after 72 hrs with exposure to the Tm and Tg combinations.

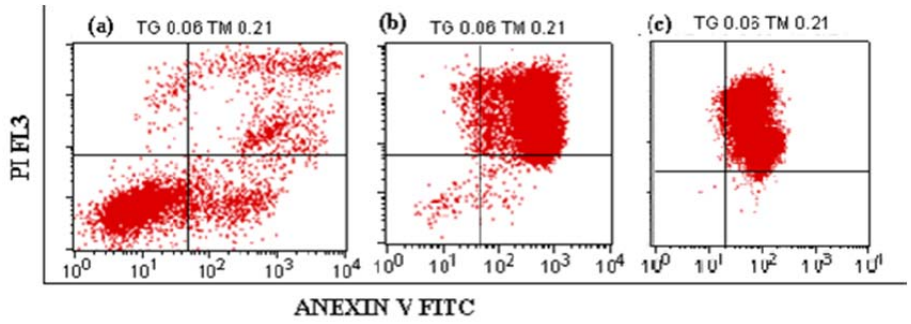

Fig. 110. Scatter plots depicting the apoptotic effect of 0.21 Tm:0.06 µM Tg combination on MCF-7.

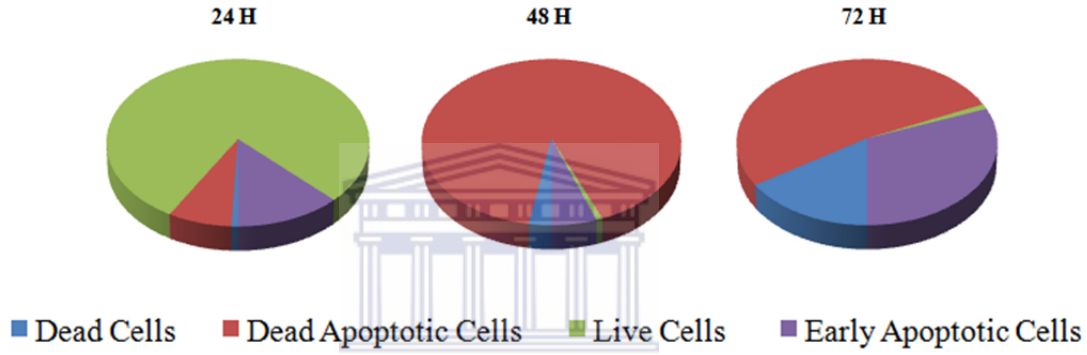

Fig. 111. The effects of 0.21 Tm:0.06 µM Tg on MCF-7 cells.

48 hrs of exposure to 0.21 µM Tm with 0.06 µM Tg resulted in an increase from 7.6% to 91.15% in dead apoptotic cell numbers, followed by a decreased of 52.4% after 72 hrs. Early apoptotic population reached 30.91% while dead cell population reached 15.8%.

CHAPTER 4

4. DISCUSSION AND CONCLUSION

Normal cells undergo growth phases in order to maintain survival, which involves alterations within the cell in order to proliferate, repair or segregate the genome (Hartwell and Kastan, 1994). Cancerous cells, however, are mutated cells that have been genetically or phenotypically altered (Dervan, 2001). Tumourigenesis is the uncontrolled growth of mutated cells due to down regulation of tumour suppressor genes (TSGs) (Xiang et al., 2012). Although progress has been made in the diagnosing and managing of breast cancer, there are still limitations with regards to early detection of this disease and therefore there is a need to further elucidate breast cancer mechanisms and identify potential anti-cancer promoting agents.

Nicotine is an alkaloid known to deregulate critical signalling pathways in different systems functions and acts as a tumour promoter and displays anti-apoptotic characteristics (Zeidler et al., 2007). In this study it was observed that viability was unaffected by nicotine exposure. Furthermore, an increase was observed between 48 and 72 hrs, with higher concentrations of nicotine resulting in a greater increase in viability. These findings indicate that exposure to this alkaloid supported cell proliferation. This was also observed and reported by Dasgupta et al., 2009. Catassi et al., 2008, Hirata et al., 2010, and Shin and Cho, 2005. They further reported that nicotine sheltered various cell lines from apoptosis when combined with compounds such as tunicamycin and doxorubicin (Zhou et al., 2007).

The increase in cell viability correlating to the increase of nicotine concentrations was also reported by Zhou and co-workers (2007). Similarly to viability, ATP and DNA proliferation in MCF-7 cells exposed to nicotine increased with the incubation periods. Nicotine was unable to induce early apoptosis at any concentration and any time interval analysed. These findings thus support previous work reporting on the tumour promoting properties of nicotine.

It has been reported that combinations of nicotine and compounds known for their potential anti-cancer properties (exhibiting extreme toxicity towards cells), may provide a therapeutic tool against tumour progression (Zhou et al., 2007). Our study investigated the combinatorial effects if nicotine and tunicamycin (Tm) as well as nicotine and thapsigargin (Tg) on MCF-7 cells in an attempt to induce apoptosis and simultaneously significantly reduce the Tm or Tg-treated toxicity. In order to determine the appropriate concentrations to be used in combination with nicotine, the effect of the single pure compounds, *viz;* Tm and Tg on cell viability had to be analysed. Thus if cell death was observed in this study, using the selected concentrations at which significant viability was still evident, it could be proposed that the cellular arrest was through apoptotic mechanisms and not as a result of toxicity.

It is known that Tm induces ER stress by interfering with the glycoslylation of newly synthesised proteins. The UPR is a homeostatic response to the accumulation of misfolded or anomalous proteins (Hiss et al., 2007). ER stress occurs when the UPR is unable to respond adequately resulting in an accumulation of perturbant proteins within the cell. In this study it was demonstrated that, Tm decreased cell viability at

24, 48 and 72 hrs. The MCF-7 cells were unable to increase viability after 48 and 72 hrs at higher Tm concentrations. However, at low concentrations, MCF-7 cells recovered from the inhibitory effects of Tm. From this study it was apparent that the effects of Tm on MCF-7 cells are dose-dependent as well as time dependent. These findings were also reported by Semmes and co-worker (1992), He et al. (2009) and Hiss et al. (2007).

Tm concentrations selected for the analyses of ATP production, resulted in a maximum of 50% cell viability. The rationale for selecting these concentrations stems from the reported ability of nicotine to suppress apoptotic properties of Tm (Zhou et al., 2007) and thus an increase in cell viability was expected when combining nicotine and Tm (discussed later in the chapter). The ATP levels in cells treated with the selected Tm concentrations were highest at 24 hrs when compared to 48 or 72 hrs. However, the various concentrations of Tm analysed, had no effect on the ATP levels produced by the MCF-7 cells, at any specific time interval. The decreased ATP concentrations observed as a result of Tm exposure may have resulted in the decreased viability reported in this study. Recently Wang et al. (2011) reported that Tm inhibited cell proliferation in $Bak^{-/-}Bax^{-/-}$ IL-3-dependent cells. They further reported that Tm inhibited glucose uptake and significantly reduced lactate production suggesting that Tm inhibited the glycolytic pathway. Studies have demonstrated that inhibition of the glycolytic pathway results in significantly depleted ATP in cancer cells (Xu et al., 2005). Thus interfering with the glycolytic pathway will affect the cell's survival rate. The abovementioned findings support the data obtained in this study, demonstrating that Tm exposure to MCF-7 cells decreased viability and ATP production possibly as a result of the inhibition of glycolysis.

DNA proliferation analysis was determined using the same selected concentrations as ATP analyses. It was observed that the MCF-7 cells' DNA concentrations were significantly low at 24 hrs. The DNA levels then noticeably increased by 48 hrs and were at substantially elevated levels at 72 hrs. However, the proliferation was not affected by the various concentrations of Tm at a specific time interval. While it was observed that Tm resulted in a decrease in cell viability and ATP production, it in contrast increased DNA proliferation. Studies have demonstrated that Tm resulted in a decrease in DNA levels and more specifically that the cells arrested in the G1 phase. However, these findings were based on exposure to Tm for less than 48 hrs (Hsu et al., 2009), while this study continued to 72 hr incubations. Moreover, studies reported that the ability to resist or reverse Tm effects were cell type dependent, in which they demonstrated that even after solid tumour variants of Chang hepatoma cells were blocked in the G1 phase by low Tm concentrations, they could still divide in the presence of Tm up to 8 days (Savage and Baur, 1983). The increased DNA proliferation observed at 72 hrs may be explainable based on the studies by Savage and Baur (1983) in which it is possible that some MCF-7 cells were blocked in the G1 phase and others were blocked in the S phase.

The effect of Tm on early apoptosis in MCF-7 cells, were determined by inducing cells with the same concentrations used for ATP and DNA analysis. The results obtained indicated that, Tm had slightly increased early apoptosis in MCF-7 cells at 24 hrs. At 48 and 72 hrs, Tm resulted in their significant induction of apoptosis. The higher cell viability and ATP observed at 24 hrs correlated with the low apoptotic effects induced at 24 hrs. Furthermore, the decreased viability and ATP concentrations noted at 48 and 72 hrs was accompanied by the significant increase in

early apoptosis. This study conclusively demonstrated that Tm induced early apoptosis in MCF-7 cells at concentrations that were not toxic to the *in vitro* cell line. Furthermore, it was established that the decreased viability was associated with a decrease in ATP production.

Thapsigargin (Tg), is known to induce ER stress by inhibiting its Ca^{2+}-ATPase and thus blocking ER's sequestration of Ca^{2+} leading to an increase in cytosolic levels. When intracellular Ca^{2+} levels increase, it elicits GRP78 expression linked to the UPR, indicating an ER stress response (Takadera et al., 2007). In this study, it was observed that viability was dose dependent, and decreased as concentrations increased, at all, time intervals. It was also noted that at lower concentrations of Tg, cell viability remained unaffected and comparable between 24 and 72 hrs. The inhibitory effects of Tg on MCF-7 viability was also reported by Thastrup et al. (1990) and Takadera et al. (2007). It must be highlighted that while this study validates previous findings, it is the first report on long term exposure to Tg.

ATP analysis of selected Tg concentrations resulting in cell viability of approximately 50%, showed that the various concentrations of Tg analysed at a specific time interval, had no effect on the ATP levels produced by the MCF-7 cells,. However, an increase in incubation periods resulted in a decrease in ATP levels for all Tg concentrations, except at the lowest concentration of 0.05 µM. The decrease in ATP production observed over time may be correlated with the decrease in viability demonstrated. Tg's mechanism of action is based on its ability to block the ER Ca^{2+}-ATPase and thus induce cellular arrest. This study demonstrated a significant decrease in ATP production and the mechanism for the decrease has been linked to enlarged

mitochondria associated with disrupted cristae structure. It was reported that as an adaptive response to ER stress, an early increase in the induction of mitochondrial metabolism occurs which may explain the significant decrease in ATP levels observed (Beaver and Waring, 1996, Bravo et al., 2011). Studies have reported that the absence of ATP supports the inhibitory effects of Tg (Thastrup et al., 1990) and thus the findings in this study further support the decrease in ATP observed.

DNA proliferation analyses in the presence of Tg saw a significant increase as incubation time increased. The DNA levels at 24 hrs were noticeably lower when compared to 48 hrs. At 72 hrs, DNA proliferation had increased by more than 2- fold when compared to 48 hrs. However, the proliferation was similar when comparing the effects of the various concentrations at the specific time intervals. The low DNA concentrations observed at 24 hrs could be correlated to the suppressed ATP and viability levels. Interestingly as was observed for Tm, the DNA concentrations between 48 and 72 hrs were significantly elevated in comparison to the markedly decreased ATP and viability. Previous findings report that Tg may result in cellular arrest at the G0 (Beaver and Waring, 1996), the G1 or G2 phase (Bourougaa et al., 2010). Bourougaa and co-workers (2010) demonstrated that the phase of cellular arrest is dependent on the cell type and their p53 isoform expressed and is thus pathway-specific. The results obtained in this study therefore suggest that the MCF-7 cells are blocked in the G2 phase. Similar to the proposed mechanism of Tm, it is possible that some MCF-7 cells were blocked in the G0/1 phase while others may have been blocked in the G2 phase with exposure to Tg and may explain the increase in DNA proliferation. This hypothesis needs further investigating.

MCF-7 cells exposed to Tg showed a high live cell percentage at 24 hrs (except at 0.4µM) and did not induce early apoptosis. The live cell population decreased at 48 and 72 hrs when compared to 24 hrs. At 48 hrs, a significant increase in dead apoptotic cell populations was observed, indicating that Tg had the ability to induce apoptosis in MCF-7 cells. At 72 hrs, early apoptotic cell populations increased and a decrease in dead apoptotic cells were observed. Thus validating that Tg did not decrease viability due to toxicity, but activated apoptotic mechanism, even at incubation periods of 72 hrs.

Utsumi and co-workers (2004) demonstrated that nicotine had protective effects in Tm-treated PC12 cells in a dose-dependent manner. Sasaya et al. (2008) further validated these findings by reporting that GRP78's expression was suppressed when Tm was combined with nicotine in PC12 cells. In this study it was observed that combinations of nicotine and Tm (ratio 1:1) concentrations significantly decreased viability of MCF-7 cells, at all selected time intervals. It was observed that 1 µM Tm resulted in 46.18±0.918 % viability at 24 hrs. In order to produce the same effect on viability, 100 µM Tm in combination with 100 µM Nic was required, demonstrating the apoptotic-suppressing effects of Nic. Nic also supported MCF-7 cells against Tm at 48 and 72 hrs, resulting in more than 100% increase in viability for the abovementioned concentration ratios. The anti-apoptotic properties of nicotine in combination with Tm, was also reported by Utsumi et al. (2004) and Sasaya et al. (2008) in PC12 cells.

The combination of Tm and Nic showed a significant decrease in ATP production at 24 hrs. Comparative analysis of the effect of 1 µM Tm at 24 and 72 hrs to the

combinatorial effects of 1 µM Tm: 1 µM Nic showed that the ATP concentrations were similar. However, at 48 hrs, ATP levels were significantly lower for the Tm:Nic combinations when compared to effects of the single compound. The decreased ATP concentrations observed correlated with the decreased cell viability as a result of Tm:Nic combinations. It is important to note that while the addition of Nic resulted in an increased viability, it did not affect the ATP concentrations when compared to the effects of the single compound, except when comparing 1 µM Tm to 1 µM Tm: 1 µM Nic at 48 hrs. To date, there is no literature reporting on the possible mechanism involving the combinatorial effects of Tm and Nic on ATP production in MCF-7 cells.

The same combination concentrations used for ATP analysis were employed in the determination of DNA proliferation. The results demonstrated that DNA levels decreased at 24 hrs while an increase in DNA concentrations were observed at both 48 and 72 hrs. It was also noted while a decreased DNA proliferation was observed at the lowest combinatorial concentrations at 48 and 72 hrs compared to subsequent concentrations, overall the combinations resulted in a significant increase in DNA proliferation between 24 and 72 hrs. Furthermore the effects of the single compound at 1 µM displayed similar DNA proliferation when compared to the 1 µM Tm: 1 µM Nic combinations.

It can be concluded that Tm:Nic (ratio 1:1) displayed a dose response where a decreased viability, ATP as well as DNA proliferation was observed at 24 hrs. At 48 hrs, cells exposed to the 1:1 Tm:Nic ratio showed that viability and ATP was inhibited while DNA proliferation was increased. Similar effects were observed for 72

hrs, however, the DNA concentrations had increased by 2-fold when compared to 48 hrs. This is the first report on the combinatorial effects of nicotine and Tm on ATP and DNA proliferation in MCF-7 cells between 24 and 72hrs.

Analysis of apoptosis demonstrated that Tm:Nic (ratio 1:1) at 24 hrs resulted in higher live cell populations at the lowest concentration, while a decreased live cell population associated with an increased early apoptotic population was observed at the higher combinatorial concentrations. At 48 hrs an elevated live population was still observed at the lowest concentrations however, the higher concentrations induced a significant percentage of dead apoptotic cells. After 72 hrs, the dead apoptotic cell numbers decreased however remained significant and the early apoptotic cell numbers also increased. These results demonstrated that Tm:Nic (1:1 ratio) significantly induced early apoptosis, resulting in cell death in the MCF-7 cells between 24 and 72 hrs. Discrepancies found between dead apoptotic and early apoptotic cells between 48 and 72 hours, where dead apoptotic cells decreased significantly and early apoptotic cells increased, remains to be explained and thus requires reinvestigation. Moreover, the cell death was due to apoptosis and not as a result of toxicity. This finding had also been previously reported by Sasaya et al. (2008).

Both the single compound and Tm:Nic combination at 1 µM resulted in comparable live and early apoptotic populations at 24 hrs. At 48 hrs, the dead apoptotic population was approximately 28% higher when exposed to the combinations when compared to the single compounds. Interestingly, at 72 hrs results showed both the single compound and combinations of Tm:Nic had similar effects on the early apoptotic populations. Therefore, from this study it can be concluded that nicotine supports cell

proliferation between 24 and 48 hrs, as previously reported (Utsumi et al., 2004, Sasaya et al., 2008). However, after 48 hrs nicotine no longer exhibits anti-apoptotic effects against Tm in MCF-7 cells.

It is reported that nicotine did not suppress the apoptotic properties of Tg in PC12 cells after 24 hrs. This study demonstrated that while Tg induced caspase 12 expression (an apoptotic enzyme associated ER stress), its combination with nicotine did not result in a decrease in caspase 12 expression (Sasaya et al., 2008). To date, no other studies have reported on the combinatorial effects of Tg and nicotine. 1:1 ratio combinations of Nic and Tg showed a dose-response where viability had significantly decreased at, all time intervals analysed, except between the lowest concentrations. Significant decline in viability between 24 and 72 hrs was only observed between intermediate and highest concentrations. Although previous studies reported that nicotine did not suppress the apoptotic effects of Tg, this study showed that Tg:Nic (1:1 ratio) at 1 µM had higher cell viabilities at, all time intervals when compared to the effects of the single compound of 1 µM Tg. It was also noted while 1 µM Tg resulted in only 3.145 ± 0.386 % viability at 72 hrs, the Tg:Nic (1:1 ratio) at 1 µM resulted in 29.013 ± 4.456% viability. Moreover, using 5 µM 1:1 ratios of Tg:Nic, viability was still at 18.187 ± 1.009 %. It must be concluded from this study that nicotine reduced the inhibitory effects of Tg on MCF-7 cell viability. Although in conflict with reports by Sasaya et al. (2008), it must be noted that this study was performed in human breast cancer cells over a period of 72 hrs, while the previous study reported on the effects for 24 hrs in PC12, prostate cancer cells.

A significant decline in ATP concentration was observed for all combinatorial (1:1 ratio) concentrations between 24 and 72 hrs. ATP levels however, remained the same

for all concentrations tested at specific time intervals. Interestingly, a comparison of the effect of 1 µM Tg compared to the combinatorial effects of the 1:1 ratio of 1 µM Tg:Nic, showed that the ATP levels were similar at 24 hrs, however, was significantly higher at 48 hrs, and then displayed similar significantly declined levels at 72 hrs. Overall, the single compound displayed similar trends to the combination of Tg:Nic.

Viability significantly decreased when cells were exposed to various Tg:Nic combinations for 24 hrs, however, the ATP levels remained the same. When the single compound of Tg at various concentrations were was exposed to the MCF-7 cells it resulted in similar viability accompanied by similar ATP levels. It is clear from previous studies that a decrease in ATP supported the inhibitory effects of Tg (Bravo et al., 2011). This study demonstrated that when Tg was in combination with nicotine, it had a greater inhibitory effect on ATP levels, particularly at 48 hrs, when compared to the effects of the single compound. It thus suggests that nicotine not only suppresses Tg toxicity but also aids in apoptotic signalling by causing significant ATP reduction.

The same combination concentrations of Tg:Nic (ratio 1:1) used for ATP analysis were employed in the determination of DNA proliferation. The results showed that DNA levels significantly decreased at 24 hrs, while a dramatic increase in DNA concentration were observed at both 48 and 72 hrs. Thus the 1:1 ratios of Tg:Nic resulted in a significant increase in DNA proliferation between 24 and 72 hrs. Exposure to various concentrations of the single compound of Tg at 48 hrs resulted in significantly lower DNA levels than compared to the levels when exposed to combinations of Tg:Nic. Overall, a decrease in viability was associated with a

decrease in ATP between 24 and 72 hrs, however, although a significantly low DNA concentration was observed at 24 hrs, the levels markedly increased after 72 hrs.

Tg:Nic (ratio 1:1) did not induce early apoptosis at 24 hrs . However, at 48 hrs, it resulted in a markedly elevated dead apoptotic cell population. Interestingly, at 72 hrs, lower combinatorial concentrations resulted in a significant increase in live cell population, while the highest concentration of 5 µM resulted in 44.53% early apoptotic cells and 39.33% dead apoptotic cells. The effect of the single compound of 0.4 µM Tg at 24 hrs, resulted in a 38.79% early apoptotic cell population whereas, the combinatorial 1:1 ratio of Tg:Nic for 1 µM resulted in only a 7.68% early apoptotic population. It is evident from this study, that nicotine decreased the pro-apoptotic effect of Tg in MCF-7 cells at 24 hrs as well as at 72hrs. At 48 hrs however, there were significantly more dead apoptotic cells as a result of the combinatorial 1:1 ratio of Tg:Nic for 0.1 µM, when compared to the single compound effect.

Our findings suggest that nicotine displayed anti-apoptotic properties against Tm and Tg at 24 hrs in MCF-7 cells. However, between 48 and 72 hrs the suppressing effects of nicotine are no longer evident. Nicotine's mechanism of action could possibly be through its ability to decrease expression of GRP 78, while Tm and Tg are known to increase its expression (Sasaya et al., 2008). This mechanism could explain the results obtained at 24 hrs, however, cannot explain the significant increase in apoptosis observed after 48 hrs in MCF-7 cells exposed to both Tm:Nic and Tg:Nic (1:1 ratio) combinations.

To date, no studies have investigated the combinatorial effects of Tm and Tg on MCF-7 cells. In an attempt to determine whether these compounds may have synergistic properties with respect to their apoptotic potential, various combinations were analysed. Initially, the IC_{50} concentrations were used in 1:1 ratios, but it resulted in 100% toxicity (data not shown). In order to demonstrate MCF-7 viability, concentrations less than 50% of the Tm and Tg IC_{50} concentrations were used for analysis. Results showed that when combining Tm at 50% (0.105 µM) of its IC_{50} with 25% of the IC_{50} of Tg (0.03 µM), viability was below 50% and did not change between 24 and 72 hrs. Combinations of Tm (0.105 µM) with 0.06 µM Tg resulted in viability that was also below 50%, and decreased significantly between 24 and 72 hrs. When combining the Tm (0.105 µM) with 0.12 µM Tg, it again resulted in less than 50% viability with a greater inhibitory effect between 24 and 72 hrs. Thus the higher the Tg concentration combined with Tm, the greater the decrease in % viability. Investigating the effect on ATP concentrations showed that all combinations had similar effects at the specific time interval analysed. The significant decrease in ATP levels observed between 24 and 72 hrs were similar for all combinations investigated. Even though higher Tg concentrations affected viability, it did not affect ATP production.

Using the same combinations for DNA proliferation analysis, it was observed that there were no differences when comparing the combinatorial concentrations at any specific time interval. The DNA concentration for all combinations significantly increased between 24 and 48 hrs followed by a significant decrease after 72 hrs.

Effects of the combinations on apoptosis showed that all concentration combinations only induced a maximum of 12.9% cells in early apoptosis with a significant population of live cells. At 48 hrs, more than 85.76% of the cells were dead due to apoptosis. While at 72 hrs, more than 31.01% were in early apoptosis and less than 57.8% were dead as a result of apoptosis. These findings demonstrated that the selected combinations of Tm and Tg induced apoptosis between 24 and 72 hrs. This study also showed that Tm in combinations with selected concentrations of Tg (below IC_{50}) resulted in decreased viability, ATP concentrations and a noticeably lowered DNA proliferation. Furthermore, increasing the Tg concentration in combination with Tm resulted in greater inhibitory effects.

Investigation into the combinatorial effects of Tg at 50% (0.06 µM) of its IC_{50} with 25% of the IC_{50} of Tm (0.0525 µM) showed that viability was below 50% and did not change between 24 and 72 hrs. The combination of Tg (0.06 µM) with 0.105 µM Tm also resulted in viability below 50%, with a significant decrease between 24 and 72 hrs. However, when combining the Tg (0.06 µM) with 0.21 µM Tm, it resulted in approximately 50% viability with a lesser inhibitory effect between 24 and 72 hrs. Thus the highest Tm concentration combined with 0.06 µM Tg, resulted in the least significantly inhibitory effect on viability between 24 and 72 hrs.

Investigating the effect on ATP concentrations showed that all combinations had similar effects at the specific time interval analysed. The significant decrease in ATP levels observed between 24 and 72 hrs were similar for all combinations investigated. Thus the decreased viability was accompanied by decreased ATP concentrations.

The DNA proliferation was analysed with the same combinatorial concentrations as for ATP analysis. Results demonstrated that no differences occurred at any specific incubation period. The DNA concentration for all combinations significantly increased between 24 and 48 hrs followed by a substantial decrease by 72 hrs. This study showed that Tg in combinations with selected concentrations of Tm (below IC_{50}) resulted in decreased viability, ATP concentrations and a significant lowered DNA proliferation.

The apoptotic effects of the combinations showed that 0.06 µM Tg in combination with 0.0525 µM Tm resulted in 40.17% dead apoptotic cell population at 24 hrs. Whereas, subsequent concentrations, resulted in an elevated live cell population and a maximum of 9.87% dead apoptotic cells at 24 hrs. At 48 hrs however, more than 89.69% of the cells were dead due to apoptosis at all concentrations analysed. 72 hrs, displayed more than 29.87% in early apoptosis while less than 52.37% were dead as a result of apoptosis for all concentrations analysed. These findings verified that the selected combinations of Tg and Tm induced apoptosis between 24 and 72 hrs.

Data demonstrated that both variations of Tm and Tg concentrations (in which a fixed concentration of Tm or Tg was used) could induce apoptosis. However, combinations of 0.105 µM Tm with 0.12 µM Tg resulted in more inhibition on cell viability than when combining 0.06 µM Tg with 0.21 µM Tm. It must be noted that the effects of the combinations of Tm and Tg on ATP, DNA proliferation and apoptosis were similar.

Of interest to note, is that cells exposed to selected concentrations of the single compounds of Tm and Tg as well as the combinations of nicotine with Tm or Tg, resulted in a significant increase in DNA proliferation and induction of apoptosis. However, cells exposed only to combinations of Tm and Tg displayed significantly lowered DNA levels and still induced apoptosis. These findings suggest that the latter combinations activate different apoptotic pathways compared to the cellular mechanisms activated when cells are exposed to the single compounds and their combinations with nicotine.

To date, there are no reports on the combinatorial effects of Tm and Tg on MCF-7 cells. This study demonstrated that combinations of Tm and Tg were synergistic in their apoptotic potential and that when Tm concentrations were increased in combination with Tg, their synergistic relationship decreased. Further studies needs to be performed in order to establish the precise concentrations that would possibly result in an antagonistic relationship.

CHAPTER 5

5. REFERENCES

5.1. CHAPTER 1

Armstrong, D.W., Wang, X., Lee J.T., (1999) Enantiomeric composition of nornicotine, anatabine, and anabasine in tobacco, Chirality, **11**, 82–84.

Balakrishnan, A., Menon, V.P., (2007) Effect of hesperidin on matrix metalloproteinases and antioxidant status during nicotine-induced toxicity, Toxicology, **238**, 2–3, 90-98.

Ball, M., Andrews, S.P., Wierschem, F., Cleator, E., Smith, M.D., Ley, S.V., (2007) Total synthesis of Thapsigargin, a potent SERCA pump inhibitor, Org Letters, **9**,4,663-666.

Benowitz, N.L., Jacob III P., Fong, I. and Gupta, S**.,** (1994) Nicotine metabolism profile in man: Comparison of cigarette smoking and transdermal nicotine, Journal of Pharmacology and Experimental Therapeutics. **268**, 296-303.

Benowitz, N.L., Hukkanen, J., Jacobs P III., (2009) Nicotine chemistry, metabolism, kinetics and biomarkers, Handbook of Experimental Pharmacology, **192**, 29-60.

Caldwell, W.S., Greene, J.M., Dobson, G.P., de Bethizy, J.D., (1993) Intragastric nitrosation of nicotine is not a significant contributor to nitrosamine exposure, Ann. N.Y. Acad. Sci. **686,** 213-328.

Chan, C. T., Metz, M. Z., Kane, S. E., (2005) Differential sensitivities of trastuzumab (Herceptin®)-resistant human breast cancer cells to phosphoinositide-3 kinase (PI-3K) and epidermal growth factor receptor (EGFR) kinase inhibitors, Breast Cancer Research and Treatment, **91,** 187–201.

Chemical structure of Thapsigargin, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=446378&loc=ec_rcs, accessed 29 January 2012.

Christensen, S. B. & Norup, E. (1985) Absolute configuration,Tetrahedron Lett. 26, 107-110.

Clayton, P., Lu, A., Bishop, L., (2010) The Pyrolysis of (2)-(S)-Nicotine: Racemization and Decomposition, Chirality, **22**, 4, 442–446.

Crowley-Weber, C.L., Dvorakova, K., Crowley, C., Bernstein, H., Bernstein, C., Garewal, H., Payne, C.M., (2003) Nicotine increases oxidative stress, activates NF-kB and GRP78, induces apoptosis and sensitizes cells to genotoxic/ xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis, Chemico-Biological Interactions, **145,** 53-66.

Dasgupta, P., Rizwani, W., Pillai, S., Kinkade, R., Kovacs, M., Rastogi, S., Banerjee, S., Carless, M., et al., (2009) Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell line, Internation Journal of Cancer, **144,** 2, 36-45.

Delom, F., Emadali, A., Cocolakis, E., Lebrun, J.J., Nantel, A., Chevet, E., (2007) Calnexin-dependent regulation of tunicamycin-induced apoptosis in breast carcinoma MCF-7 cells, Cell Death and Differentiation, **14**, 586–596.

Dervan, P.A., (2001) Understanding breast cancer. McFarland and Company, Inc. Publishers, North Carolina.

Drew, P.D., Krichau, N., Reichwald, K., and Henrik Toft Simonsen, H.T., (2009) Guaianolides in apiaceae: perspectives on pharmacology and biosynthesis, Phytochemistry Reviews, **8**, 3, 581-599.

Ferlay, J., Shin, H.R.S., Bray, F., Forman, D., Mathers, C., Parkin, D.M.. GLOBOCAN (2008) v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://globocan.iarc.fr, accessed on 23/March/2012.

Gross, P.E., Ingle, J.N., Alés-Martínez, J.E., Cheung, A.M., Chlebowski, R.T., Wactawski-Wende, J., McTiernan, A., Robbins J., et al., (2011) Exemestane for Breast-Cancer Prevention in Postmenopausal Women, New England Journal of Medicine, **364**, 25, 2381-2391.

Hasegawa, A., Osuga, Y., Hirota, Y., Hamasaki, K., Kodama, A., Harada, M., Tajima, T., Takemura, Y., et al., (2008) Tunicamycin enhances the apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand in endometriotic stromal cells, The European Society of Human Reproduction and Embryology, **24**, 2, 408-414.

He, L., Kim, S.O., Kwon, O., Jeong, S.J., Kim, M.S., Lee, H.G., Osada, H., Jung, M., et al., (2009) ATM blocks tunicamycin-induced endoplasmic reticulum stress, Federation of European Biochemical Societies, **583**, 903-908.

Hecht, S.S., Hochalter, J.B., Villalta, P.W. and Murphy, S.E., (2000) 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: Formation of a lung carcinogen precursor, PNAS**, 97**, 23, 12493-12497.

Hsu, J.L., Chiang, P.C., Guh, J.H., (2009) Tunicamycin induces resistance to camptothecin and etoposide in human hepatocellular carcinoma cells: Role of cell cycle arrest and GRP 78, Naunyn Schmiedebergs archives of pharmacology, **380,** 373-382.

http://www.ansci.cornell.edu/plants/toxicagents/sesqlactone/sesqlactone.html accessed 4 May 2012.

http://www.nature.com/nrc/journal/v4/n12/fig_tab/nrc1505_F1.html accessed 28 February 2012.

http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001911/ accessed 3 April 2012.Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., (2011) Global cancer statistics, CA: A Cancer Journal for Clinicians, **61**, 2, 69–90.

Jung, Y.H., Lim, E.J., Heo, J., Kwon, T.K., Kim, Y.H., (2012) Tunicamycin sensitizes human prostate cells to TRAIL-induced apoptosis by upregulation of TRAIL receptors and downregulation of cIAP2, Internation Journal of Oncology, 40, **6**, 1941-1948.

Kane, J.K., Konu, O., Ma, J.Z., Li, M.D., (2004) Nicotine co-regulates multiple pathways involved in protein modification/degradation in rat brain, Brain Research. Molecular Brain Research, **132**, 2, 181-91.

Klein, T.E., Chang, J.T., Cho, M.K., Easton, K.L., Fergerson, R., Hewett, M., Lin, Z., Liu, Y., et al., (2001) Integrating genotype and phenotype information: An overview of the PharmGKB project, The Pharmacogenomics Journal, **1**, 167-170.

Li, J., Jianze Lee, A.S., Amy, S., (2006) Stress induction of GRP 78/BiP and its role in cancer, Current Molecular Medicine, **6**, 1, 45-54.

Liu, J.C., Voisin, V., Bader, G.D., Deng, T., Pusztai, L., Symmans, W.F., Esteva, F.J., Egan, S.E., Zacksenhaus, E., (2012) Seventeen-gene signature from enriched Her2/Neu mammary tumor-initiating cells predicts clinical outcome for human $HER2^{+}$:$ER\alpha^{-}$ breast cancer, PNAS, 109, **15**, 5832-5837.

Löwy, I., (2010) Preventive strikes: women, precancer, and prophylactic surgery. The John Hopkins University press, Baltimore.

Nakayama, H., Zhao, J., El-FAKhrsany, A., Isosaki, M., Satoh, H., Kyotani, Y., Yoshizumi, M., (2009) Neuroprotective effects of pramipexole against Tunicamycin-induced cells death in PC12 cells, Clinical and Experimental Pharmacology and physiology, **36**, 1183- 1185.

Onoda, N., Nehmi, A., Weiner, D., Mujumdar, S., Chrsisten, R., Los G., (2001) Nicotine affects the signalling of the death pathway, reducing the response of head and neck cancer cell lines to DNA damaging agents, Head and Neck, **23**, 10, 860-870.

Paleari, L., Negri, E., Catassi, A., Cilli, M., Servent, D., D'Angelillo, R., Cesario, A., Russo, P., Fini, M., (2009) Inhibition of nonneuronal α7-nicotinic receptor for lung cancer treatment, American Journal of Respiratory Critical Care Medicine, **17**, 1142-1150.

Price, N.P.J., Tsvetanova, B., (2007) Biosynthesis of the Tunicamycins: A Review, Japan antibiotics research assosciation, Journal of Antibiotics, **60**, 8, 485- 491.

Sasaya, H., Utsumi, T., Shimoke, K., Nakayama, H., Matsumura, Y., Fukunaga, K., Ikeuchi, T., (2008) Nicotine suppresses tunicamycin-induced, but not thapsigargin-induced, expression of grp78 during ER stress-mediated apoptosis in PC12 Cells, Journal of Biochemistry, **124**, 1, 36-45.

Schematic diagram of nicotine metabolism in the liver http://www.pharmgkb.org/pathway/PA2011, accessed on 20 January 2012.

Schröder, M., (2007) Endoplasmic reticulum stress responses, Cellular and Molecular Life sciences, **65**, 826-894.

Shimoke, K., Kishi, S., Utsumi, T., Shimamura, Y., Sasaya, H., Oikawa, T., Uesato, S., Ikeuchi, T., (2005) NGF-induced phosphatidylinositol 3-kinase signalling pathway prevents Thapsigargin-triggered ER stress-mediated apoptosis in PC12 cells., Neuroscience Letters, **389**, 124-128

Shu, C.W., Sun, F.C., Cho, J.H., Lin, C.C., Liu, P.F., Chen, P.Y., Chang, M.D., Fu, H.W., Lai, Y.K., (2008) GRP78 and Raf-1 cooperatively confer resistance to endoplasmic reticulum stress-induced apoptosis, Journal of Cell Physiology, **215**, 3, 627-635.

Takadera, T., Fujibayashi, M., Kaniyu, H., Sakota, N., Ohyashiki, T., (2007) Caspase-dependent apoptosis induced by Thapsigargin was prevented by Glycogen Synthase Kinase-3 inhibitors in cultured rat cortical neurons, Neurochemical Research, **32**, 1336-1342.

Takadera, T., Yoshikawa, R., Ohyashiki, T., (2006) Thapsigargin –induced apoptosis prevented by glycogen synthase kinase -3-inhibitor in PC12 cells, Neuroscience letters, **408**, 124-128.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., Dawson, A.P., (1990) Thapsigargin, a tumour promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, Biochemistry, Procedure of Natural Academic Science. USA, **87,** 2466-2470.

Tsvetanova, B.C., Kiemle, D.J., Price, N.P., (2002) Biosynthesis of tunicamycin and metabolic origin of the 11-carbon dialdose sugar, tunicamine, Journal of Biological Chemistry., 277, **38** , 35289-96

Wang, G., Yang, Z.Q., Zhang, K., (2010) Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential, The American Journal of Translational Research, **2**, 1, 65-74.

Xiang, T., Li L., Yin, X., Yuan, C., Tan, C., Su, X., Xiong, L., Putti, T.C., et al., (2012) The ubiquitin peptidase UCHL1 induces G0/G1 cell cycle arrest and apoptosis through stabilizing p53 and is frequently silenced in breast cancer. PLoS ONE, **7**, 1, e29783.

Xu, C., Bailly-Maitre, B., and Reed, J.C., (2005) Endoplasmic reticulum stress: cell life and death decisions, The Journal of Clinical Investigation**, 115**, 2656–2664.

Yildiz, D., (2004) Nicotine, its metabolism and an overview of its biological effects, Toxicon, **43**, 619-623.

Yoshida, H., (2007) ER stress and diseases, FEBS Journal, **274**, 3, 630-658.

Yuan, L., Cao, Y., Knöchel, W., (2007) Endoplasmic reticulum stress induced by tunicamycin disables germ layer formation in Xenopus laevis embryos, Developmental dynamics, **236**, 2844-2851.

Zeidler, R., Albermann, K., Lang, S., (2007) Nicotine and apoptosis, Apoptosis, **12,** 11, 1927-1943.

Zhang, H.Y., Du, Z.X., Liu, B.Q., Gao, Y.Y., Meng, X., Guan, Y., Deng, W.W., Wang, H.Q., (2009) Tunicamycin enhances TRAIL induced apoptosis by inhibition of cyclin D1 and the subsequent downregulation of survivin; Experimental and molecular medicine; **41**, 5, 362.

Zhang, L.J., Li, Z.Q., Yang, Y.P., Li, X.W., Ji, J.F., (2009) Tunicamycin suppresses cisplatin-induced HepG2 cell apoptosis via enhancing p53 protein nuclear export, Molecular and cellular Biochemistry, **327**, (1-2), 171-82.

Zhou, Y., Gu, X., Ashayeri, E., Zhang, R., Sridhar, R., (2007) Nicotine decreases the cytotoxicity of Doxorubicin towards MCF-7 and Kb-3.1 Human Cancer Cells in Culture, Journal of the National Medical Association, **99**, 4, 319.

5.2 CHAPTER 2

Mosmann, T., (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, **65**, 1–2, 55–63.

5.3 CHAPTER 4

Beaver, J.P., and Waring, P., (1996) Thapsigargin induces mitochondrial dysfunction and apoptosis in the mastocytoma P815 cell line and in mouse thymocytes. Cell Death and Differentiation, 3, 4, 415-425.

Bourougaa, K., Naski, N., Boularan, C., Mlynarczyk, C., Candeias, M.M., Marullo, S., Fa'hraeus, R., (2010) Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47, Journal of Molecular Cell, **38**, 78-88.

Bravo, R., Vicencio, J.M., Parra, V., Troncoso, R., Munoz, J.P., Bui, M., Quiroga, C., Rodriguez, A.E., et al., (2011) Increased ER-mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress, Journal of Cell Science, **124**, 2143-2152.

Catassi, A., Servent, D., Paleari, L., Cesario, A., Russo, P., (2008) Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: Implications on lung carcinogens, Journal of Mutation Research/Reviews in Mutation Research, 659, 221-231.

Dasgupta, P., Rizwani, W., Pillai, S., Kinkade, R., Kovacs, M., Rastogi, S., Banerjee, S., Carless, M., et al., (2009) Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell line, Internation Journal of Cancer, **144,** 2, 36-45.

Dervan, P.A., (2001) Understanding breast cancer. McFarland and Company, Inc. Publishers, North Carolina.

Hartwell, L.H., Kastan, M.B., (1994) Cell cycle control and cancer. Science Magazine, **266**, 5192, 1821-1828.

He, L., Kim, S.O., Kwon, O., Jeong, S.J., Kim, M.S., Lee, H.G., Osada, H., Jung, M., et al., (2009) ATM blocks tunicamycin-induced endoplasmic reticulum stress, Federation of European Biochemical Societies, **583**, 903-908.

Hirata, N., Sekino, Y., Kanda, Y., (2010) Nicotine increases cancer stem cell population in MCF-7 cells, Journal of Biochemical and Biophysical Research Communications, **403**, 138-143.

Hiss D.C., Gabriels G.A., Folb P.I., (2007) Combination of tunicamycin with anticancer drugs synergistically enhances their toxicity in multidrug-resistant human ovarian cystadenocarcinoma cells. Cell Cancer International, **7**, 1, 5.

Hsu, J.L., Chiang, P.C., Guh, J.H., (2009) Tunicamycin induces resistance to camptothecin and etoposide in human hepatocellular carcinoma cells: Role of cell cycle arrest and GRP 78, Naunyn Schmiedebergs archives of pharmacology, **380,** 373-382.

Sasaya, H., Utsumi, T., Shimoke, K., Nakayama, H., Matsumura, Y., Fukunaga, K., Ikeuchi, T., (2008) Nicotine suppresses tunicamycin-induced, but not thapsigargin-induced, expression of grp78 during ER stress-mediated apoptosis in PC12 Cells, Journal of Biochemistry, **124**, 1, 36-45.

Savage, K.E., Baur, P.S., (1983) Effect of tunicamycin, an inhibitor of protein glycosylation, on division of tumour cells *in vitro*, Journal of Cell Science, **64**, 295-306.

Semmes O.J., Sztein M.S., Bailey J.M., Merritt W.D., (1992) Tunicamycin inhibits function and expression of the high-affinity IL-2 receptor in a murine IL-2-dependent cell line. International Journal of Immunopharmacology. 14, 4, 583-93.

Shin, V.Y., Cho, C.H., (2005) Nicotine and gastric cancer, Alcohol, **35**, 259-264.

Takadera, T., Fujibayashi, M., Kaniyu, H., Sakota, N., Ohyashiki, T., (2007) Caspase-dependent apoptosis induced by Thapsigargin was prevented by Glycogen Synthase Kinase-3 inhibitors in cultured rat cortical neurons, Neurochemical Research, **32**, 1336-1342.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., Dawson, A.P., (1990) Thapsigargin, a tumour promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, Biochemistry, Procedure of Natural Academic Science. USA, **87,** 2466-2470.

Utsumi, T., Shimoke, K., Kishi, S., Sasaya, H., Ikeuchi, T., Nakayama, H., (2004) Protective effect of nicotine on tunicamycin-induced apoptosis of PC12h cells, Neuroscience Letters, **370**, 244-247.

Wang, X., Eno, C.O., Altman, B.J., Zhu, Y., Zhoa, G., Olberding K.E., Rathmell, J.C., Li, C., (2011) ER stress modulates cellular metabolism, Biochemical Journal, **435**, 285-296.

Xiang, T., Li L., Yin, X., Yuan, C., Tan, C., Su, X., Xiong, L., Putti, T.C., et al., (2012) The ubiquitin peptidase UCHL1 induces G0/G1 cell cycle arrest and apoptosis through stabilizing p53 and is frequently silenced in breast cancer. PLoS ONE, **7**, 1, e29783.

Xu, R.H., Pelicano, H., Zhou, Y., Carew, J.S., Feng, L., Bhalla, K.N., Keating, M.J., Huang, P., (2005) Inhibition of Glycolysis in Cancer Cells: A Novel Strategy to Overcome Drug Resistance Associated with Mitochondrial Respiratory Defect and Hypoxia. Cancer Research, **65**, 613.

Zeidler, R., Albermann, K., Lang, S., (2007) Nicotine and apoptosis, Apoptosis, **12,** 11, 1927-1943.

Zhou, Y., Gu, X., Ashayeri, E., Zhang, R., Sridhar, R., (2007) Nicotine decreases the cytotoxicity of Doxorubicin towards MCF-7 and Kb-3.1 Human Cancer Cells in Culture, Journal of the National Medical Association, **99**, 4, 319.