ANGLIA RUSKIN UNIVERSITY

FACULTY OF SCIENCE AND ENGINEERING

Differential expression of long and short isoforms of tissue transglutaminase 2; Implications for cisplatin resistance in an *in-vitro* MCF-7 breast cancer model

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Anglia Ruskin University

Abstract

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Differential expression of long and short isoforms of tissue transglutaminase 2; Implications for cisplatin resistance in an *in-vitro* MCF-7 breast cancer model

Ebidor Ufoumanefe Lawani-Luwaji

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Despite significant advances in surgery and biology, cancer remains a major health problem because of tumour cell metastasis and resistance to chemotherapeutic drugs. Cisplatin has been described as 'the penicillin' of cancer and was the first platinum-based chemotherapeutic drug developed for cancer treatment. However, it has the unfortunate downside of chemoresistance, thus limiting its applicability to the treatment of the more responsive hormone-negative, rather than hormone-positive, metastatic breast cancer.

Tissue transglutaminase 2 (TG2), a multifunctional Ca^{2+} enzyme, belongs to a family of related genes involved in various cellular processes such as the cell cycle, adhesion, protein crosslinking, endocytosis and apoptosis. It is overexpressed in cancer and chemoresistant cells and implicated in encouraging resistance to anti-cancer drugs. The enzyme exists in structurally different isoforms, which are thought to have opposing functions. The full length TG2-L supports cell survival; while the truncated TG2-S form promotes cell death. However, the mechanism has not been investigated in breast cancer.

The effect of altering the expression of TG2-L and TG2-S isoforms was investigated in cisplatin-sensitive and cisplatin-resistant hormone-positive MCF-7 breast cancer cells. The cells were treated with non-toxic doses of retinoic acid up to 72 hours in order to raise TG2 expression, while inhibition of TG2 was achieved with cystamine and specific anti-TG2 silencing RNA (siRNA). MTT assay measured cellular viability; flow cytometry measured levels of apoptosis and necrosis, and Western blot analysis measured TG2 isoform expression.

The results generated from this study reveal for the first time that retinoic acid treatment of both wild type and cisplatin-resistant cell lines, induced overexpression of TG2-L but had little or no effect on TG-S expression, promoting cell survival and increasing cisplatin chemoresistance in all cases. Conversely, TG2 inhibition with cystamine and siRNA treatment selectively suppressed TG2-L expression (with little effect on TG2-S expression) and reduced cisplatin resistance to basal control levels without affecting cell viability, in all cells tested.

Collectively, the results suggest that targeting TG2-L by controlling levels of dietary retinoid consumption, could be a potential therapeutic tool in combating cisplatin resistance in hormone-positive breast cancer patients where, currently, rapid induction of resistance is a barrier to effective treatment and cisplatin therapy.

Keywords: Retinoic acid, TG2, Cisplatin, Cancer.

Conference presentations

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Declaration

This thesis is a presentation of my original work, and I declare that the figures, graphs and results have not been previously submitted elsewhere.

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Abbreviations

AKT	Protein kinase B
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BRCA	Breast cancer gene
c-SRC	Proto-oncogene c-Src
CCK-8	Cell Counting Kit-8
CDK	Cyclin-dependent kinase
cIAP1	Cellular inhibitor of apoptosis protein 1
cIAP2	Cellular inhibitor of apoptosis protein 2
DNA	Deoxyribonucleic acid
ER+ve	Oestrogen-positive
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
HER2 +ve	Human epidermal receptor 2 positive
IGFBP-3	Insulin-like growth factor-binding protein 3
$p21^{Cip1}$	CDK-interacting protein 1
p53	phosphoprotein p53, tumour suppressor p53
Prb	Retinoblastoma protein
PR+	Progesterone-positive
PTEN	Phosphatase and tensin homolog
PKM2	Pyruvate kinase muscle isozyme
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7
mi-R205	microRNA
MTT	Cell Proliferation Kit I
Mtor	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell

PI3K	Phosphoinositide 3-kinases
RHO	Ras homologous protein
RIPA buffer	Radioimmunoprecipitation assay buffer
SLC	Solute carrier proteins
TG1	Transglutaminase 1
TG2	Tissue transglutaminase 2
TG3	Transglutaminase 3
TG4	Transglutaminase 4
TG5	Transglutaminase 5
TG6	Transglutaminase 6
TG7	Transglutaminase 7
TNBC	Triple-negative breast cancer
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1: Introduction

1.0 Cancer

Cancer is a prominent multifactorial disease and amongst one of the leading causes of death worldwide. These group of diseases arise from genetic and epigenetic alterations of healthy cells, leading to irregular proliferation (Senthebane *et al*., 2018). The process begins when healthy cells change from their normal state to a hyper-proliferative state, exhibiting characteristic features: an unlimited ability for growth, disregard to programmed cell death, activating invasion and metastasis (figure 1.10). These changes are often caused by genetic mutations that either deactivate tumour suppressor genes or activate oncogenes (Coleman *et al*., 2008). A vital factor in the development of cancer is the inactivation of programmed cell death (Brown and Attardi, 2005) and defective apoptotic signalling, which results in several human diseases, including cancer (Johnson, 2013).

1.1 Apoptosis

Apoptosis is a highly regulated and conserved form of cell death and plays a considerable physiological aspect in embryonic development as well as in ageing. Also, proliferation and cell death must be controlled to maintain tissue homeostasis (Elmore, S., 2007). Cancer is an example where the normal machinery of the cell cycle regulation is defective, with either decreased removal of abnormal cells and/or an overproliferation of cells (King and Cidlowski,1998). Apoptosis is activated by a variety of stimuli which include: oxidative stress, irradiation, chemotherapeutic agents and endoplasmic reticulum stress. The caspase family of cysteine proteases act as effector molecules in apoptosis; they are initially synthesised as inactive proenzymes but on activation can cleave various molecules in the nucleus and cytoplasm, which eventually leads to the morphological features seen in apoptotic cell death such as DNA fragmentation, nucleus shrinkage and loss of cell shape (Fulde *et al*., 2009).

Caspases are activated by the tumour necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL), which results in receptor aggregation and the recruitment of fas associated death domain (FADD) and finally pro-caspase-8. A death-inducing signal complex is formed with the activation of caspase-8 and the resultant initiation of apoptosis by directly cleaving downstream effector caspases. Another mechanism by which caspases are activated is through the mitochondria pathway initiated by the apoptosis-inducing factor (AIF), cytochrome c, second mitochondria-derived activator of caspases (SMAC) and Diablo IAP-Binding Mitochondrial Protein direct (DIABLO) homolog protein. When cytochrome c is released into the cytosol by cellular stress, it results in the formation of a cytochrome c/ apoptotic protease activating factor 1 /caspase 9 apoptosome complex with the resultant activation of caspases. Activation of caspases has to be regulated because of the detrimental effect it has on cell survival if inappropriately activated (Fulde *et al*., 2009).

The Bcl-2 (B-cell lymphoma 2) gene family of proteins, regulates proteins that either inhibit or promotes apoptosis. The pro-apoptotic proteins include Bcl-2 homologous antagonist/killer (Bak), apoptosis regulator (Bax), Bcl-2 associated agonist of cell death (Bad), and B-cell lymphoma short-form (Bcl-xs), while Bcl-2 and B-cell lymphoma long-form (Bcl-xL) are anti-apoptotic. Overexpression of anti-apoptotic and underexpression of pro-apoptotic proteins result in reduced cell death, which is synonymous with cancers. Bcl-2 is expressed in approximately 80% of breast cancers and correlates with the expression of progesterone and oestrogen receptors (Cory *et al*., 2003).

Other proteins that inhibit apoptosis include, X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein 1(cIAP1), and cellular inhibitor of apoptosis protein 2 (cIAP2) which prevent apoptosis by inhibiting caspases (Parton *et al*., 2001).

Indeed both increased and defective apoptosis have different pathological outcomes, while increased apoptosis causes atrophy, defective apoptosis results in unrestrained cell proliferation, such as cancer (Favaloro *et al*., 2012).

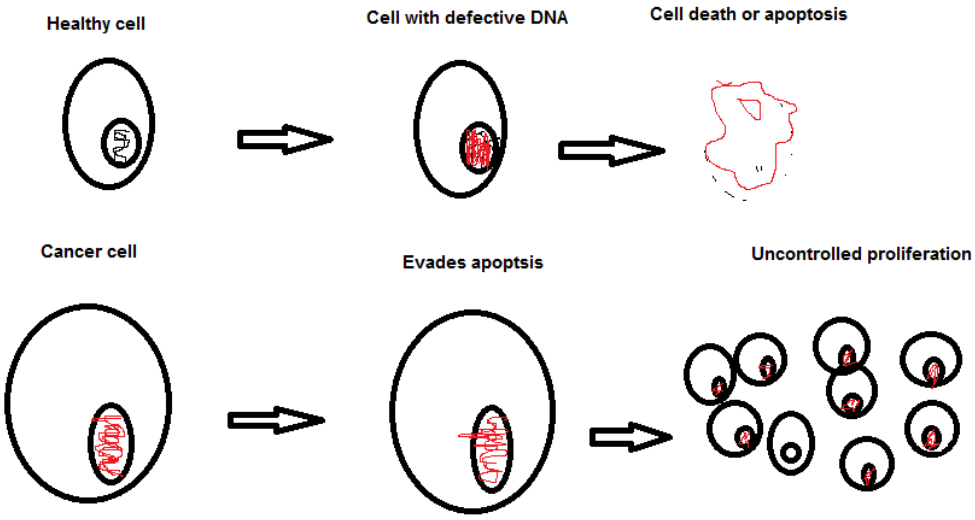


Figure 1.1: Schematic diagram showing apoptosis in the healthy breast cell and defective apoptosis in the breast cancer cell

1.2 Human breast carcinoma

Amongst women worldwide, breast cancer is the most common and principal cause of cancer-related deaths after lung cancer and the 2nd cause of death in the UK, according to the 2016 cancer statistics. The human breast is characterised by connective tissues, fat, glandular tissues and is divided into lobes with a web of ducts spreading to the nipples from the lobes (see figure 1.1). Breast cancers consist of two main types: *in situ* and invasive carcinoma. *In situ* breast carcinoma arises from either the lobular or ductal epithelium and is confined to these spaces without invading the basement membrane, which constitutes the boundaries of the mammary epithelium. When the malignancy from the lobes or ducts extends past the basement membrane, such malignancy is seen as invasive carcinoma. The main cause of death from breast cancer is mainly due to metastasis of the invasive disease (Richie and

Swanson, 2003), and approximately 10% of diagnosed breast cancer is of the invasive lobular type (Cancer Research UK).

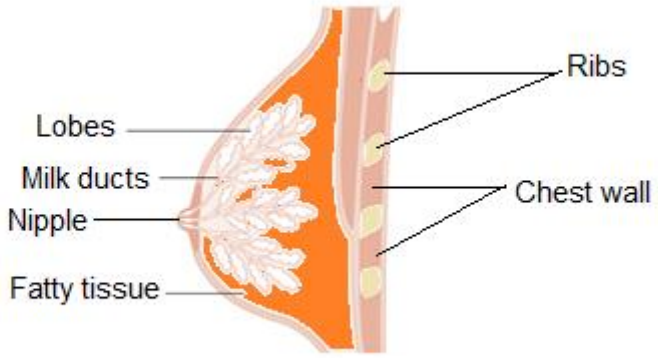


Figure 1.2: Anatomy of the human breast.

Composition of the human breast showing the lobes, ducts and fatty tissues.

Table 1.1 shows a list of the different types of breast cancer depending on the specific cell type of origin.

Table 1.1: Breast cancer types (Cancer Research UK)

	Breast cancer type	Cell type of origin
1.	Invasive breast cancer	Lobular cells
2.	Invasive lobular breast cancer	Lobular cells
3.	Triple-negative breast cancer	Cells without oestrogen, progesterone or HER2+ receptors.
4.	Inflammatory breast cancer	Cells lining the lymph channel
5.	Angiosarcoma of the breast	Cells that line the blood or lymphatic vessels.
6.	Ductal carcinoma in situ (DCIS)	Cells that line the ducts of the breast tissue
7.	Lobular carcinoma in situ (LCIS)	Cells inside the breast lobules
8	Male breast cancer	Cells that line up the ducts of the breast tissue
10.	Paget's disease of the breast	Nipple or areola

The risk factors for developing breast cancer are numerous, and these include gender, age, genetics, use of hormonal replacement therapy (HRT), breast density and family history of breast cancer. Other risk factors include exposure to radiation treatment, race and ethnicity, obesity, use of oral contraceptives, smoking, alcohol and lack of breastfeeding. Statistics available predict 1 out of 7 women will have breast cancer during their lifetime; out of this number, 23% of these cases would have been preventable (Cancer Research UK).

In humans, the hormones oestrogen, progesterone and the human epidermal growth factor type 2 are needed for healthy breast cells to grow and divide and also some cancer cells. Receptors for these hormones are found in or on cells and attach to the hormones and serve

as biomarkers to detect the type of breast cancers, and are used for breast cancer testing (Conger *et al.*, 2016). Table 1.2 characterises breast cancer based on their receptor status. Oestrogen-positive (ER+ve) cancer grow in response to the hormone oestrogen; HER-2 positive tumours overexpress the HER-2 receptor; and triple-negative breast cancer does not express surface receptors for the ligands of HER2 or the hormones oestrogen or progesterone (Duffy, 2006).

Table 1.2: Types of breast cancer

Hormone	Type of breast cancer	Receptor
Oestrogen	Oestrogen-positive (ER+)	Oestrogen receptor (ER)
Progesterone	Progesterone-positive (PR+)	Progesterone receptor (PR),
Human epidermal growth factor type 2	HER2 +ve	The human epidermal growth factor receptor-2 (HER2).
Absence of Oestrogen, Progesterone and Human epidermal growth factor type 2	Triple-negative cancer	No receptor expressed
Presence of Oestrogen, Progesterone and Human epidermal growth factor type 2	Triple positive cancer	All receptors expressed

Oestrogen exists commonly in three forms: oestrone (E1), 17β-oestradiol (E2), and oestriol (E3). In premenopausal women, E2 is the predominant oestrogen produced while E3 is produced after menopause (Lipovka et al.,2016). Oestrogen promotes the growth of sexual organ maturation and secondary sexual characteristics in females and is synthesised in the ovaries, mesenchymal cells of the adipose tissues of osteoblasts, chondrocytes, vascular endothelium and the breasts (Simpson, 2003). Both oestrogen(E2) and oestrogen receptors (ER) have been implicated in the progression of breast cancer (Saha and Vadlamudi., 2012). Figure 1.3 shows the involvement of E2 and ER in tumorigenesis and metastasis.

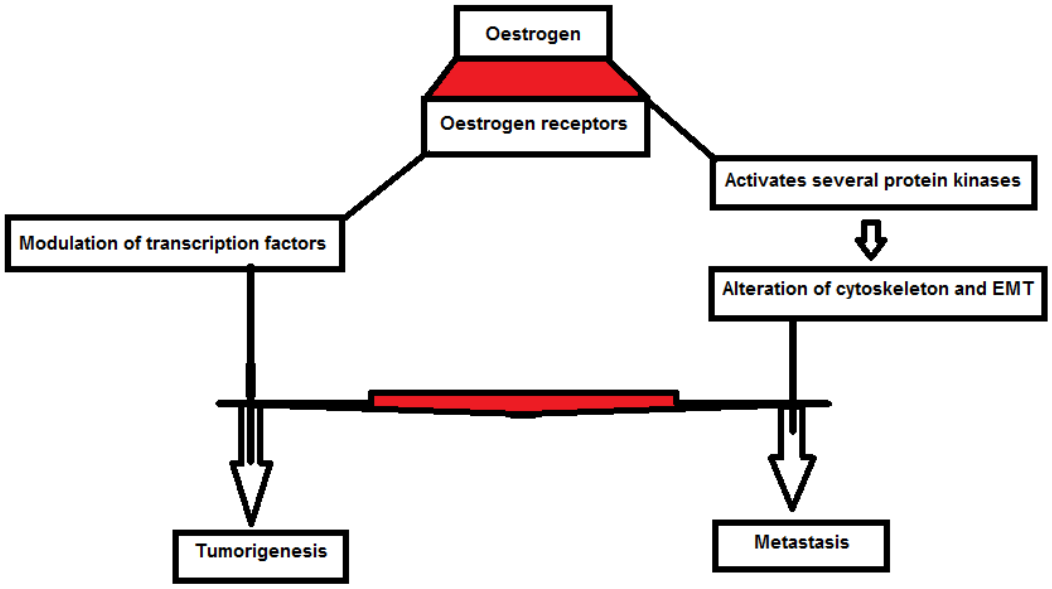


Figure 1.3: Schematic representation of oestrogen regulation of both tumorigenesis and metastasis

1. 2.1 Oestrogen receptor-positive breast cancer

The physiological functions of oestrogen are facilitated mainly via the oestrogen receptors (ER), which has two subtypes oestrogen receptor alpha (Erα) and oestrogen receptor beta (Erβ) with distinct characteristics and expression patterns. ERα is expressed more ubiquitously throughout the body, whereas the pattern of ERβ is limited to the ovary, thymus and testis. Both ERα and ERβ receptors are found in the normal breast, expression of ERβ seems to be the receptor widespread in mammary tissues (Speirs *et al*., 2002) and expressed in epithelial and stromal cells while ERα is expressed in a subcategory of epithelial cells (Li *et al.,* 2010).

Both isoforms are essential because they control multiple physiological processes, such as cellular growth, differentiation, and function of the reproductive mammary gland. It has also been implicated in several diseases, especially breast cancer (Duffy, 2006) owing to some signalling pathways it controls which worsen the progression of ER-positive tumours (Lipovka and Konhilas, 2016).

The receptors are over-expressed in around 70% of breast cancer cases, and evidence suggests that binding of oestrogen to the ER receptors encourages the propagation of mammary cells, resulting in increased DNA replication and cell division. The pathology also involves the interruption of the normal cell cycle, DNA repair and apoptosis and, consequently leads to tumour formation (Deroo and Korach, 2005).

1.2.2 MCF-7 breast cancer cell line (hormone positive)

The human MCF-7 breast cancer cell line, which expresses the oestrogen receptor, was obtained from a patient who had metastatic disease in the 1970s, and it has been the standard system for studying oestrogen-positive breast cancer (Simstein *et al*., 2003). Since its isolation, the MCF-7 breast cancer cell line has been extensively studied, and results obtained have contributed immensely towards breast cancer research and the improvement of the patient treatment outcome (Lee *et al*., 2015).

1.2.3 Triple-negative breast cancer (TNBC)

Triple-negative breast cancer is characterised by the absence of oestrogen receptors, HER2 and progesterone receptor, and is typically a basal-like breast cancer (Foulkes *et al*., 2010). TNBC occurs in about 10–20% of breast carcinomas and mostly affects younger women, women with high parity, high body mass index, and lack of breastfeeding, those with a mutation in the BRCA1 gene and specifically those of the Hispanic and African – American race (Dawood *et al*., 2010). Owing to the absence of receptors (ER.PR and HER2), TNBC is unresponsive to hormonal and HER2 targeted treatments, and cytotoxic therapies have been the choice for chemotherapy. Research such as identifying MAPK/ERK pathway inhibitors, Phosphoinositide 3-kinases (PI3Ks) inhibitors, Heat shock protein (HSP-90) inhibitors, programmed death 1 (PD-1) inhibitors and histone deacetylase inhibitors. These are some agents being considered/currently under investigation clinically against this triple-negative breast cancer (Abrahamson *et al*., 2014)

1.2.4 HCC 1806 breast cancer cell line (hormone negative)

The human HCC 1806 breast cancer cell line (TNBC) is categorised by the loss or reduced expression of the progesterone receptor, oestrogen receptor and HER2 protein. Triple-negative breast cancer type is typically aggressive owing to its invasive nature, the lack of receptors on the tumour makes conventional breast cancer treatments that target the receptors, hormone therapy ineffective (Rorie *et al*., 2012). There has been slight progress in the formation of drug treatments to target this breast cancer subtype, though no precise therapies exist presently for TNBC cytotoxic chemotherapy; research is currently ongoing to identify possible targets for improved therapy (Al-Bahlani *et al*.,2018).

1.2.5 Breast cancer treatments

Six types of breast cancer treatments are currently available: chemotherapy, surgery, radiation therapy, hormone therapy, targeted therapy and immunotherapy. Chemotherapy treatment depends on the type of breast cancer involved; oestrogen-positive breast cancers require treatments that prevent the hormones from binding to the receptors. An example is tamoxifen used mainly in postmenopausal women, and this drug inhibits proliferation activities of oestrogen by binding to the oestrogen receptors. Targeted cancer therapy is used to treat HER2+ve breast cancer. Trastuzumab (monoclonal antibody), is an example of a chemotherapeutic drug used to treat HER2 +ve breast cancers, the drug attaches to and interferes with the HER2 receptor proteins on or in cancer cells. Drugs that target BRCA (breast cancer gene) DNA repair gene mutation are used to treat triple-negative cancers (Cancer Research UK).

1.2.6 Regulation of apoptosis in the mammary gland

Development of the breast is in two physiological stages, puberty and pregnancy, where there are noticeable changes in differentiation and proliferation. Before the onset of pregnancy, terminal cells of the developing mammary ducts remain mitotically quiet, but during pregnancy, there is a rapid proliferation including lobular alveoli growth and ductal branching (Javed and

Lteif, 2013). Then, after the end of lactation, restructuring and apoptosis occur, returning the breast to its primary structure. When pregnancy does not happen, the cells continue resting while the menstrual cycle is active until menopause, when apoptosis occurs in the terminal duct and lobular duct. For healthy development and homeostasis, a balance exists between differentiation, proliferation and cell death. Thus, variations that could cause the increase of cellular proliferation and decrease of apoptosis might allow the build-up of mutations resulting in breast cancer (Green and Streuli, 2004; Javed and Lteif, 2013). Variation of the B-cell lymphoma 2 family of apoptotic regulatory proteins occurs partly due to the influence of the hormone oestrogens and progesterone. Also, defective cellular processes with functions to detect damaged DNA contributes to the acquisition of cancer (Parton *et al*., 2001).

Cells respond to stress either in the activation of cell survival pathways or through cell death with the elimination of the damaged cell. To a large extent, whether cells display a protective or destructive response depends mostly to the duration and nature of the stress and cell type involved. Also, there is evidence for cross-talk between the two cellular responses that finally decides the outcome of the stressed cell. The mechanism of cell death, whether it occurs by apoptosis, necrosis, pyroptosis or autophagy hinges on the cells ability to deal with the stress and also on several exogenous factors (Fulde *et al*., 2009).

Apoptosis is a form of organised/programmed cell death. This form of cell death is accompanied by cell rounding up, pyknosis (reduction of cellular volume), chromatin condensation, karyorrhexis (nuclear fragmentation), plasma membrane blebbing but the integrity is maintained until phagocytes engulf the cell (Baehrecke, 2002; Kroemer *et al*., 2009).

Necrosis is not an organised programmed death but a rupture of the plasma membrane and loss of cellular contents. This form of cell death is characterised by oncosis (gain in cell volume), swelling of organelles and finally cell rupture (Kroemer *et al*., 2009). Necrosis is seen as a form of uncontrolled cell death, but accumulating evidence suggests a set of signalling

pathways may regulate it. For example, Toll-like receptors (TLR3 and TLR4) and death domain receptors have been observed to provoke necrosis (Festjens *et al*., 2006).

Autophagy is described as a 'self-eating' form of cell death, and the process is characterised by the vesicular sequestration and degradation of organelles. It is a regulated, orderly mechanism that eliminates dysfunctional or unnecessary cellular components and allows for recycling (Kroemer *et al*., 2009).

1.3 Transglutaminases

Transglutaminases consist of a nine-member family of both intracellular and extracellular enzymes, which are Ca^{2+}-dependent and catalyse the posttranslational modification of proteins. These family members are extensively distributed in a wide range of organisms from bacteria to plants and animals (Bergamin *et al*., 2007; Siegel and Khosla 2007). As enzymes, they exert their function through the modification of protein targets, and themselves undergo several post-translational modifications including nitrosylations, phosphorylation, proteolytic cleavages and fatty acylation reactions (Esposito and Caputo, 2005).

The transglutaminase family (Table 1.3) is made up of transglutaminase 1 (TG1), transglutaminase 3 (TG3) and transglutaminase 5 (TG5) are intracellular proteins and expressed in the epithelial tissues. Tissue transglutaminase 2 (TG2) is found in both intracellular and extracellular locations and expressed in a diverse range of body tissues; transglutaminase 4 (TG4) is expressed in the prostate gland and factor XIII which is expressed in the blood. The other members, transglutaminase 6 (TG6) and transglutaminase 7 (TG7) are ubiquitous, while the enzymatically inactive band 4.2 is an erythrocyte membrane-associated protein that maintains the integrity of the erythrocyte membrane (Esposito and Caputo,2005; Siegel and Khosta, 2007).

Table 1.3: The nomenclature and distribution of transglutaminases family members (Siegel and Khosta, 2007).

Transglutaminases	Alternative names	Tissue	Location
Transglutaminase 1	TG1, TGK, keratinocyte TG, particulate TG	Epithelia	Cytosolic, membrane
Transglutaminase 2	tTG, TGC, TG2, Tgase	Ubiquitous	Cytosolic, nuclear, mitochondria, extracellular
Transglutaminase 3	Callus TG, Epidermal TG, hair follicles, bovine snout TG	Epithelia	Cytosolic
Transglutaminase 4	Type 4 TG/ prostrate TG.	Prostate	Extracellular
Transglutaminase 5	Type 5 TG, TG5, TGX.	Epithelia	Cytosolic
Transglutaminase 6	TG6. type 6 TG and TGY	Ubiquitous	Extracellular
Transglutaminase 7	Type 7 TG, TG7/TGZ	Ubiquitous	Membrane
Transglutaminase factor VIII	Plasma TG, factor XIII A, fibrin stabilising factor.	Blood plasma, platelets	Extracellular
Band 4.2	B4.2	Erythrocytes	Membrane

1.4 Tissue transglutaminase 2 (TG2)

Discovered in 1957 by Waesch and Heinrich, TG2 was first detected in mammalian liver homogenates, and It is the most studied and biologically characterised member of the transglutaminase protein family (Thomazy and Fesus, 1989). TG2 is also known as tTG, type II transglutaminase or ‘liver transglutaminase’ because of its high abundance in the liver. With

a molecular weight of 75 kD, it consists of 687 amino acids and is involved in several cellular processes, including the cell cycle, endocytosis, protein crosslinking, cellular adhesion and apoptosis. The protein has been linked with several diseases such as coeliac sprue, Alzheimer's disease, Huntington's disease (Hoffner and Djian, 2005), breast cancer (Herman *et al*., 2006), pancreatic cancer (Akar *et al*., 2007), colorectal cancer (Miyoshi *et al*., 2010) and several other cancer types (Mangala and Mehta, 2005; Siegel and Khosta, 2007), where it is typically overexpressed (Mehta, 1999).

TG2, like other family members, is involved in posttranslational modification of proteins, which occurs at the amide moiety of glutamyl-side chains of protein substrates (Bergamin *et al*., 2007), sometimes introducing isopeptide bonds within and/or between the glutamyl and lysine polypeptide chains (Chen and Mehta, 1999). Such bonds have tremendous physiological significance because of their stable and resilient nature to mechanical and proteolytic breakdown and can only be broken through total proteolytic degradation such as lysosomal proteolysis of the proteins involved (Chen and Mehta, 1999).

1.5 Structure of TG2 and the conformational control of TG2 activity

Human TG2 displays the four-domain structure peculiar to other transglutaminases (figure 1.4a) which includes an N-terminal β-sandwich domain that consists of 130-140 amino acids and folds into β-strands and an α-helix (Hitomi *et al*., 2016).

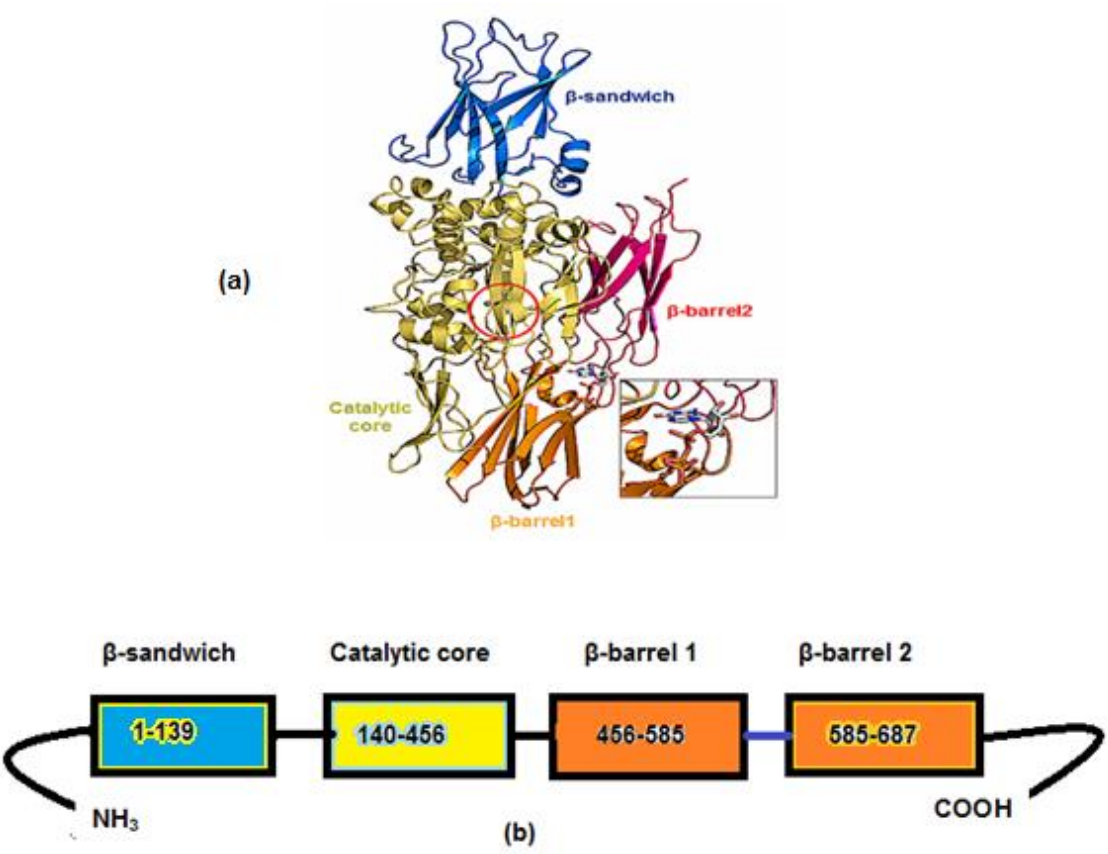


Figure 1.4: (a) Crystal Structure of tissue transglutaminase 2 (Laszlo and Piacentini, 2002) and schematic representation (b) of the four different protein domains and the number of amino acids.

The first domain, the β sandwich domain, is made up of 139 amino acids, the catalytic core domain contains a catalytic. A catalytic core is made up of a set of three amino acids found near the active site, and for TG2, it consists of the triad Cys-277, His-335, and Asp-358, arranged in a charge-relay system. The catalytic core is involved in acyl transfer and catalytic reactions while the other two domains are the β-barrel domains. These domains have binding sites for several cellular compounds, e.g., fibronectin and integrin-binding sites are located on the N-terminal β-sandwich, and a phospholipase C binding site is found in the C-terminal (as reviewed by Onyekachi and Coussons 2013).

The highly preserved catalytic triad of Cys277-His335-Asp358 shared by other enzymatically active members is involved in catalysing transamidation, transamination and protein cross-

linking reactions, but unlike other transglutaminases, TG2 requires Ca^{2+} for its activation. Upon activation by Ca^{2+}, cysteine 277 becomes extremely reactive and mediates the catalytic step towards peptidyl-glutamyl substrate(s). This reaction forms a thioester intermediate that is stabilised by interaction with an additional essential amino acid, tryptophan residue (Bergamini *et al*., 2011).

The availability of Ca^{2+} ions in the cellular milieu controls the activity and the conformational state of TG2. Two major types of structural conformation exist (fig 1.5) based on which cofactor is bound to the catalytic core. A 'closed' or 'compact' conformation is formed when the guanotide molecules (GTP) bind to it, and in this state, the β-barrel domain folds over the catalytic triad and access to Cys 277 is obstructed by the C-terminal β-barrels. Cys 277 is a significant regulator of the activity of the enzyme, and in this conformation, it is inactive. Liu *et al*.,(2002), put forth the hypothesis that when GTP/GDP binds to TG2, it stabilises or secures the conformation, preventing substances from accessing the catalytic site. However, when bound by Ca^{2+} ions, the β-barrel shifts away from the catalytic triad allowing substances into the active site and forming the 'open' or 'active' conformation of the enzyme. In summary, TG2 enzyme activity depends on the conformation of the protein, the presence of either calcium ions or nucleotides (GTP or GTP). This activity is also dependent on the spliced state of the gene product (section 1.10).

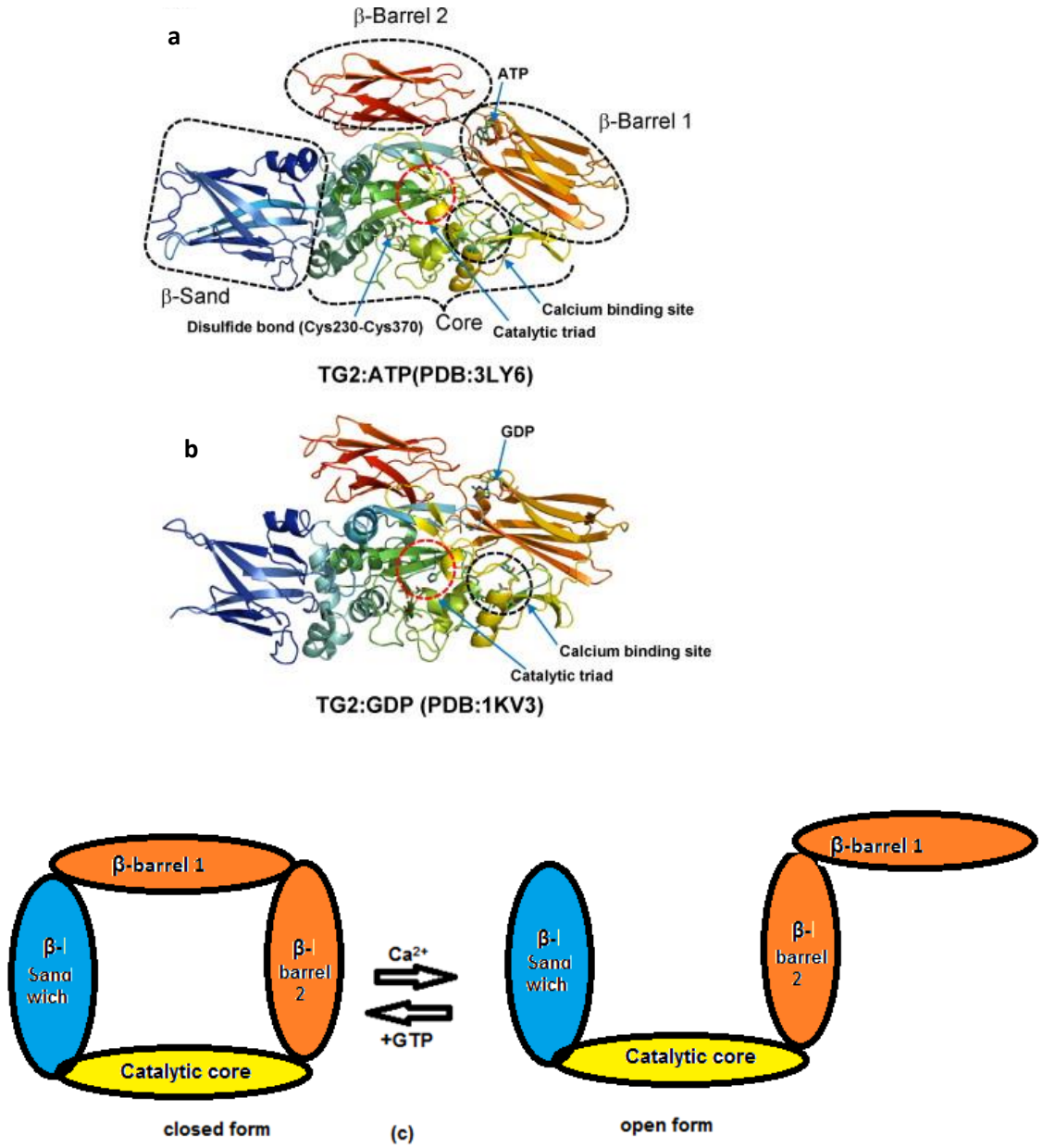


Figure 1.5: Crystal structure of the closed and open conformation of TG2 (Han et al.,2010) and a schematic representation showing the closed (a) and open (b) form based on the availability of either calcium of GTP and schematic representation (c) of the crystal form.

There is a competition between the guanotides and Ca^{2+} to change the conformation to either the 'closed' or the 'open' state. Intracellular TG2 is assumed to be in the 'closed' state because of the high GDP/GTP levels with only a small proportion being in the open state. Conversely, extracellular TG2 is presumed to be in the 'open' state because of the low GTP and high Ca^{2+} concentrations (Datta *et al*., 2006, Singh *et al*., 2016; Katt *et al*., 2018).

1.6 Functions of TG2

The enormous array of tissue transglutaminase functions in the cell are both enzymatic and non-enzymatic.

1.6.1 Deamination function

In deamination reactions catalysed by TG2, an amide functional group in the glutamyl side chain of a protein is replaced with water, and the result is glutamic acid (fig 1.6a). Introduction of such into proteins affects their activity, conformation and ability to interact with other proteins. Such reactions are encouraged by low pH, low substrates and TG2 concentrations. An example is in that of the wheat protein gliadin, where TG2 catalysed deamination reactions are linked to celiac disease (as reviewed by Nurminskaya and Belkin, 2012).

1.6.2 Transamination function

Another enzymatic function of TG2 is its transamination activity, which facilitates the addition of an amine group into an existing protein molecule. The amino nitrogen from the substrate for the reaction (donor) is transferred to a keto acid which serves as the receptor (Lorand and Graham, 2003). Such an addition results in one of two outcomes: a post-translational modification, which changes the unique characteristics of the protein, or the formation of an iso-peptide bond, which crosslinks two proteins. Such bonds are resistant to proteolytic chemical and physical forces. These bonds have been proposed to stabilise the extracellular matrix, thus averting the release of apoptotic cells' content into the environment (Gundemir *et al*., 2012).

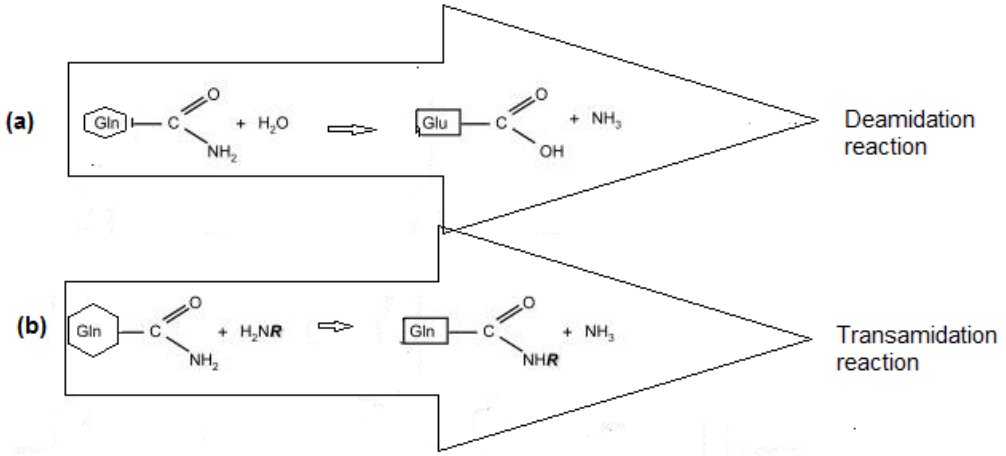


Figure 1.6: Transglutaminase catalysed reactions (a) deamidation and (b) transamidation

1.6.3 Protein-Protein cross-linking/ GTPase / ATPase function

Protein cross-linking is one of the various and most studied enzymatic reactions catalysed by this enzyme. TG2 crosslinks itself to other cellular proteins through its lysine residues and to substrates containing glutamyl, such as the gluten peptides and extracellular matrix proteins (fibrinogen and fibronectin). The simultaneous existence of both reactive lysine and glutamyl residues facilitates TG2 to create isopeptide crosslinks between molecules that can affect the interaction, conformation and stability of proteins; perhaps significantly these isopeptide bonds are usually very resilient to polymerisation (Nurminskaya and Belkin. 2012). TG2 is involved in GTP-binding and also functions as a G-protein receptor (Nakaoka *et al*., 1994) and has identical α subunits with the GTP binding protein Ghα, and its GTPase, cross-linking activity is under the control of cofactors Ca^{2+} and GDP.

1.6.4 Protein phosphorylation

Protein phosphorylation is also a post-translational modification caused by the covalent attachment of a phosphoryl group to a protein side-chain(s), usually tyrosine, threonine or serine. Such modifications play an essential role in regulating protein-protein interactions and functions (Wang *et al*., 2012). TG2 has been observed to have intrinsic serine/threonine kinase

activity and involved in several phosphorylation reactions. Of potential importance to the control of apoptosis, TG2 phosphorylates p53 at serine residues 15, and 30, which are essential interaction sites with Mdm2. p53 is a tumour suppressor protein which when activated by phosphorylation induces genes involved in DNA repair and cell cycle arrest, especially from the G2 and M phases. Activation of p53 involves destabilising the interaction between Mdm2 and p53, which results in cell cycle arrest and apoptosis (section 1.15.2). Thus, the TG2- induced phosphorylation decreases the capacity to interact with Mdm2 (Mishra and Murphy 2006).

Histones are also known substrates for TG2 phosphorylation and cross-linking activity, and such modification alters chromatin structures, transcription, replication and gene expression (Sato et al.,2003). Histones H1, H2A, H2B, H3 and H4 phosphorylation has been shown to promote gene transcription (Dunn, 2003), and the phosphorylation of H1 mainly was associated with chromatin change in breast cancer cells (Mishra et al., 2006) and retinoblastoma proteins (pRb), a tumour suppressor protein (Mishra et al., 2007).

1.7 Other functions of TG2

Multiple physiological roles have been linked to TG2, as depicted in the 'TG2 tree of life' (figure1.7). Intracellularly, TG2 is thought to play opposing roles: a pro-apoptotic and an anti-apoptotic part, depending on the cellular context and biological cues (Scondy and Fesus, 2005). Extracellular TG2 is involved in cell adhesion (Gaudry et al., 1999; Gaudry et al., 1999b), matrix assembly (Collighan and Griffin, 2009), wound healing (Telci 2006), receptor signalling and diverse other cellular activities.

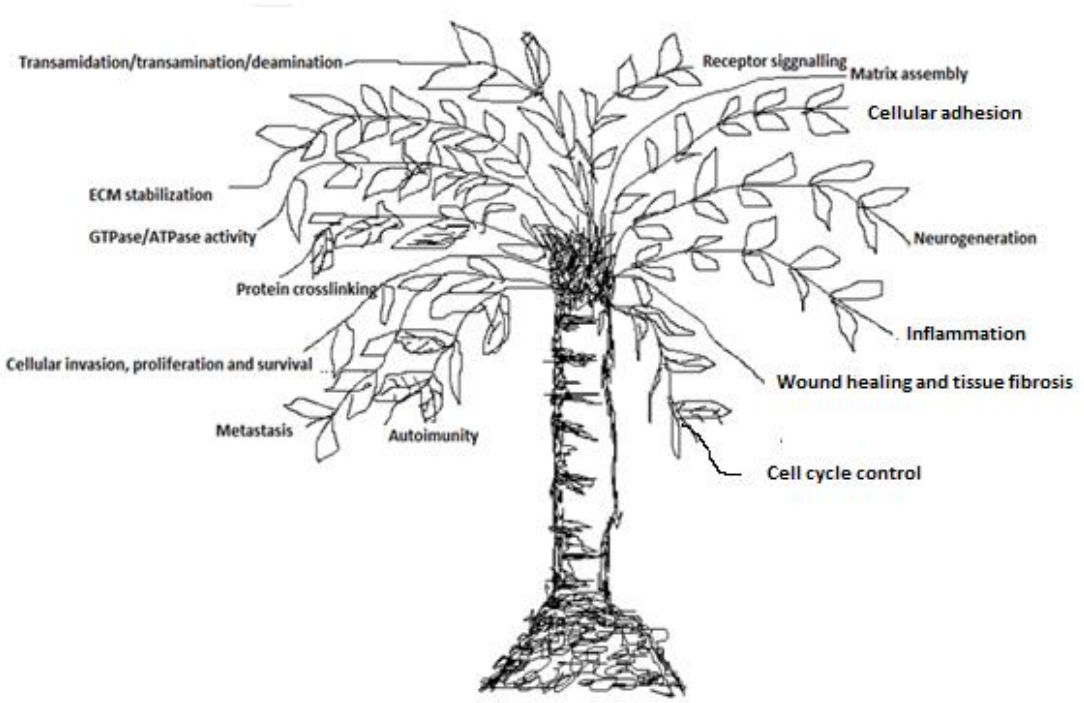


Figure 1.7: Tissue transglutaminase 2 'tree of life.'

An illustration of the diverse functions of tissue transglutaminase 2, shown as a tree of life with different branches depicting the multifunctional nature of the protein.

1.8 Localisation and distribution of TG2

TG2 is expressed abundantly in various organs such as the heart, liver, and intestine, as well as erythrocytes (Placentini and Fesus, 2002), where it is found in both intracellular and extracellular locations (Park *et al*., 2010). Intracellularly, TG2 is principally in the cytosol though it is also present in the nucleus, mitochondria and endoplasmic reticulum. Transamination activity is perceived to be latent in the cytosol due to the low calcium ion concentrations. However, it can be triggered by cellular stressors, which activate an influx of extracellular calcium or intracellular release from calcium stores and specific stimuli can cause the translocation of cytosolic TG2 into the nucleus. Approximately 5% of cellular TG2 is found in that compartment, where it functions as a G-protein or cross-links histones via its transamidation function (as reviewed by Onyekachi *et al*., 2015).

TG2 contained in the extracellular matrix (ECM) is shown to play an essential role in cell adhesion, migration, ECM organisation and turnover. TG2, therefore, contributes to routine wound healing, tissue regeneration, inflammation, and fibrosis. Due to its multifunctional, ubiquitous nature and interaction with different substrates, TG2 has been implicated in several pathological conditions, including multiple forms of human cancer (Zemskov *et al.,* 2007).

1.9 TG2 substrates

As a ubiquitous protein, the array of TG2 substrates is enormous; this might account for its involvement in several cellular processes. TG2 substrates are distributed widely in the cytosol, nucleus, mitochondria and on the extracellular surface of the cell. Table 1.4 shows a list of TG2 substrates in the ECM with emphasis on substrates involved with signal transduction and cellular processes that can lead to cancer. A list of other substrates can be found in the appendix section.

Table 1.4: Some TG2 substrates in cells grouped by functions

Substrate	Involved in
Nf-kβ PTEN AKT Histones p53 Caspases Epidermal growth factor Latent TGF-beta binding protein-1 (LTBP-1) Cystatin 6 (M/E) Galectin 3 Small GTPases Keratin, type II cytoskeletal 1 Retinoblastoma Histones PKM2	Cancer biology
Osteonectin Osteopontin (extracellular matrix cell adhesion protein) Nidogen (entactin) Galectin 3 Fibrinogen A alpha Fibronectin Collagen alpha 1(III) Cell adhesion molecule C-CAM SLC proteins	Extracellular matrix-cell interaction and stabilization
Loricrin Band 3 Clathrin heavy chain Solute carrier proteins Focal adhesion kinase	Membrane structure/function and membrane traffic
Rho A Phospholipase A2 Nucleotide(s) binding/hydrolysing Insulin-like growth factor-binding protein-3 (IGFBP-3) Insulin-like growth factor-binding protein-1 Integrins Fibronectin Osteoclasts	Signal transduction
Vimentin Tubulin Troponin T Microtubule-associated protein tau - Isoform Tau-F (Tau-4)	Cytoskeleton regulation

1.10 TG2 isoforms

Structurally, there are several isoforms of TG2, which are generated from alternative splicing of the mRNA transcript from the TG2 gene. Alternate splicing during gene expression offers the opportunity to make protein products with different functions from the parent gene and/or product. The main alternatively, spliced forms of TG2 gene products include variants in the amino acid sequence of the C-terminal, resulting in the formation of a long variant (TG2-L) and a short variant (TG2-S). Other shorter variants have also been discovered (TG2-S_2, TG2-S_3 and TG2-S_4), which were produced by alternative polyadenylation, generating proteins with different C-termini; (Gentile *et al.,*1999; Phatek *et al.,* 2013) the short variant (TG2-S) being highly expressed in Alzheimer's disease brains, and it *was* proposed to be responsible for the noticeable increase in Ca^{2+}-dependent TG2 crosslinking measured in AD brains (Citron et al. 2001).

Figure 1.8: shows the full-length protein with 687 amino acids and a molecular weight of 75 kDa (Gentile et al., 1991); TG2-S which has a molecular weight of 52 kDa with 540 amino acid residues; and TG2-S_2, which has a molecular weight of 38kDa with 349 amino acid residues respectively (Fraij and Gonzales 1996). These differentially spliced copies have a different function (Begg *et al*., 2006).

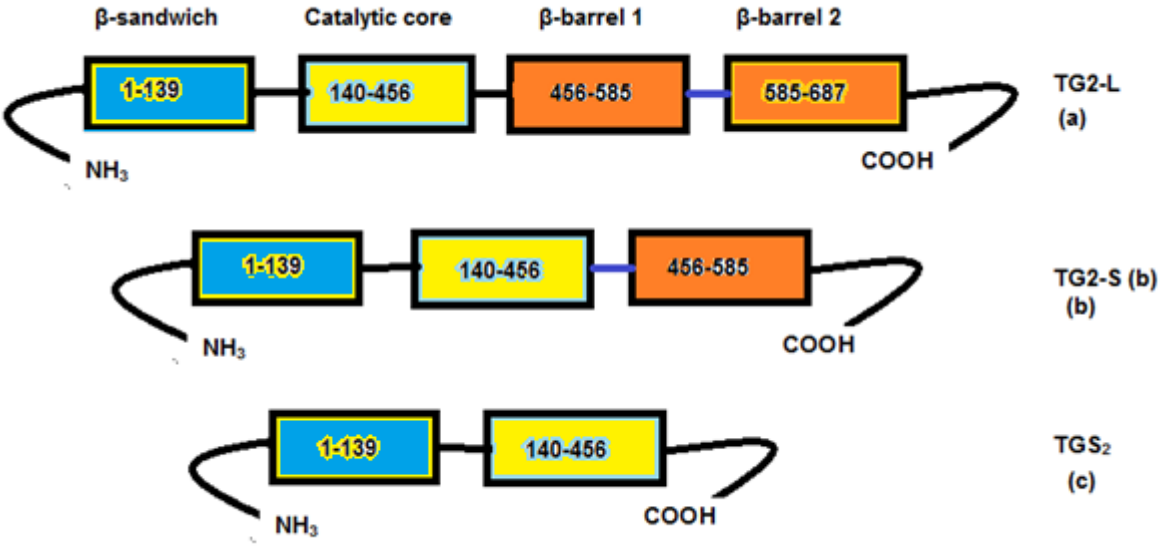


Figure 1.8: Schematic diagram of TG2 isoforms, (a) TG2-L (b) TG2-S (c) $TG2S_2$. The numbers indicate the amino acids in each domain.

Singh *et al*., (2010) demonstrated the effect of the different conformations of TG2 on the viability of cells and discovered that the disruption of the hydrogen bonds between the C-terminal β barrel and the catalytic core maintains the protein in an 'open state' which causes cells to enter into apoptosis promoting cell death. In contrast, the 'closed-form' encouraged cell survival. TG2-S was seen to have a cytotoxic effect on NIH3T3 cells (Antonyak *et al*., 2006; Hitomi *et al*., 2015) and on neuroblast cells; it was seen to have opposite effects to TG2-L (Tee *et al*., 2010).

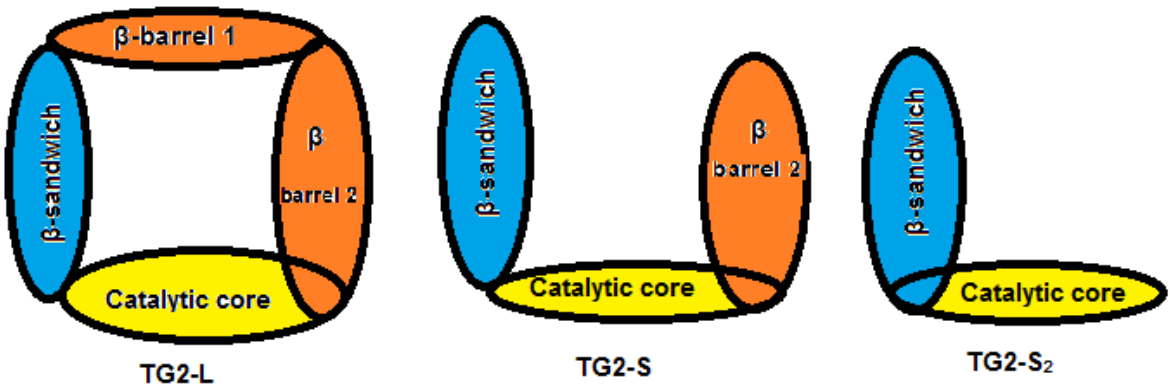


Figure 1.9: Schematic diagram of the conformational states of the full length and truncated TG2-S variants. The truncated variants supposedly are always in the 'open state'.

The expression of the shorter variant (TG2-S) is usually lower than the full-length variant (TG2-L) as seen in healthy cells, but there is more variation in the expression of the isoforms in cancer cells. However, TG2-L contributes approximately 50-60 % of the total TG2 expression showing the variability of TG2 splicing among cell lines (Phatek *et al*., 2013).

1.10.1 TG2-L and TG2-S in cell proliferation and cell death.

The multiple biological functions of TG2 include the propagation of signals that encourage the survival of cells in some circumstances, and cell death in others. The possible association between TG2-facilitated cell malignancy and survival is relevant since increasing evidence has shown that inhibition of TG2 enhances apoptosis in cancer cells (Budillon *et al*., 2013).

The expression of TG2 variant forms is altered in several cancers (Phatek et al., 2013), which may suggest that the differential levels expressed may play a significant role in the cellular crosstalk involved in driving cells into apoptosis or survival. From previous findings, TG2-L expression in fibroblast, as well as in cancer cells, protects against apoptosis while the expression of TG2-S induced apoptosis. The pro-apoptotic action of TG2-S was not due to the GTP-binding activity because it exhibits a weak transamidation activity, owing to the truncated C-terminal, which removes the guanine binding site. The apoptotic ability of TG2-S was seen to be due to a rare capacity that the isoform has to displays a complex form of

abnormal aggregation, such abnormal oligomerisation has been seen as a mechanism for cell death induction. Hence, mutated, misfolded or posttranslational modified proteins that attain the capacity to oligomerise randomly can accumulate in cells, disrupting normal cellular processes and thus lead to cell death (Antonyak *et al*., 2006). Based on the conformation shown in figure 1.9, TG2-S is always in the 'open state', emphasising the significance of the last 30 amino acids, including Arg 579 which plays a vital role in cell division (Frezza and Mauro, 2015) and whose absence may be responsible for TG2-S cytotoxic nature.

Using fibroblast cells with a Myc-tagged form of TG2, Antonyak *et al*.,(2011), showed that TG2-L actively protected the cells from apoptosis due to serum starvation, by inducing increased levels of the mammalian target of rapamycin/viral accessory protein p13 (mTor/P13complex 1, p70-S6-kinase pathway and proto-oncogene c-Src (c-SRC) binding which promotes cell survival. When cells were treated with inhibitors that block TG2 function and c-Src kinase activity, the mTor/P13complex 1, p70 S6-kinase pathway was interrupted, eliminating the TG2-mediated survival of cells. TG2-L was also observed to encourage the survival of malignant mesothelia in hypoxia (Zonca *et al*., 2017).

1.11 The cell cycle

Cell differentiation, division and cell death are major biological processes that control homeostasis in multicellular organisms; intricate pathways regulate genomic integrity, growth and survival of cells. In these pathways, DNA repair, programmed cell death and cell cycle checkpoints have critical roles, and interruption or poor regulation of cell death pathways may lead to abnormal cell growth (Wiman and Zhivotovsky, 2017).

Malfunction of the cell cycle plays significant roles in the development of several disorders such as cardiovascular and neurodegenerative diseases, and cancer. Increased understanding of the mechanisms of these diseases has directed the development of new therapeutics for pathology linked with cell cycle irregularities (Wiman and Zhivotovsky, 2017).

Cyclins and cyclin-dependent kinases (CDKs) are specific proteins responsible for driving the cell forward in the cell cycle phases G1, S, G2, and M (Malumbres and Barbacid, 2009). A vital step in the cell cycle that is carefully regulated is the G1 to S transition because as soon as the cell enters the S phase, it is destined to go through the subsequent phases to give rise to daughter cells (Wiman and Zhivotovsky, 2017). CDK complexes can be inhibited by p^{21} (cyclin-dependent kinase inhibitor 1), and p^{21} was demonstrated to be induced by miR-184 which are short (18-25) nucleotides of non-coding RNA (Richardson et al., 2018). Inhibition of the CDK complexes will affect the progression of the cell cycle and cell division.

Two hallmarks of cancer, as shown in figure 1.10, are the disregard for signals of programmed cell death and continuous proliferation (Hanahan and Weinberg, 2011). Abnormal growth at the molecular level is often due to the activation of oncogenes such as Ras and Myc and the disruption of the retinoblastoma protein (pRB) controlled regulation of G1 to S phase. pRB is a tumour suppressor protein whose role in the cell cycle is to prevent excessive replication and acts as the 'brake' in the cell. Indeed many tumours display a genetic change that alters the function of pRB. Recent progress in the knowledge of the cell cycle and cell death has shown that molecular targeting of key biomarkers may provide new targets for enhanced cancer treatment (Wiman and Zhivotovsky, 2017).

pRB is a substrate for TG2 (Table 1.4), and it was observed that TG2 phosphorylates pRB at Ser^{780} which resulted in the destabilisation of the pRb·E2F1 complex, an essential requirement for the progression of the cell cycle (Lian *et al*., 2010).

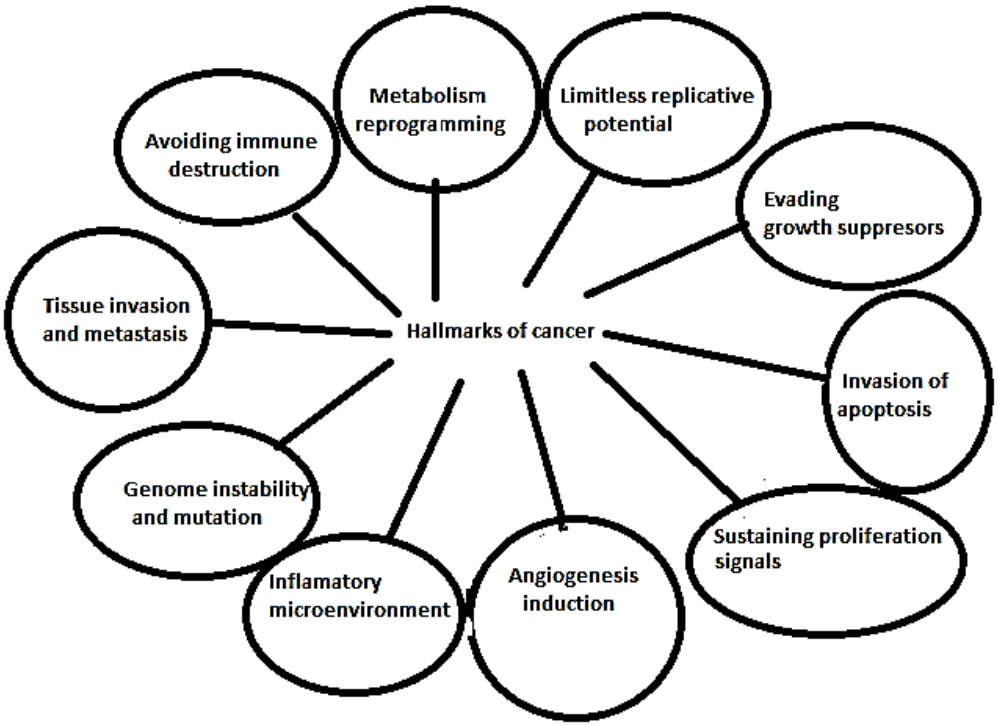


Figure 1.10: The ten hallmarks of cancer

The defence mechanisms exhibited by cancer cells which serve as a framework to understand tumour pathogenesis.

1.12 Cancer and chemoresistance

Chemoresistance is defined as the resistance of cells to the action of a specific therapeutic agent(s). (Stedman, 2005). Tumours can be innately resistant preceding chemotherapy or develop chemoresistance during the treatment process, to develop a phenotype that is cross-resistant to a variety of related-chemotherapeutic agents (Gore *et al*., 2015). Chemoresistance is the primary cause of treatment failure and is more pronounced in advanced metastatic disease, in the majority of patients (Longley *et al*., 2006).

In a similar way to how alterations in bacteria produce infections that are resistant to antibiotics, changes in cancer cells produce tumours that are resistant to treatments that were previously effective . Relapse in cancer patients is believed to be due to the growth of a small percentage of cells that escape death and mutate into these resistant forms (Forsythe,2010). Initially, the patient may show significant improvement until the new form of cancer

establishes. Such resistance to anti-cancer drugs is a primary problem encountered and a leading cause of treatment failure, in addition to other factors (fig 1.11), such as pharmacological and cellular factors (Gore *et al*., 2015). Despite targeted therapeutics and specific treatment approaches, it seems likely that resistance will continue to be a significant clinical problem (Gatti and Zunino, 2005) because cancer cells still develop resistance to novel chemotherapeutic drugs.

1.13 Molecular mechanisms of chemoresistance

The problem of chemoresistance is multidimensional, and several factors affect tumour drug sensitivity. However, evidence has emerged from research over recent decades that can partially explain the mechanisms of resistance (Longley *et al*.,2006). These processes include the reduction of active drug concentration at the target level, due to activation of transporter proteins, detoxification mechanisms within the cell, alterations affecting drug-target interactions and induction of factors that influence cellular responses that affecting tumour cell survival (Fig 1.11).

A key drug-resistance mechanism in cancer cells is the ability to decrease drug concentration at the drug target. Two possible mechanisms capable of reducing drug concentration at the target site includes reduced drug uptake or enhanced drug efflux. Drug transporters aim to remove chemotherapeutic drugs from the cell, and this mechanism is a natural defence by which means the tumour cells can evade the action of the drug (Kachalaki *et al*., 2013).

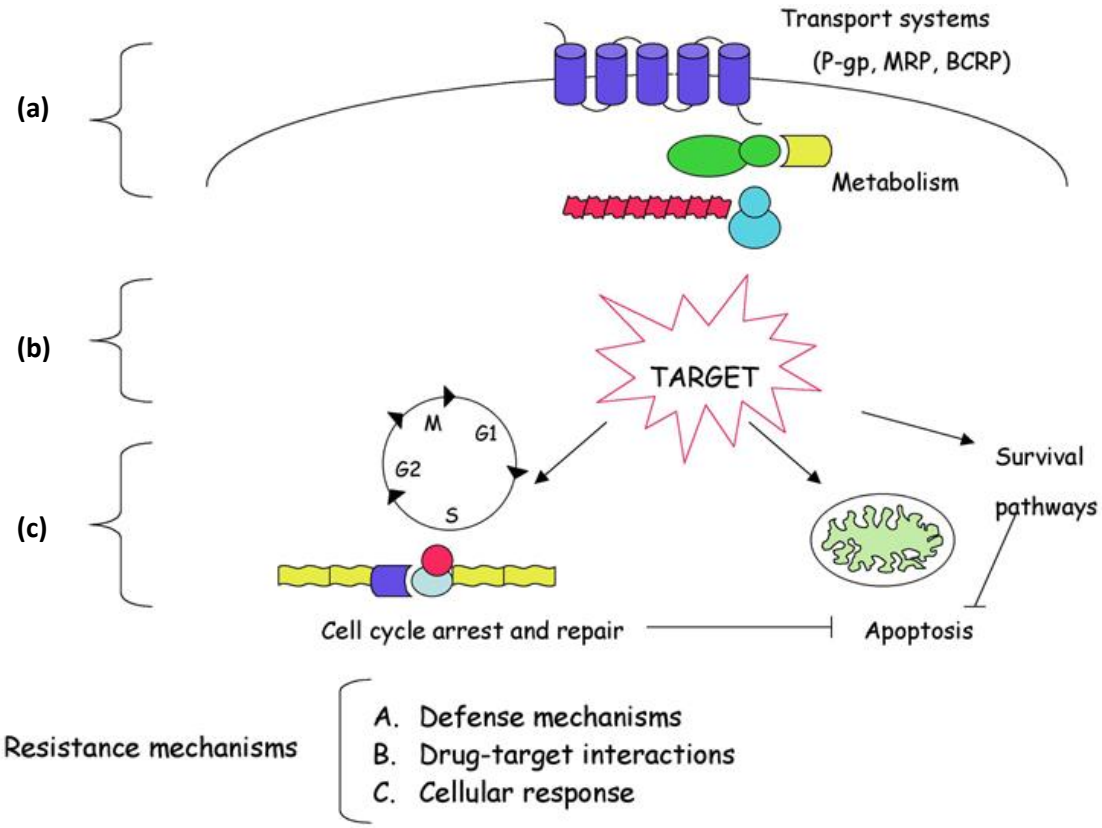


Figure 1.11: Mechanisms of chemoresistance. (Adapted from Gatti and Zunino, 2005)

An illustration of the resistance mechanisms by cells. (a) represents the drug transporters P-glycoprotein 1 (P-gp), multidrug-resistant associated protein (MRP) and breast cancer resistance protein (BCRP). (b) the drug target site such as the DNA; (c) cellular response to the drug: cell cycle arrest, initiation of apoptosis or cell survival.

1.13.1 Resistance mechanisms of cancer cells

The strategies employed by cancer cells to evade the effect of anti-cancer drugs are described in this section.

1.13.2 Drug transporters

Most drugs are transported from the digestive tract into the portal blood vessel, but drug bioavailability at the site of action is dependent upon local cellular active transport processes (Zhou, 2010). Based on metabolic needs or the presence of toxic substances, membrane transporters relocate substances in and out of cells (Cucillo *et al*., 2016).

Several cytotoxins found naturally and used in chemotherapy as drugs enter cells by passive diffusion, and these amphipathic drugs are hydrophobic enough to diffuse through a lipid bilayer but also hydrophilic enough to reach their target (Gatti and Zunino, 2005).

A decrease in drug influx, as well as increase of efflux from the cells, may reduce the accumulation of drug inside the cell, as most chemotherapeutic drugs move into cells via passive diffusion through the plasma membrane. Thus, changes in the cell membrane structure associated with oncogenes will also affect drug influx (Bush and Li, 2002).

The ATP-binding cassette (ABC) family of proteins is responsible for influencing the intracellular concentration of drugs and several compounds in cells and tissues (Gatti et al., 2005). Approximately 49 ABC transporter genes have been discovered in the human genome, which are expressed naturally in a diversity of tissues remove xenobiotics from the body (Kachalaki *et al*., 2013).

Drug transporters typically are composed of two transmembrane domains that recognise and translocate substances through the plasma membrane and two other nucleotide-binding domains, which help to generate the energy needed, by the hydrolysis of ATP (Kachalaki *et al*., 2013).

1.13.3 Modifications of drug targets

Any alteration or modification in the expression levels of the drug target during patient treatment can lead to resistance. An example of such change is with the antimetabolite drug 5-Fluorouracil, which inside the cell is converted to the compound fluorodeoxyuridine monophosphate (FdUMP). FdUMP is a strong inhibitor of the enzyme thymidylate synthase (TS) which is needed for DNA synthesis (Longley *et al*., 2003). An increase in TS affects the quantity of 5FU required to inhibit all targets, and if insufficient, cancer cells will continue multiplying (Kachalaki *et al*., 2016). Research has shown that patients with elevated levels of TS were resistant to chemotherapy when compared with responsive patients (Leichman *et al*., 1997).

Another example is that of decreased oestrogen receptor expression in oestrogen positive breast cancer cells which leads to the failure of tamoxifen, a major drug of choice for treatment to subdue cell growth (Miller, 2004; Campos 2004).

1.13.4 Cellular response to chemotherapeutic agents

There are several different cellular responses to chemotherapeutic agents, and the initiation of DNA repair processes is considered to be the first and most frequent response of cells to toxic damage. Cells have developed complex signalling pathways to stop cell cycle progression in the presence of DNA damage, thus facilitating DNA repair (Zhou *et al*., 2000; Friedberg, E. C. 2003). However, when the impact of cellular insult surpasses cellular ability to repair, cells enter into apoptosis (Cory and Adams, 2002).

1.13.5 Alterations in DNA damage repair

Cells can develop chemoresistance following recovery from DNA damage probably caused by oncosis. The excision repair cross-complementing protein group 1 (ERCC1), which is a DNA-repair protein, has been shown to be prominent in platinum-based drug resistance and the overexpression of this protein correlates with platinum drug resistance in certain cancers, such as non-small cell lung cancers, ovarian and gastric cancers (Chang *et al*. 2005; Olaussen *et al*., 2006; Bouwman and Jonkers, 2012).

1.13.6 Problems with cell cycle regulation

The tumour suppressor protein (p53) is encoded in humans by the homologous gene TP53 and functions as a tumour suppressor gene by regulating the cell cycle, DNA repairs and inducing apoptosis (Khoury and Bourdon, 2003). p53 controls apoptosis and cell cycle arrest after DNA damage and prevents the production of new daughter cells with damaged DNA (Lowe *et al*., 2004; Kachalaki *et al*., 2016). p53 controls the G1 to S phase and also at the G_2/M phases, and its activation promotes cell cycle arrest and encourages DNA repair or triggers apoptosis. Therefore, its inactivation has dire consequences in cells, leading to the emergence of several cancers (Kapil and Fok 2007).

Previous reports have revealed that in healthy growing cells, p53 translocates between the nucleus and cytoplasm, but when the cell is under stress such as in cases of hypoxia, UV irradiation or in the presence of chemotherapeutic drugs, p53 stabilises its location in the nucleus (Mesaeli and Philipson, 2003). In the nucleus, p53 binds to DNA stimulating the production of $p21^{Cip1}$ (CDK-interacting protein 1) which interacts with cdk2, a cell division stimulating protein. The interaction between $p21^{Cip1}$ and cdk2 prevents the cell from advancing to the next cell division stage with the resultant cell cycle arrest and apoptosis. So, a mutant p53 will be unable to bind to DNA effectively with the resultant consequence that $p21^{Cip1}$ cannot act with cdk2 to stop the cell proliferation. Thus, cell division continues uncontrollably leading to the production of abnormal daughter cells resulting in tumours (NCBI 1998; Zukerman *et al*., 2009).

Increased expression and/or mutation of the p53 gene has been linked with poor breast cancer prognosis, chemotherapy radiotherapy. Cancer is an example where the normal machinery of the cell cycle regulation is defective, with either decreased removal of abnormal cells and/or an overproliferation of cells (King and Cidlowski,1998) and, the tumour suppressor gene is a significant player (Pucci *et al*., 2000).

Another essential regulator of cell cycle progression is the phosphatase and tensin homolog (PTEN) protein which is also involved in tumour suppression activities, such as inhibition of cell growth, migration, invasion and focal adhesion (Kapil and Fok 2007). It induces cell cycle arrest by upregulating $p27^{KIP1}$ (a member of the universal cyclin-dependent kinase inhibitor (CDKI) family), a cell cycle inhibitor, and collaboration between p53and $p27^{KIP1}$ appears critical in cancer development (Minami *et al*., 2016).

1.13.7 Changes in B-cell lymphoma 2 (Bcl-2) regulation

The Bcl-2 family of proteins regulates and activates caspases, which are the essential effectors of apoptosis. The Bcl-2 family contains both anti-apoptotic and pro-apoptotic proteins, and the pro-apoptotic members initiate the activities of the caspases leading to

apoptosis. Previously, a study has shown an association between high expressions of Bcl-2 in cancers with reduced response to anti-cancer agents (Kachalaki *et al*., 2016).

1.14 Overcoming chemoresistance

Resolving the problem of chemoresistance is essential in cancer treatment, and different approaches have been investigated to solve this problem. The suppression of genes involved in chemoresistance through the silencing of specific mRNA was found to be an effective method and is perhaps amongst the most advanced techniques currently used. Notably, the method was used to increase the sensitivity of human leukaemia cells (HL-60) to Etoposide (a chemotherapy medication used for the treatments of leukaemia) by using specific siRNA to suppress the multidrug resistance gene -1 (MDR-1 gene) (Kachalaki *et al*., 2015).

TG2 has been linked with several pathological processes including cancer and chemoresistance, and the biological functions of TG2 are based on the capability to catalyse crosslinking reactions in the presence of Ca^{2+} ions (Mehta and Han, 2011).

Under normal physiological conditions, due to the low Ca^{2+} and high GTP levels in cells, TG2 acts as a scaffold protein involved in critical signalling pathways. Activation of these pathways (NF-kβ, AKT and focal adhesion kinase) results in the survival of cells (Lee *et al*.,2004; Chhabra *et al*.,2009; Kim *et al*.,2006; Mehta *et al*., 2010).

Conversely, under trauma, stress or other extreme cellular conditions with a resultant loss of Ca^{2+} homeostasis, TG2 is activated, which results in its crosslinking of proteins and apoptosis of cells. Thus, TG2 functions in either cell survival or cell death and acts as a double-edged sword (Mehta and Han, 2011). Downregulation of proteins which promotes chemoresistance could revert resistance rendering tumours more sensitive to anti-cancer drugs.

1.15 TG2, cancer and chemoresistance

The wide range of TG2 biochemical activities accounts for its involvement with a variety of proteins and cellular processes (as reviewed by Nurminskaya and Belkin (2012). Most

oncogene/tumour suppressor genes produce proteins that have a role(s) in the control of cell cycle progression. One such family of proteins are the integrins, a family of proteins which allow cells to be sensitive to signals in the environment from the ECM. These signals are vital for most cell types about proliferation, adhesion, survival, migration and differentiation. Indeed, integrin-arbitrated signals are essential at the checkpoint where cells advance from the G1 to the S phase (see cell cycle section 1.11) and send signals for growth (Moreno-Layseca *et al*., 2014).

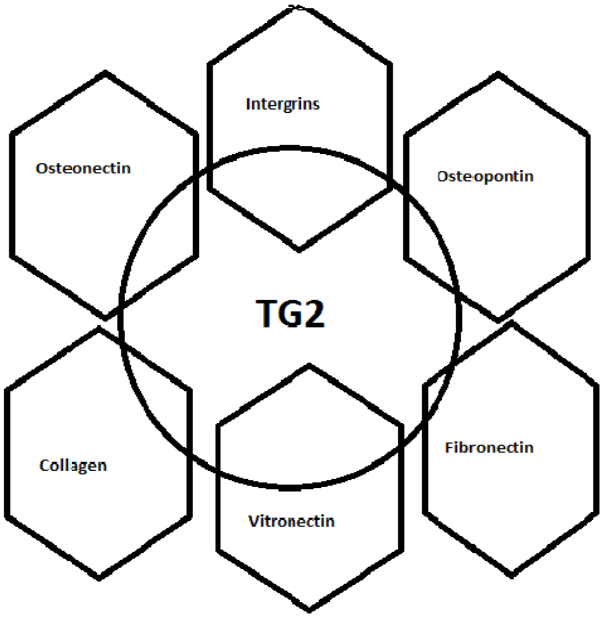


Figure 1.12: Some proteins modulated by TG2 whose alteration has a negative effect on proliferation

Depending on the type of protein interacting with integrins, the signalling function and binding activity can be changed, which in turn can lead to a change(s) in the behaviour of the cell (Verma and Mehta, 2007). TG2 is such a modulating protein (fig 1.11), which forms complexes with a large percentage of integrins on cell surfaces and with other ECM proteins such as osteonectin, osteopontin, collagen, vitronectin and fibronectin, proteins which are also involved in apoptosis, signal transduction, invasion, metastasis and cell adhesion. These cell

cycle molecules either directly or indirectly are linked with the cell cycle and cell homeostasis, and any disturbance to the equilibrium gives rise to a dysfunctional system and pathogenesis (Mangala and Mehta, 2005).

The interaction of TG2 with a significant number of ECM protein, fibronectin (figure 1.11), which are involved in cell survival signals has been shown to stimulate focal adhesion kinase leading to the activation of numerous signalling pathway that promotes the development of resistant phenotypes in breast cancer cells (Herman *et al*., 2006).

1.15.1 TG2 and inflammation

Inflammation which means ‘set on fire’ is a complicated biological response which is elicited by harmful stimuli. Inflammation involves a series of events such as cell proliferation, migration, neovascularisation, stabilisation of the ECM, and apoptosis which are all required for tissue repair and wound healing (Mehta and Han, 2011).

Inflammation is a component of tumour progression, and indeed it is possible that many cancers stem from chronic irritation, infection and inflammation. The tumour microenvironment is composed mainly of inflammatory cells such as interleukins, tumour necrosis factor, interferon-gamma and the granulocyte-macrophage colony-stimulating factor which participates in the tumorigenic process encouraging survival, proliferation and migration. Also, tumours have adapted some signalling molecules such as chemokines and selectins from the innate immune system for migration, invasion, and metastasis (Coussens and Werb, 2012). For example, the body’s response to cancer has many similarities to inflammation pathology. Inflammatory cytokines and cells found at tumour sites are more inclined to aid proliferation and the progression of tumours than stimulating an anti-tumour response. Susceptibility and severity of cancer may be linked with changes of the inflammatory cytokine genes because inhibition or deletion of these genes inhibited the development of cancer (Ballenul and Mantovani, 2001).

Proinflammatory cytokines induce TG2, and interferon-Ɣ (IFN-Ɣ) and this synergism between IFN-Ɣ and TG2 have been suggested to aggravate coeliac disease (Bayardo *et al*., 2012).

1.15.2 TG2 and p53

The translocation of p53 between the cytoplasm and nucleus is regulated by the murine double minute gene (Mdm^2), and a defect in this interaction affects p53 function. Though it is a cytosolic protein, TG2, can translocate under certain situations to the nucleus and has been observed to phosphorylate p53 at Ser^{13} and Ser ^{20,} reducing the ability of p53 to interact with Mdm^2 (Mesaeli and Philipson,2003). Ser^{13} and Ser ^{20} residues are essential in the Mdm^2-p53 interaction (Mishra and Murphy. 2006).

1.15.3 TG2, NF-κB and breast cancer

The pleomorphic nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein family plays a vital role in inflammation and the immune system. Due to its essential role in controlling pro-inflammatory genes by protecting abnormal cells from apoptosis, it has been linked to cancer initiation and progression (Hoesel *et al*., 2013), and is involved in mammary tissue development. NF-κB plays a significant role in breast cancer, owing to its ability to activate the expression of multiple anti-apoptotic and angiogenic genes directly (Brown, 2012). Increased TG2 expression has been correlated with increased expression of proteins involved with aggressive breast cancer such as NF-κB, hypoxia-inducible factor and protein kinase B (Kumar and Mehta, 2012). A relationship has also been observed between elevated TG2 levels and increased NF-κB levels and activity in pancreatic and breast cancer cells (Agnihotri *et al*., 2013).

1.15.4 TG2 and metastasis

Metastasis is a common reason for cancer-linked deaths, including breast cancer-related deaths (section 1.2). The stages leading to metastasis are intertwined, cells detach from original sites, invade blood vessels lumen and proliferate in other tissues different from the

original tumour site. The association of TG2-L with integrin and fibronectin promotes both adhesion and cell motility of cancerous cells, and the interaction promotes cell survival by rendering them resistant to apoptosis (Mangala and Mehta, 2005).

In MDA-MB231, a metastatic breast cancer cell line, TG2 was demonstrated to interact with integrins B1, B4 and B5 and fibronectin (figure 1.12) which facilitate ECM adhesion. The increased expression of TG2 was also seen to increase the invasion of breast cancer cells attached to fibronectin-coated surfaces. The authors concluded that TG2 expression in breast cancer cells plays a vital role in the development of the metastatic phenotype (Mangala *et al*., 2007). TG2 was then shown to induce EMT in different tumours, and the GTP binding activity was demonstrated to be responsible in ovarian cells (Shao et al., 2009; Cao *et al*., 2012) and mammary epithelial cells (Kumar *et al*., 2012).

miRNA is involved in various cellular processes and has been linked with the progression of cancer. It has been demonstrated to have roles in the regulation of tumour cell survival and proliferation (Hu *et al.,* 2016). Furthermore, Seo *et al*. (2019) demonstrated that TG2 induces EMT by inhibiting miRNA-205 (miR-205).

1.15.5 TG2 in autophagy

TG2 plays varying roles in activating the different forms of programmed cell death: necrosis, apoptosis and autophagy (figure 1.13). Autophagy is a mechanism of metabolic and chemotherapeutic stress tolerance, where cells devour fragments of themselves to persist in the face of starvation and stress (Kroemer *et al*., 2009). Its induction has both pro-apoptotic and pro-survival roles which contribute to the efficacy of chemotherapeutic agents as well as drug resistance. Overexpression of pro-apoptotic Bcl-2/Bcl-xL proteins. autophagy can be induced after ischemia, and anti-apoptotic Bcl-2 inhibits autophagy under nutritional stress (Degenhard *et al*., 2006).

A study with MCF-7 cells revealed that epirubicin (EPI) a cancer medication, induced autophagy and the EPI-induced autophagy gave protection to the MCF-7 cells from EPI-induced apoptosis (Sun *et al*., 2013). Defective autophagy may contribute to chemoresistance, and research has shown that inhibiting the expression of TG2 in pancreatic cancer cells by siRNA leads to the initiation of autophagy, and finally to cell death (Akar *et al*., 2002).

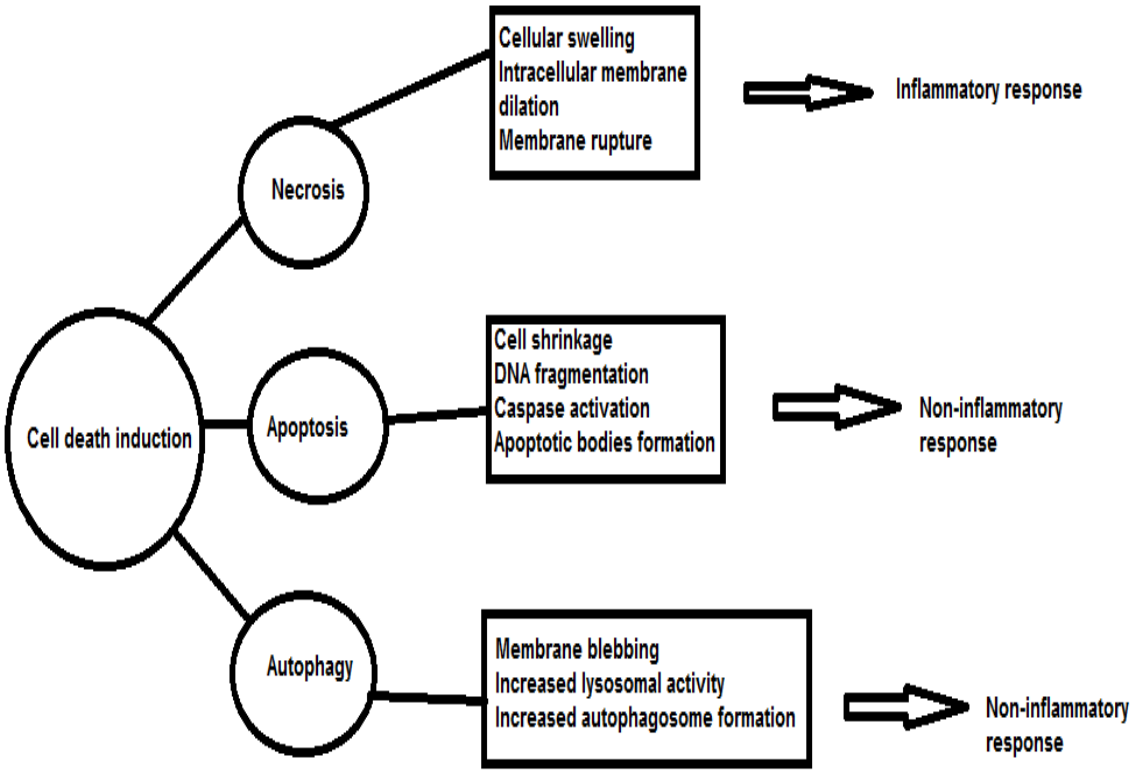


Figure 1.13: Different types of programmed cell death with cellular response

1.15.6 TG2 in apoptosis

It has been proposed that the transamidating activity of TG2 is a vital controller between autophagy and apoptosis (figure 1.14). Cho *et al*., 2010, showed that the knockdown of endogenous TG2 caused a significant increase of caspase-3 activity and poly (ADP-ribose) polymerase (PARP) cleavage in mouse embryonic fibroblasts (MEF) cells exposed to apoptotic stimuli, and the same cells displayed the build-up of light chain (LC3) following

autophagy induction. The authors suggested that the transamidating activity of TG2 confers a protective role in MEF cells response to death stimuli because inactive C277 mutant TG2 suppressed caspase 3 and PARP but catalysed the final stages of autophagosome formation (Cho *et al*.,2010). Increased TG2 expression was also seen to inhibit apoptosis through suppression of the activity of caspase 3 and 9, which aids the release of cytochrome c into the cytosol and mitochondria membrane depolarisation (Rossin *et al*., 2011).

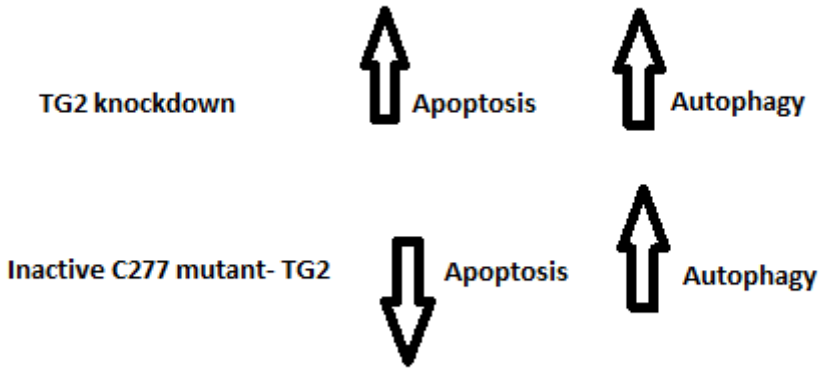


Figure 1.14: Effect of TG2 transamidating activity on apoptosis and autophagy.

An illustration showing the transamidating activity of TG2 is essential for the induction of apoptosis**.**

1.15.7 TG2 and endocytosis

Extracellular materials are taken into the cells by vesicles located on the plasma membrane through a process known as endocytosis. The uptake of fluids or macromolecules by vesicles into the cells is known as pinocytosis, while phagocytosis refers to the engulfment of larger particles. TG2 has been shown to interact with intracellular transport vesicles and is involved in endosomal trafficking, phagocytosis and pinocytosis (Kawabe *et al*., 2012). Also, drug delivery vectors play roles in the pharmacokinetics and toxicity challenges associated with non-viral intracellular delivery. Membrane trafficking is an important route for drug delivery into the cytosol, and such systems could be exploited for cytosolic drug delivery (Dyer *et al*., 2011).

A previous study with hepatocarcinoma cells (Meshram et al., 2017), provided evidence that elevated TG2 levels hindered the uptake of cisplatin into cells with a change in isoform ratios, which was associated with chemoresistance. The authors demonstrated that the isoform predominant in the cytosol was the long-form (TG2-L) while the membrane fraction of the cell is made up mainly of the short form (TG2-S). This confirms an earlier study by Milakovic *et al*., (2004) who showed that TG2 is predominant in the cytosol, present in the nucleus and associates with the plasma membrane. Thus the intracellular location of TG2 isoforms could have a bearing on its functions and cellular interactions with other proteins.

1.16 TG2 compensation by other family members

Though the functions of TG2 are diverse, no foetal abnormality was observed when TG2 was silenced in mice, which suggests that there may be a form of compensation by other members of the family. Also, there is no human disease traceable to the deficiency of TG2 though there are suggestions of possible compensation by other members of the transglutaminase family (Deasey *et al*., 2013).

Characterisation of the compensation mechanism of catalytically active transglutaminase was analysed from seven different tissues/organs obtained from both wild type and TG2-knockdown mice. The data obtained revealed tissue-specific compensation based mechanism for the loss of TG2. A transcriptional compensational mechanism was seen in the liver and heart, and a functional compensation was observed in the skeletal/cartilaginous tissues while there was no form of TG2 compensation in the skeletal muscle (Deasey *et al*., 2013).

1.17 Phytochemicals, diet and cancer

Phytochemicals are naturally-occurring plant-derived compounds that provide plants with colour and flavour, deter predators and offer protection. These compounds are known to provide health benefits to man owing to their anti-inflammatory, anti-oxidant and anti-proliferating properties. Furthermore, epidemiological evidence suggests that a fruit-rich diet containing high levels of phytochemicals, may reduce cancer risk, and it was proposed that

purified phytochemicals may, therefore, have therapeutic properties (Budisan *et al*., 2017). Sulforaphane (SFN), a natural compound obtained from cauliflower and broccoli, has been shown to have anti-cancer activities (Zhang *et al*., 1992; Surh *et al*., 2003). SFN also protected against drug-induced hepatotoxicity induced by drugs such as cisplatin, microstatin, aflatoxin B1 and triptolide (Lu *et al*., 2014; Li *et al*., 2014; Gaona-Gaona *et al*., 2011). It has also been shown to protect human hepatocytes, and mice liver from cadmium selenium (CdSe) quantum dots (QDs) induced cytotoxicity used in disease diagnosis (Wang *et al*., 2015).

Notable amongst such phytochemicals is vitamin A, a category of fat-soluble retinoid which includes retinal, retinyl esters and retinal. Vitamin A occurs in two forms: vitamin A and provitamin A carotenoids. Natural vitamin A is found in animal food sources such as fish, dairy and meat (highest in the liver) as retinoids, while in plants as carotenes (alpha-carotene, β-carotenes and β-cryptoxanthin). Provitamins and the natural vitamin A precursors are converted by the body intracellularly into retinol and finally broken down to retinoic acid. Vitamin A can also be taken as a dietary supplement in the form of a multivitamin as retinyl palmitate or retinyl acetate, or preformed vitamin (Bruoso, 2019).

Published reports exist on the relationship between diet and cancer, and these studies often focus on the likelihood of specific dietary components having a link with an increased or decreased cancer risk. Some of these studies have proven that certain compounds have pro- or anti-carcinogenic properties (NCI, 2015). A meta-analysis of dietary patterns and cancer risks suggests a potential role of diet in certain cancers, though the evidence was not conclusive (Guiseppe *et al*., 2015). Intake of dietary fat increased blood oestrogen levels, with the possibility of encouraging the development of breast cancer in women (Wu *et al*., 1999).

Other studies suggested that retinoids could reduce cancer risk and the consumption of a diet rich in vitamin A, including β-carotene reduced risk in larynx, lung, bladder, mouth and cervical cancers. Conversely, other situations have linked retinoids to increased cancer risk under certain conditions (as reviewed by Graham, 1984). Retinoid could, therefore, be a two-edged sword reducing cancer risk in some cancers and increasing the risk in others.

From epidemiological studies, it was observed that there was a negative association between vitamin A rich food and the occurrence of several human cancers. However, high intake of dietary preformed Vitamin A was observed to result in teratogenicity in babies whose mothers consumed more than 10000 UI (3000 ug) of preformed of vitamin A daily (Rothman *et al*., 1995).

1.17.1 Retinoids and cancer

RA activities in cells are coordinated by two families of nuclear receptors the retinoic acid receptors and the retinoid X receptors. These receptors function as ligands that bind to retinoic acid response elements on DNA and induces transcription factors that boost the transcription of specific genes (Zhu *et al*., 1997).

Retinoids are generally used to treat dermatological and visual diseases though their role in cancer treatment and prevention has been investigated. Owing to their anti-proliferative, differentiation, anti-apoptotic and pro-apoptotic properties, they have been used as possible chemopreventive or chemotherapeutic agents (as reviewed by Bushue and Wan, 2010). Low vitamin A intake was seen to result in a much higher risk of developing cancer while altered retinoic acid receptor was associated with malignant transformation of cultured cells or animal tissues (Sun *et al*., 2002). Also, retinoids have been shown to suppress carcinogenesis in tumorigenic animal models of lung, skin, breast, ovarian and prostate diseases. Conversely, retinoids reversed premalignant human epithelial cells lesions and induced myeloid cells differentiation (as reviewed by Bushue and Wan, 2010).

1.17.2 Retinoids and breast cancer

There have been several clinical trials with retinoids either by themselves or in combination with oestrogen inhibitors and interferons to prevent or treat breast cancer progression (Recchia *et al*., 2009; Veronesi *et al*.,2006; Zanardi *et al*.,2006). Retinoids inhibited mammary gland cancer in both animal models and human trials and were seen to be useful, especially at the early stages of the tumour progression, but with more aggressive tumours, the

effectiveness was reduced (Bushue and Wan, 2010). Expression of retinoic acid receptors (RARs) varies in lactating and post-lactating glands, likewise in healthy and malignant epithelial cells. RARs have also been linked to carcinogenesis (Zanardi *et al*.,2006).

The expression of retinoic acid receptors (RARα, RARγ, RXRα, and RXRβ) are the same amongst hormone-dependent breast cancer and hormone-independent breast cancer cells (Zujewski *et al*.,1999). The expression of RARβ gene is upregulated in healthy mammary cells upon retinoic acid treatment, and deficiency of its expression decreased treatment efficiency in aggressive breast cancers. From the reports available, RA inhibition of breast cancer is linked with the expression of RARβ. Equally, In animal cell models, retinoids in combination with anti-oestrogens inhibit proliferation of cells in oestrogen receptor-positive metastatic breast cancer but are not effective in oestrogen receptor-negative metastatic cancers (as reviewed by Bushue and Wan, 2010).

1.17.3 The argument for and against vitamin A in cancer progression

Vitamin A is an essential requirement, as deficiency results in night blindness, and increased maternal mortality in pregnant women (Sommer, 2001). The required dietary allowance varies amongst children, men and women (see Table 1.5) and hypervitaminosis A has been linked with bone disorders, and alteration to the metabolism of some fat-soluble vitamins (Olson *et al*., 1987; Trumbo *et al*., 2001).

There was an ongoing argument in the 1990s regarding the role played by vitamin A in cancer treatment and progression. Vitamin A was seen to amplify gene expression in breast cancer cells, encouraging stem cells surrounded by a tumour to transform into endothelial cells. These transformed cells were reported to generate blood vessels which link up to the main blood supply, encouraging further tumour growth (GUMC, 2008).

Beta-carotene and vitamin A was seen to accelerate the *progression* of lung cancer during a clinical trial (Omenn *et al.,* 1996), while a retinoid analogue fenretinide, was seen to prevent

breast cancer reoccurrence in pre-menopausal women, but elevated the risk in their postmenopausal counterparts (Veronica *et al*., 1999).

Divergent views on studies with human prostate cancer revealed that inhibiting the breakdown of retinol to retinoic acid with VN/14-1 (retinoic acid metabolism blocking agent), decreased the tumour volume. The authors suggested that vitamin A precursors are essential for anti-cancer activities in cells or retinoic acid is pro-oncogenic (Khandelwal *et al*., 2007). Another study revealed that the combination of vitamin A and vitamin C on cultured breast cancer cells inhibited growth by 75.5% when compared to the untreated control. The authors also proposed that vitamin C aids vitamin A inhibitory effect on the cells (Kim *et al*., 2006; Andrew,2008).

Andrew W. Saul of the Orthomolecular Medicine News Service on August 20, 2008, had this to say:“ *Vitamin A is very far from being a cancer "promoter." Rather, it is very near to the cancer solution*.” The results generated from this present study, however, does not conform with this statement because RA was seen to be a cancer promoter than a solution (fig 5.7, fig 5.8 and fig 5.9)

The recommended dietary intake of vitamin A is shown in Table 1.6.

Table 1.5: Recommended vitamin A daily allowance (Trumbo et al., 2001).

Age (years)/Gender	Required dietary allowance (µg)
Male /9-13	600
Female/9-13	600
Male /19-50	900
Female/19-50	700

1.18 Chemotherapeutic drugs

There are different classes of chemotherapeutic drugs, based on their mechanism of action. These include the antimetabolites, DNA-interactive agents, hormonal therapies, monoclonal antibodies and molecular targeting agents (MacDonald, 2012).

The mechanism of action of chemotherapeutic drugs belonging to the antimetabolites group operates through interruption of critical biosynthetic pathways. Structural equivalents of purines or pyrimidines may be incorporated into the cell which interrupts nucleic acid synthesis. An example in this class is 5-Fluorouracil (Nussbaumer *et al*., 2011).

DNA-interactive agents constitute one of the largest and key anti-cancer drug families. They damage the DNA of cancer cells by the attachment of alkyl groups to the DNA bases. Another means by which they carry out their action on cells is by forming cross-links between the DNA strands, thus preventing replication (Nussbaumer *et al*., 2011) and platinum complexes such as cisplatin and oxaliplatin belong to this category. Some chemotherapeutic drugs also act as intercalating agents where they insert and bind to the bases of the DNA of the cancer cell. This union causes damage to DNA and interrupts replication. Examples in this group are the anthracyclines, which include doxorubicin and epirubicin (Thurston, 2006).

Other classes of anticancer drugs include topoisomerase inhibitors, which inhibit the function of topoisomerases upsetting the geometry and function of DNA (Maulik et al., 2014). DNA-cleaving agents cause strand cleavage at binding sites, and anti-tubulin agents, which inhibit microtubule dynamics throughout the cytoplasm and disrupt critical cellular functions (Thurston, 2006).

1.18.1 The drug cisplatin

In 1965, Barnett Rosenberg discovered cisplatin, described as the ‘penicillin of cancer’, because it was the first platinum-based chemotherapy drug to be developed, and still one of the most effective chemotherapy drugs widely used for cancer treatment. Discovering cisplatin ([PtCl2(NH3)2] or CDDP]), cis-[Pt (II)(NH(3))(2)Cl(2]) (figure 1.15) was a milestone in cancer

chemotherapy, which generated interest in the use of platinum(II) and other metal-containing compounds as potential anticancer drugs. Since the introduction of cisplatin into clinical trials, there has been a tremendous impact in cancer chemotherapy, which has altered the course of therapeutic management of various cancers, including those of the ovaries, bladder, testes, oesophagal, breast, cervical, stomach, prostate cancers, head and neck. Also, cisplatin is used in the treatment of metastatic ovarian cancer, metastatic testicular cancer and advanced bladder cancer. It is used in different aspects of chemotherapy, such as an antineoplastic chemotherapeutic drug, in adjuvant therapy and used in combination with radiation therapy (Florea and Besselberg, 2011).

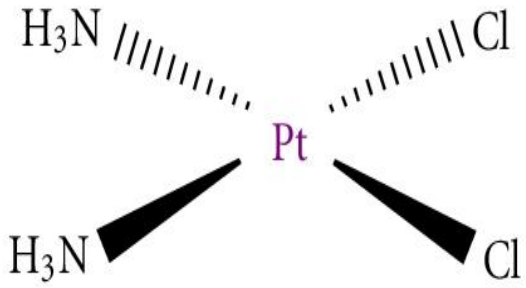


Figure 1.15: Cisplatin (Gómez-Ruiz *et al*., 2012)

Chemical structure of cisplatin showing platinum flanked by two molecules of chlorine on the right and by ammonia on the left.

Shapewise, cisplatin is flat and square, and its covalent bonds enable it to readily exchange molecules with other ligands in the body including water. Understanding the mode(s) of action of cisplatin has assisted in developing refined therapeutic methods that have improved the antitumor effect of the drug. However, the knowledge is far from complete, as the mechanisms underlying cells developing resistance, which significantly restricts the use of this class of drugs clinically, still represents a significant gap in our knowledge (Siddik, 2003).

1.18.2 Mode of action

The mechanism of action of cisplatin was the principal reason for its extensive use in cancer chemotherapy. CDDP is considered chiefly as a DNA-destructive chemotherapeutic drug,

where it forms platinum complexes inside the cell. Research shows that cisplatin loses its two chloride ions, and replaced by a hydroxy group making it a reactive species that readily binds to DNA bases at sites where guanine and adenine sit close to each other on the same strand (figure 1.16). It also forms cross-links between DNA strands and these changes on the strands interfere with cell function, especially with DNA replication and repair mechanism, which results in an arrest of the cell cycle followed by induction of apoptosis (Florea *et al*., 2011).

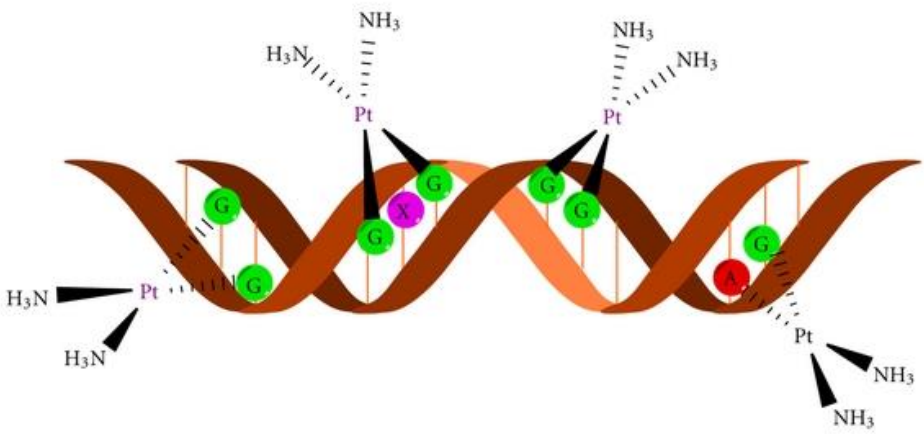


Figure 1.16: Cisplatin´s mechanism of action, forming crosslinks with DNA (Gómez-Ruiz et al., 2012).

Cisplatin showed significant single-agent activity as front-line therapy in metastatic breast cancer (MBC) in a trial where twenty consecutive patients were treated with cisplatin 30 mg/m^2/d for four days every three weeks (Sledge *et al*., 1988). Also, preclinical data support the notion that BRCA1 associated breast cancers may be sensitive to anti-cancer agents that cause DNA damage such as cisplatin (Gronwald *et al*.,2012).

1.18.3 Cellular uptake of cisplatin

Cisplatin is administered as 75mg/m2 intravenously every three weeks for 30-120 minutes in a sterile intravenous solution of sodium chloride, and it enters the bloodstream still intact because of the high concentration of chloride ions (approximately 100 mM) (Silver *et al*., 2010). Cisplatin enters into cells through active uptake though some cisplatin molecules are

trafficked by passive diffusion owing to the low concentration of chlorine in the cell (approximately 3-20 mM). Figure 1.17 shows some of cisplatin cellular targets in the cell: RNA; DNA; methionine; mitochondria, sulphur-containing enzymes such as glutathione; metallothionein and some endogenous proteins. (Siddik *et al*., 2003).

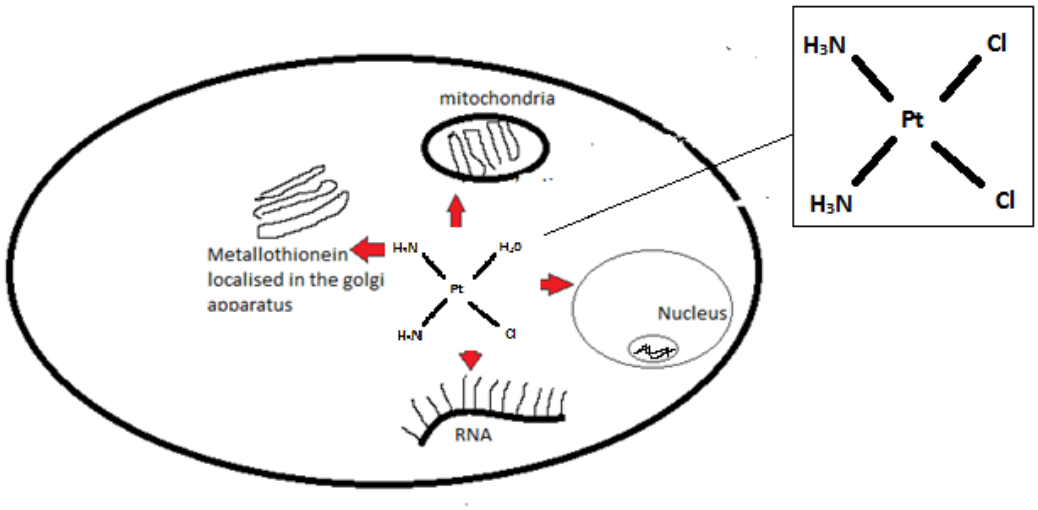


Figure 1.17: Figure 8 Cellular uptake of cisplatin indicating targets (red arrow) mitochondria; RNA; DNA, and metallothionein inside the Golgi apparatus.

1.18.4 Cisplatin resistance in breast cancer

When accompanied by surgery, adjuvant and neoadjuvant chemotherapies in breast cancer are an effective treatment. Amongst them, cisplatin is used widely as a cell cycle phase nonspecific agent in advanced breast cancer treatment, because of its known cytotoxic mode of action. Though cisplatin has been established to be an active anti-cancer agent for treating several types of cancers and an effective inducer of apoptosis, (Ormerod *et al*., 1996; Henkels and Turchi, 1997), the efficiency has not been seen in breast cancer often because of issues relating to the rapid development of chemoresistance (Siddik, 2003).

Resistance to cisplatin has been seen to develop when the tumour cells do not experience apoptosis at relevant clinical drug concentrations. Thus, the treatment of these tumours often requires drug concentrations that are cytotoxic to healthy cells. Resistant cells may be inhibited by cisplatin at levels as high as 50–100-fold more than the required concentration needed to inhibit sensitive tumour cells (Hills et al., 1989; Kelland et al.,1995; Hagopian et al.,1999). High doses can lead to severe toxicities in different organs, particularly the kidneys, bone marrow, and irritation to the digestive tract (Brabec *et al*., 2005).

Cisplatin chemoresistance can be attained through prolonged exposure to the drug, or as a result of an intrinsic phenomenon (Saddik, 2003). From clinical studies, a two-fold increase in resistance was observed after the standard clinical dose was doubled (Perez, 1998). This effect is thought to arise due to intracellular variations that inhibit DNA damage signals from activating apoptosis and hindering cisplatin from forming interactions with DNA (Saddik, 2003). Acquired cisplatin resistance, therefore, appears to be a multifaceted phenomenon that proves a considerable obstacle in the design of effective patient therapy (Xiu Chen *et al*., 2016).

The development of resistance and ways to overcome resistance has been the object of much ongoing research. Indeed, there could still be other mechanisms of cisplatin resistance not established, and this study aims to add to the growing wealth of knowledge by studying the roles TG2 isoforms play in the development of resistance to cisplatin.

1.19 Studying chemoresistance

One approach to studying chemotherapeutic drug resistance is the development of an experimental drug-resistant model, using the method of stepwise increases in drug concentration (Mc Dermont, 2014), a low-dose intermittent increase stimulus (Godwin *et al.,* 1992) or the pulsed treatment method, where cells can recover in a drug-free medium, thus imitating the clinical pattern of chemotherapy (Stordal and Davey, 2009). As chemoresistance develops during drug administration, the exposure of cell lines to chemotherapeutic drugs can

serve as a beneficial tool for studying the dynamics fundamental to drug resistance (Yan *et al.*, 2007).

1.20 Research question of this project

Previous research has suggested that maybe TG2 mechanism is involved in chemoresistance, but knowledge is scanty about relative expression and levels of the TG2 isoforms in these processes. This research seeks to understand the relationship between TG2 isoforms and chemoresistance, and could lay the foundation for novel anti-cancer treatments using TG2 short and long forms as specified targets.

1.21 Research objectives

1. To generate cisplatin-resistant cells from the MCF-7 breast cancer cell line. Immobilised analysis of chemoresistant cell lines will help to establish the relationship between the relative levels of expression of the isoforms and development of chemoresistance.
2. To induce the overexpression of TG2 with retinoic acid, and to determine which isoform is induced, and determine the effect on the sensitivity of cells to cisplatin in both wild-type and resistant cells.
3. To inhibit TG2 enzyme activity with cystamine in both sensitive and resistant cells, and determine the effect of inhibition on resistance.
4. To use siRNA techniques to silence the TG2 gene in both wild-type and resistant cells with TG2-specific siRNA and determine the isoform(s) silenced and the effect of silencing on resistance.
5. To compare the levels and ratio of TG2 isoforms in oestrogen receptor-positive (ER +ve) cells and the triple-negative breast cancer cell line, (HCC 1806) cells.

Chapter 2: Materials and Methods

2.0 Pharmacologic agents

All reagents were obtained from Sigma Aldrich unless otherwise stated.
Cisplatin was dissolved in double-distilled water to prepare a 2 mM working stock solution. A fresh cisplatin solution was prepared in the dark and sterilised by passing through sterile filters for each experiment because the solution is unstable (Siddik et al., 2003).

Cystamine dihydrochloride was dissolved in double-distilled water to make a 2 mM working solution. The solution was sterilised with sterile filters and stored at -20^0 C in aliquots.

A 10 mM retinoic acid stock solution was prepared by dissolving 3 mg/ml in absolute ethanol RA is UV, air and oxidising agents sensitive, the stock solution was prepared under subdued lighting in a glove bag under Aliquots were then stored at -20^{0}C.

A 2mM stock solution of monodansylcardavarine was prepared by dissolving in 100µl methanol and made up with culture media, protected from light and stored in aliquots of 1ml in tubes at -20^{0}C.

2.1 Cell lines and culture media

All media used for this study was obtained from ThermoFisher Scientific and supplemented with 10% (50mls) of fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, UK) unless stated otherwise.

The human MCF-7 breast cancer cell line (oestrogen positive) used for this study was obtained from the American Type Culture Collection (ATCC) and grown in DMEM medium., UK).

The HCC1806 breast cancer cell line was obtained from the ATCC, grown in RPMI 1640 medium.

The human hepatocellular carcinoma (HEPG2) cell line, which expresses high levels of tissue transglutaminase 2, was also included to serve as a positive control. This cell line was

obtained from the European Collection of Animal Cell Cultures (ECACC) and grown in RPMI 1640 medium.

The cell cultures were maintained in an incubator at 37^0 C and 5% CO_2 and regularly observed, changing the media every 2 days. Once cells were between 70-80% confluent, cell cultures were transferred to a new culture flask by dissociating cells from the flask with trypsin-EDTA (trypsinisation), centrifuged and transferred into a new flask labelled with the name of cells, passage number and date.

2.2 Cell viability assay/ IC_{50} toxicity assay

The viability of cells was determined using sensitive colourimetric assays for the determination of cell viability and cell proliferation. Cells were seeded in triplicate in 96-well plates at a concentration of 10^5 per well and allowed to adhere by incubating overnight in a humidified atmosphere at 37^0C and 5% CO_2. The response of cells to chemotherapeutic agents was found by determining the minimum inhibitory concentration (IC_{50}), which is the concentration of the drug required to inhibit 50% of the cells from growth (Benoit et al., 2012). The IC_{50} is measured using a dose-response curve.

2.2.1 CCK-8 assay

The Cell Counting Kit-8 (CCK-8) is made up of Dojindo's highly water-soluble tetrazolium salt-8 (WST-8). The soluble tetrazolium salt is reduced by dehydrogenase activities in viable cells to give a yellow-coloured formazan dye, which is soluble in the tissue culture media. The amount of formazan dye is proportional to the number of living cells in the assay (Dojindo, 2012). Using the cell counting kit CCK-8, (Sigma Aldrich) and following the manufacturer's protocols, 10 µl of CCK-8 was added to the wells containing the cells and chemotherapeutic agent(s) and incubated for 4 hours under constant culture conditions. Absorbance was read at 450 nm with an automated microplate reader ELx 800 (BioTek UK). The mean of the triplicates was calculated from three independent experiments and expressed as a percentage of the absorbance of the control cells, which had no drug treatments.

2.2.2 MTT assay

Another type of cell viability assay uses the yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. NAD(P)H-dependent oxidoreductase enzymes in viable cells reduce the soluble yellow tetrazolium salts to an insoluble purple formazan and, the insoluble formazan compound is dissolved with a solubiliser SDS (sodium dodecyl sulfate). Cells were treated with increasing cisplatin concentration(s) for 24 hours before 25 µl of Cell Proliferation Kit I (MTT) was added to the wells. After 2 hours incubation, 100 µl of 10% SDS was also added to the wells and samples were incubated overnight. The absorbance was subsequently measured and read at 620 nm with the automated microplate reader ELx 800 (BioTek UK). The mean of the triplicates was calculated from three independent absorbance readings and expressed as a percentage of the absorbance of the control cells, which had no drug treatment.

$$\% \text{ of viable cells} = \frac{\text{Absorbance (sample)} - \text{Absorbance (blank)}}{\text{Absorbance (control)} - \text{Absorbance (blank)}} \times 100$$

2.3 Development of cisplatin-resistant cell lines.

Drug-resistant cell lines were developed using a single dose exposure method (McDermott et al., 2014), modified by Meshram et al., (2017), with further modifications. Wild type cells were exposed to cisplatin for 24 hours at the $IC_{50,}$ and the growth medium was replaced with the drug-free medium until the cells were able to attain 80% confluence. This process was repeated twice, and surviving resistant cells were subsequently maintained in drug-free media with appropriate supplements until they attained confluence.

2.3.1 Test for resistance

The IC_{50} of the wild type cells was compared with the resistant clones by the method described in section 2. 2. The disparity between the IC_{50}'s of the resistant lines and those of the wild type cell lines indicates the level of resistance. The increase in resistance known as fold resistance was determined by the following equation: **Fold resistance = IC_{50} of resistant cell line/IC_{50} of the wild-type cell line** (McDermott *et al*., 2014).

2.4 Preservation of cells

A freezing medium made up of 90% growth medium, and 10% dimethylsulphoxide was prepared for cryopreservation of the cells. At 70% confluency, cells were harvested with trypsin, centrifuged for 5 minutes at 1500, rpm and pellets were loosened with the freezing medium. Approximately 1 x 10^6 cells were transferred into sterile freezing vials, placed in 'Mr frosty' (isopropanol solution), incubated in -80^0C overnight, and finally stored in liquid nitrogen.

2.5 Flow cytometric analysis of apoptosis with propidium iodide and Annexin V

Flow cytometric analysis was carried out using Annexin V-FITC assay (BD Biosciences, San Diego) using a BD Accuri C6 flow cytometer (BD Biosciences, Europe). The Annexin V-FITC assay determines the level of phosphatidylserine (PS) on cell surfaces, and PI determines DNA levels. During apoptosis, PS translocates to the outer membrane, and fluorescently-labelled Annexin V-FITC binds to it and is detected at an excitation of 488 nm and a wavelength of 525 nm using the flow cytometer. The percentage of cells at different phases is measured when PI is plotted against Annexin V fluorescence. The cell populations are grouped into healthy, early apoptotic, late apoptotic and necrotic categories. Cells were grown to about 70% confluence, trypsinised, seeded at 1 × 10^5 cells in a 6-well plate, and allowed to adhere to the plates for 24 hours. Cells were then treated with either cisplatin, cystamine or retinoic acid, and incubated for the specified hours under physiological conditions of 5% CO_2, 37°C. After 24 hours, cells were trypsinised, washed and prepared for analysis. The cells were then analysed for apoptosis following the manufacturer´s directives (Annexin V-FITC assay) by adding 5 µl of Annexin V- FITC and 5 µl propidium iodide (PI) to the cells in the binding buffer before being incubated in the dark for 15 minutes. After the incubation period, 300 µl of binding buffer was added and the samples were analysed. Gating (selection of an area on the scatter plot with a specific population of cells) was used to locate viable single-cell events using the forward scatter (FSC), and side scatter (SSC) on the scatter plot. The gate was drawn to exclude events with low FSC, which represent cellular debris, and high SSC, which represents doublets from the analysis (see figure 2.1).

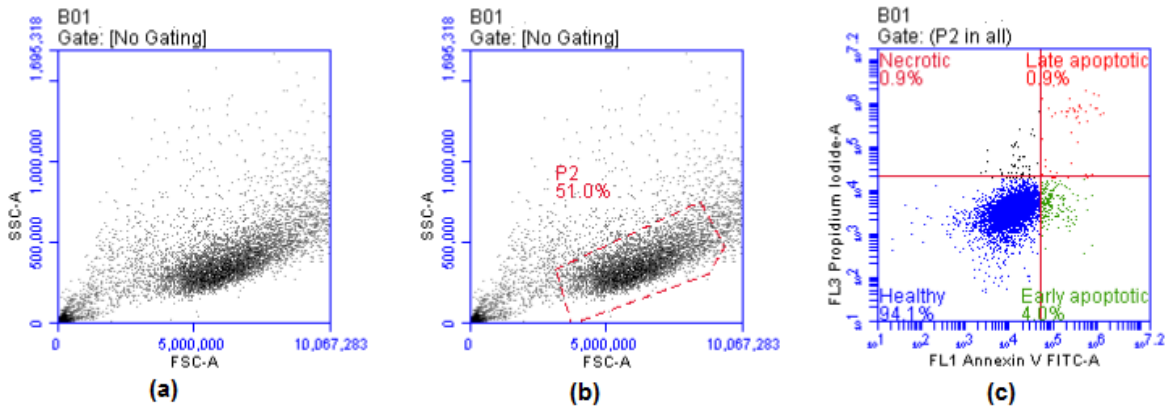


Figure 2.1: Gating limits for apoptosis by cytometry

Illustration of the gating strategy, figure (a) shows how cell debris was excluded by comparing side scatter area (SSC-A) against forward scatter height (FSC-A). Figure (b) demonstrates the identification of single cells by comparing forward scatter height (FSC-H) against forward scatter area (FSC-A) which gates out cell doublets. Figure (c) displays propidium iodide against Annexin V with cell population classified by colours, blue (healthy/ live cells); green (early apoptotic); red (late apoptotic) and black (necrotic).

2.6 Protein extraction

1 x 10^6 cells from both resistant and sensitive MCF-7 cells were lysed in the culture flasks with ice-cold radioimmunoprecipitation assay buffer (RIPA buffer) and protease inhibitor (Sigma Aldrich UK). The cell lysates were centrifuged at 4°C for 15 minutes (13,000 rpm), and the supernatant(s) containing the total protein lysate(s) were transferred to sterile Eppendorf tubes and stored at -80ºC. Determination of protein concentrations was by the Bradford protein estimation method described by Lin *et al.,* (2011).

2.6.1 Bradford assay for protein quantification

Protein concentration was determined using the Bradford assay kit (Abcam, ab102535), which is a fast and straightforward procedure to determine protein concentration in a sample. This method uses an enhanced Coomassie blue reagent, which interacts with the protein in the sample to produce a blue colour that is measured spectrophotometrically at 620 nm. A calibration curve is generated from a protein standard (BSA 1 mg/ml), and the concentration of the unknown sample extrapolated from the curve. According to the manufacturer's protocol,

5 µl of both Bradford reagent and sample were pipetted in duplicates into a 96-well plate and allowed to stand for 10 minutes at room temperature. After incubation, the absorbance of the samples was read with the automated microplate reader ELx 800 (BioTek UK), the intensity of the blue colour being proportional to the concentration of protein.

2.7 Western blot analysis to determine TG2 protein expression

Western blot analysis was performed following the protocol described by Kumar et al., (2011). Briefly, 50 µg of total protein from each sample were mixed with SDS sample buffer in Eppendorf tubes and placed on a heating block for 10 minutes at 80°C. Protein electrophoresis was performed using 10-12% NuPAGE Bis-Tris mini gels with MES Running Buffer (Invitrogen) at 150 V and 100 mA. After separation, gels containing the proteins were transferred onto nitrocellulose membranes at a voltage of 15 V and 60 mA for 1 hour and then blocked with 2% w/v bovine serum albumin solution for an hour. The membranes were washed thrice with TBS-Tween 20 (TBST) before probing with TG2 primary antibody (anti-TGM2 mouse) at a dilution of 1:4000 at 4°C overnight (Table 2.1). Membranes were then washed thrice with TBST before further incubation with secondary antibodies (goat anti-mouse) at a dilution of 1: 4000 for 1 hour at room temperature. Membranes were subsequently washed with TBST before development with horseradish peroxidase (HRP) to show the different protein bands. Anti-beta actin antibody was used as a loading control at a 1:5000 dilution.

Table 2.1: TG2 antibodies

TG2 antibody	Dilution
Anti-Transglutaminase 2 antibody (CUB 7402). Primary monoclonal antibody.	**1:3000** **1:4000**
Goat Anti-Mouse IgG H&L (HRP) (ab07023). Secondary antibody	**1:3000**
Anti-beta actin antibody (AC-15) (ab6276)	

2.7.1 Chemiluminescent blot detection

Membranes were developed with the Clarity Western ECL Substrate (Biorad), which is compatible with HRP-conjugated antibodies ideal for film-based or digital imaging. The membrane was covered with equal volumes of both Clarity Western peroxide reagent and clarity Western luminol/Enhancer reagent and left at room temperature for 3 minutes. Excess fluid was drained off, and the membrane air-dried for digital imaging with the syngene GBOX CHEMI XRQ.

2.7.2 Quantification of bands

Bands from Western Blotting were quantified using the syngene GBOX CHEMI XRQ GeneTools analysis software to analyse and compute the intensity/density. Band density was subsequently represented as bar graphs.

2.8 RNA extraction and reverse transcription PCR (RT-PCR) analysis

The RT-PCR analysis detect the RNA expression of the TG2 isoforms. Total RNA was extracted from a million cells from both sensitive and resistant cell lines respectively using the Qiagen Rneasy Mini kit following the manufacturer's protocol. Residual genomic DNA removed by the DNA elimination step in the protocol. The quantity and quality of RNA were determined spectrophotometrically with the NanoDrop 2000, and 3 µl of RNA was used in the reverse transcription PCR with TG2 primers from Eurofins Genomics (Table 2.2) and the iTaq universal SYBR® green one-step kit. Reverse transcription and PCR reaction were performed in one reaction in a LightCycler (LCS4 4.1.1.21). The PCR reactions were as follows: reverse transcription was for 10 mins at 50° C; denaturation 95°C for 5 min; amplification (40 cycles) at 95°C for 30 s; 60°C for 30 s and 72°C for 30 s. A melting curve, which checks the homogeneity of the amplified transcripts, was run between 72 and 95°C. The analysis was performed in triplicate(s) with a negative control (sample without reverse transcriptase enzyme) to rule out possible contamination, and normalisation was done with the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Table 2.2: Oligonucleotide sequence for TG2 isoforms and the housekeeping gene GAPDH.

TG2 isoform name	Primer sequence (5′ → 3′)	Reference
TGM2_v1 (TG2-L)	F CCTTACGGAGTCCAACCTCA R CCGTCTTCTGCTCCTCAGTC	Phatek et al., 2011
TGM2_v2 (TG2-S)	F ACCGCTGAGGAGTACGTCTG R TCAACAAATGCTCCAGGAA	Phatek et al., 2011
TG2	F TAAGAGATGCTGTGGAGGAG R CGAGCCCTGGTAGATAAA	Lingbao et al., 2008.
GAPDH	F ACCACAGTCCATGCCATCAC R TCCACCACCCTGTTGCTGA	Lingbao et al., 2008

Absolute quantifications was performed using the $2^{-\Delta\Delta Ct}$ quantification method (Livak and Schmittgen 2001), and Table 2.3 shows how the values were computed. The average Ct values for the housekeeping gene, test gene (TG2 short and long forms) in both experimental and control conditions were computed as Gene being Tested Experimental (TE), Gene being Tested Control (TC), Housekeeping Gene Experimental (HE), and Housekeeping Gene Control (HC) (Phatek *et al*.,2013).

Table 2.3: 2−ΔΔCt quantification method

Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)
TE	HE	TC	HC	ΔCTE	ΔCTC

The ΔCt value was computed from the difference between TE and HE, TC and HC.

$$\Delta Ct = TE_{\text{Ct value of TG2 isoform}} - HE_{\text{ Ct value of housekeeping gene}}$$

The ΔCt values calculated were for the experimental (ΔCTE) and control (ΔCTC) conditions, independently.

The difference between the experimental ΔCTE and control ΔCTC was computed to obtain the double delta Ct value (ΔΔCt):

$$\Delta\Delta Ct = \Delta CTE - \Delta CTC.$$

Finally, the expression fold change was calculated by $2^{-\Delta\Delta Ct}$, as all calculations are in logarithm base 2 (Livak and Schmittgen, 2001), and presented as bar graphs (fig 4.9).

2.9 Tissue transglutaminase enzyme activity assay

2.9.1 Specific tissue transglutaminase colorimetric microassay (NBP1-37008)

The Cov test uses biotinylated TG2 substrate-peptide (Biotin-pepT26) as the first substrate (amino acceptor acyl donor) and an amine donor acyl-acceptor as a second substrate. In the presence of tissue transglutaminase, the Ɣcarboxamide of the glutamyl residue on the Biotin-pepT26 is incorporated into the amine substrate to form a biotinylated-Isopeptide bond. The quantity of biotin incorporated is related to the TG2 activity, and is revealed by a peroxidase colourimetric reaction measured at 450 nm. Samples for the assay were prepared according to the manufacturer's protocol, 150 µl of 1X wash buffer was dispensed in each well and incubated for 15 mins at 37^0C. After 15 mins, the wash buffer was removed and replaced with 50 µL of cold assay mixture, while in the negative control, 10 µL of EDTA were dispensed. Then, 50µL of cold protein sample was added to the test wells and incubated for 30 mins at 37^0C with gentle shaking at intervals of 5 minutes. At the end of the incubation period, the wells were washed once with 200 µL of wash buffer, then washed again with 200 µL of 0.1M NaOH. Wells were subsequently washed twice with 200 µL of 1X Wash Buffer, and 100 µL of SAv-HRP solution was dispensed into each well and incubated at 37^0C for 15 minutes with mild agitation every 5 minutes. At the end of the incubation period, the wells were washed with 200 µL of 1X Wash buffer, and 100 µL of HRP substrate was dispensed into each well and left at room temperature for 3 minutes. 100 µL of blocking reagent was dispensed into each well, and the optical density was measured at 450 nm using the automated microplate reader ELx 800 (BioTek UK).

2.9.2 Fluorescent dansylcadaverine incorporation assay

TG2 activity was also determined *in situ* using labelled monodansylcardavarine (DSV), a TG2 fluorescent substrate which is easily detected using the BD accuri C6 flow cytometer at a wavelength of 533 nm. Following the protocol by D'Arcy 2017, 5 x10^5 cells were grown in a six-well plate, treated with retinoic acid and incubated between 24 and 72 hours. Media was then replaced with 50 ul of a 2 mM stock solution of dansylcadaverine. The activity of the enzyme was determined via FL-H relative fluorescent unit changes (RFU). Before cells were analysed, gating was performed to remove cell doublets and debris that could generate errors (figure 2.3).

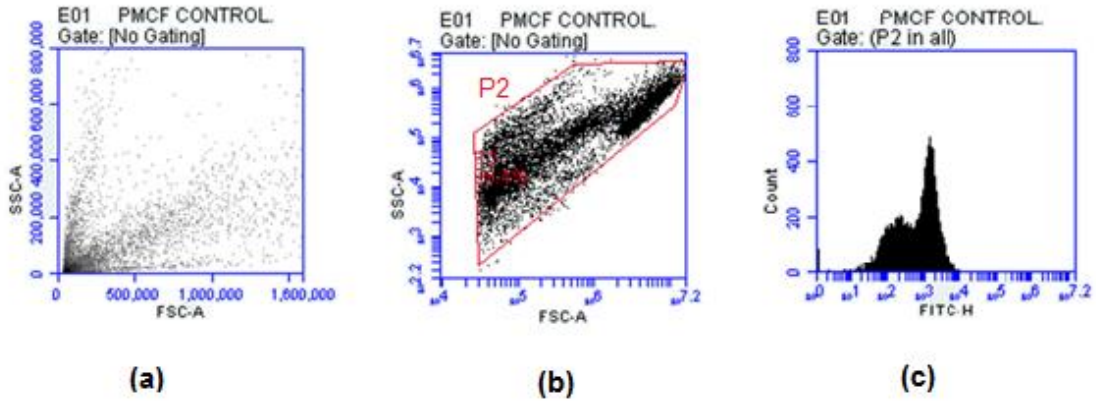


Figure 2.2: Gating limits for determining TG2 activity by cytometry

The gating limits shows (a) cell debris was excluded by comparing side scatter area (SSC-A) against forward scatter height (FSC-A) (b) Single cells were identified by comparing forward scatter height (FSC-H) against forward scatter area (FSC-A). Comparing FSC-H/FSA-A gates out cell doublets. (c) From the single cells on the y-axis and FITC-H on the x-axis, a histogram was generated which measures the fluorescence of dansylcadaverine. The mean of FITC-H generated from each sample was compared to determine the enzyme activity between samples.

2.10 TG2 inhibition by siRNA

TG2 inhibition with specific anti-TG2 siRNA (AM16708, siRNA ID # 111472) was performed using pre-designed siRNA

(5'GGCCCGUUUUCCACUAAGATT3', 3'UCUUAGUGGAAAACGGGCCTT) and transfection with Lipofectamine 2000 transfection reagent. Both reagents were obtained from Life Technologies UK. Cells were seeded into 96-well plates (1 x 10^4 per well) for viability assays and 6-well plates (1 x10^5 per well) for protein extraction, and allowed to adhere to plates. After 24 hours, according to the manufacturers' guidelines, media was replaced by Opti-MEM™ medium, with reduced FBS because serum can affect transfection results, media was replaced with DMEM for another 24 hours before analysis was carried out. The concentration of the reagents used for the silencing assay is shown in Table 2.4.

Table 2.4: Concentration and volume of reagents used for silencing

	Corning TC multiple well plates	The total volume of media used	Volume of lipofectamine	The final concentration of siRNA
1.	96-well plate	100 µl	0.3 µl	30 pmol
2.	6-well plate	600 µl	5 µl	30 pmol

Cells were incubated for 48 hours and then subjected to cell viability assay and Western blotting analysis.

2.11 Wound-healing ‘scratch’ assay

A wound-healing assay (Liang et al., 2007) was performed by growing approximately 5 x 10^5 cells in a 6-well plate for 24 hours. Media was replaced with fresh media and cells allowed to grow until they became confluent.

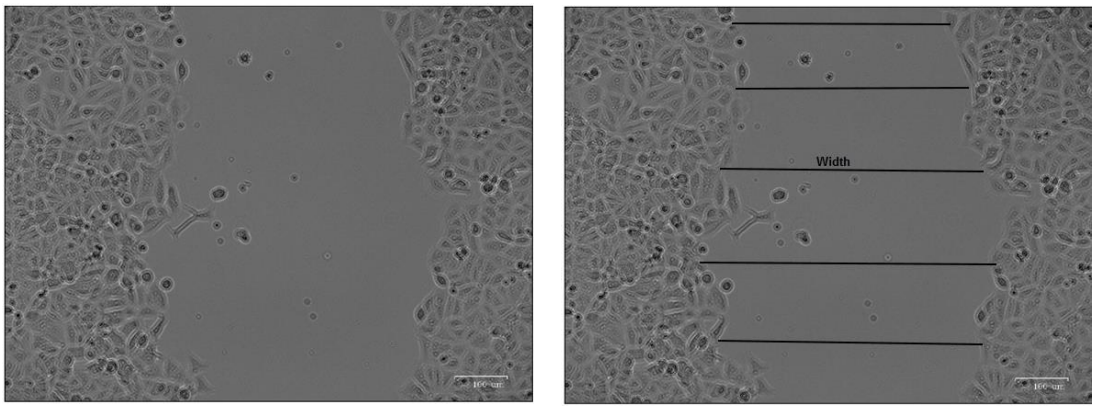


Figure 2.4: Strategy for wound healing assay measurement

(a) Scratch-made with a 10 µl pipette tip (b) width size was measured with the image j software.

A ‘scratch’ or ‘wound’ was made on the cell monolayer, using a 10 µl pipette tip and images of cells were captured at different time points as the ‘scratch’ healed for 24hours, and the width was measured at each time point. Images were captured at 0. 2. 4. 8. 12, and 24 hours with the ZOE™ Fluorescent Cell Imager (Bio-Rad). The rate of cellular migration was computed by measuring ‘wound’ width in micrometres (µM) with the Image J software.

2.12 Statistical analysis

All data generated from the experiments were analysed with the GraphPad Prism software version 7.0. Results analysed were from three or more independent experiments.

Chapter 3: Optimisation of methods

3.1 Use of HepG2 cells as a positive control

The ability to reproduce data obtained by previous colleagues in the laboratory helped to develop and optimise the techniques needed for investigating TG2 expression. Since hepatocarcinoma (HepG2) cells have previously been shown to express high TG2 levels, they were considered a suitable cell line for optimising the techniques that would subsequently be applied in this research.

3.2 Establishment of IC_{50} of HepG2 cells to cisplatin

The HepG2 cell line was treated with an increasing series of concentrations of cisplatin for 24 hours to determine the inhibitory concentration (IC_{50}). This was done to establish the concentration of cisplatin required to induce cell death and was subsequently compared with the IC_{50} determined for the MCF-7 cells (fig 4.2).

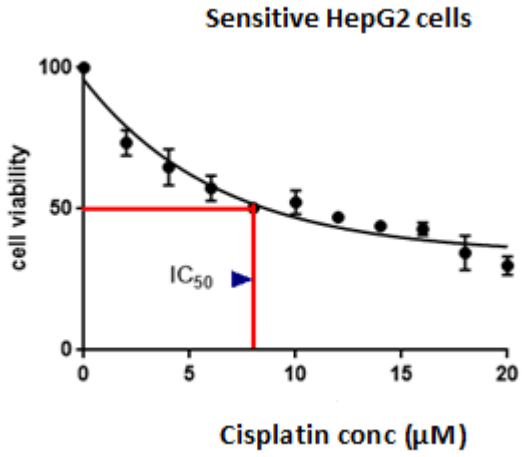


Figure 3.1: Cytotoxicity of cisplatin to HepG2 cells

Cytotoxicity of cisplatin to HepG2 cells determined with the CCK assay, following 24 hours incubation. Data is expressed as mean ±SEM represent from at least three independent experiments.

From fig 3.1, the IC_{50} of cisplatin on the HepG2 cells was calculated to be 8 µM indicating the concentration of cisplatin required to induce 50% cell death. Thus, to develop resistant cells,

wild type cells were subsequently grown in 8 µM of cisplatin for 24 hours, media replaced with fresh media without cisplatin, and the cells allowed to grow until they attained confluency (see section 2.3). This process was repeated, and the IC_{50} of the resistant cells was determined, to established resistance to cisplatin. The IC_{50} of the resistant phenotype was then compared with that of the wild type cells. The resistant cells were subsequently stored in liquid nitrogen (section 2.4).

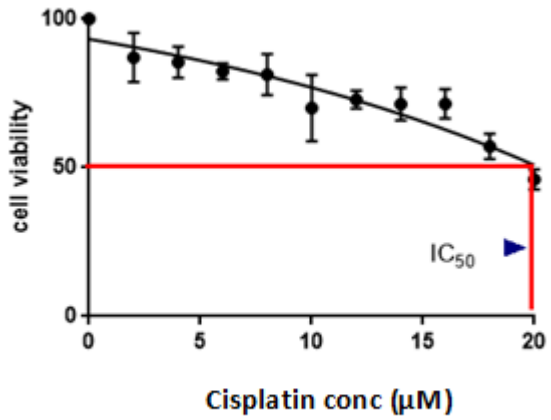


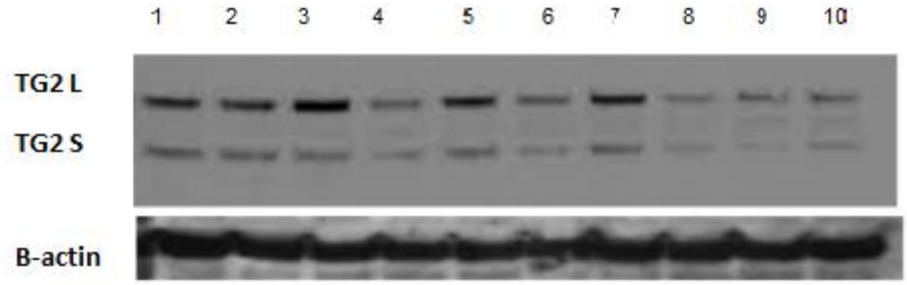
Figure 3.2: Cell viability assay of cisplatin-resistant HepG2 cells

Cytotoxicity of cisplatin on the resistant HepG2 cells determined with the CCK-8 assay after 24hours. Data is expressed as mean ±SEM represent from at least three independent experiments.

The inhibitory concentration of the resistant phenotype was calculated to be 20 µM (fig 3.2), and thus, the cisplatin-resistant cells were seen to have developed a 2.3-fold increase in resistance to cisplatin.

3.3 Effects of cisplatin on TG2 protein expression

After developing the resistant cells, the effect of cisplatin on TG2 expression was determined by Western blotting. Both wild type and resistant cells were exposed to a series of increasing concentrations of cisplatin for 24 hours. After the incubation period, total protein was extracted for analysis (see section 2.7).



Key
W- wild type
R- resistant cells
µM- cisplatin concentration

Figure 3.3: Cisplatin reduces TG2 expression in HepG2 cells

Western blot analysis of both sensitive and resistant cells treated with a series of increasing cisplatin concentration for 24 hours. Band 1: represents control for the wild type cells; band 2: (control for resistant cells); band 3: (4 µM wild type); band 4: (4 µM resistant cells); band 5: (6 µM wild type); band 6: (6 µM resistant cells); band 7: (8 µM wild type); band 8: (8 µM resistant cells); band 9: (16 µM wild type); band 10: (16 µM resistant cells). β-actin is used as a loading control.

From figure 3.3, there is a generally reduced expression of the protein in both phenotypes, indicating that as the concentration of cisplatin increases, levels of TG2 decreases. This suggests that cisplatin has a regulatory effect on TG2 levels in cells. The expression of TG2-L in both cell lines was higher than that of TG2-S, and also, there was more TG2-L expressed in the wild type cells than the resistant cells.

3.4 Discussion

Optimisation of methods and technique reduces costs and improves productivity and minimises waste of laboratory reagents. It is also a means of building up confidence and usage of laboratory operating procedures, reagent protocols and equipment usage. The result presented in this chapter conform to those of a previous study by Meshram et al., (2017) showing a reduction in TG2 expression mediated by cisplatin. The validated reagents were subsequently utilised for experimental procedures on models of breast cancer resistance.

Chapter 4: Development of a cisplatin-resistant breast cancer cell model

4.0 Introduction

The foundation of this study is the development of a drug-resistant breast cell model. Results in this chapter aim to confirm that the treated cells developed resistance to cisplatin. Cells were exposed to the chemotherapeutic drug cisplatin until they became resistant, as described in section 2.3. Cell viability and apoptosis were measured by spectrophotometry and flow cytometry, respectively, using the CCK-8 and Annexin-V assays.

4.1 Establishment of IC_{50} of MCF-7 cells to cisplatin

Cells were incubated with an increasing series of cisplatin concentration(s) for 24 hours to determine the concentration of drug to inhibit 50% of cells from growing. Cell viability was determined by spectrophotometry with the CCK-8 assay. The inhibitory concentration (IC_{50}) was computed from a dose-dependent response curve.

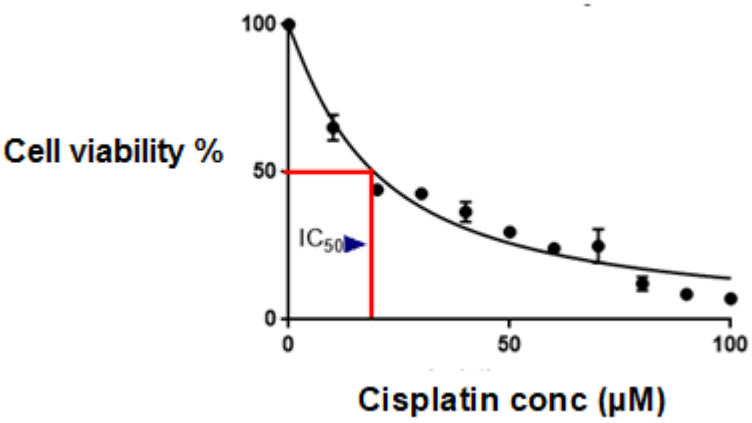


Figure 4. 1: CCK-8 assay of the viability of sensitive MCF-7 cells

CCK-8 cell viability assay performed on wild type MCF-7 cells in response to cisplatin after 24 hours incubation. The data were computed and presented as mean ± SEM from at least three independent experiments.

Figure 4.1 shows the inhibitory concentration of cisplatin on MCF-7 cells to be 18 µM, which is the concentration of cisplatin needed to inhibit 50% of cells from growing.

4.2 Effect of cisplatin on MCF-7 cells

The characteristics and state of cells after exposure to cisplatin was determined by flow cytometry, as described in section 2.5, which confirms the result obtained in figure 4.1.

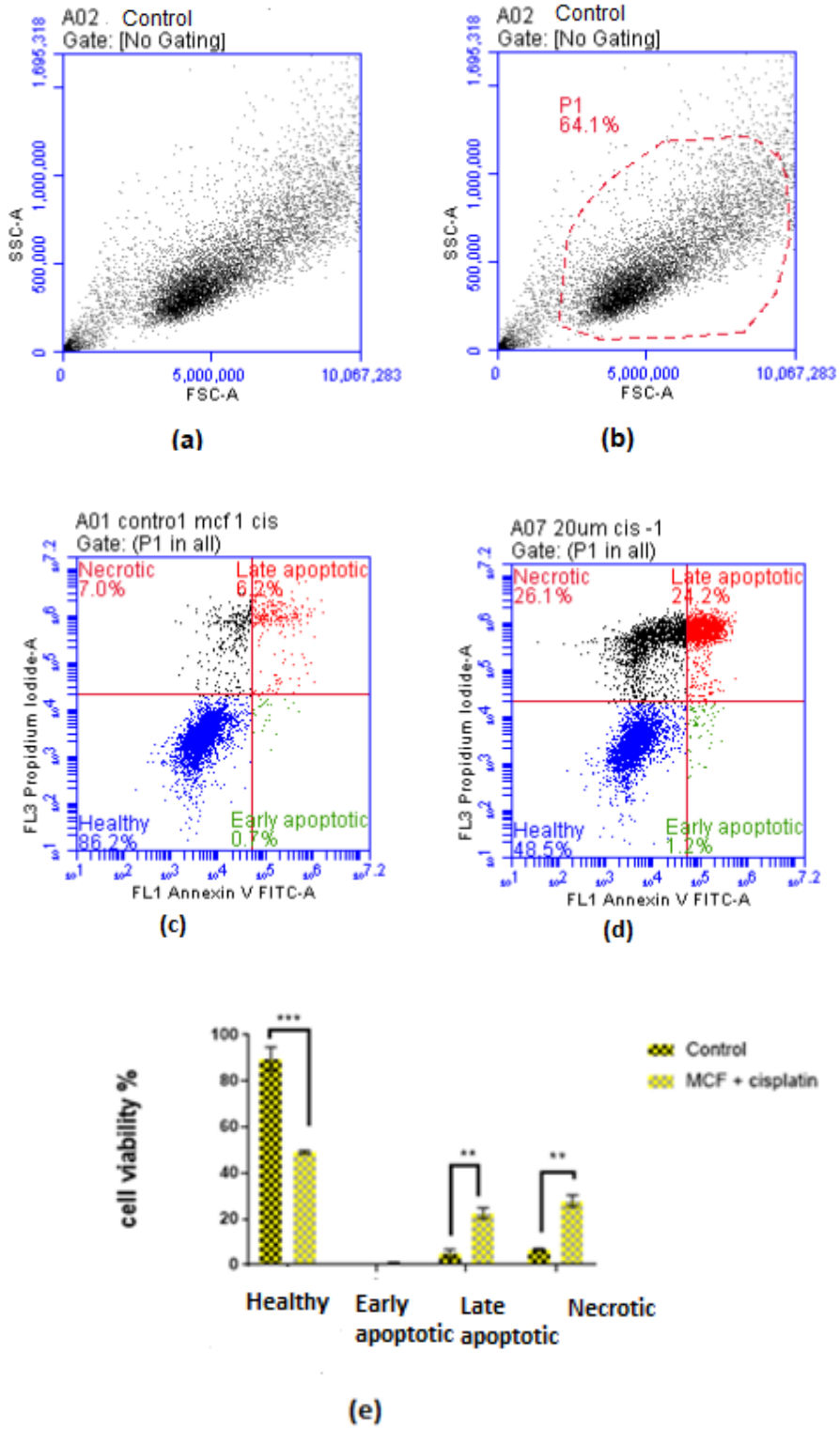


Figure 4. 2: Effect of cisplatin on MCF-7 cells

Representative cytogram of cells treated with cisplatin. (a) SSC-A/FSCA to collect single cells, (b) gating to exclude cell debris and doublets (c) MCF-7 untreated control, (d) cisplatin-treated cells at 20 µM. Data are presented as mean ± SEM, and the histogram (e) shows the difference between the control and treated cells (± SEM) and significance is denoted by *** (0.0008), ** (0.0024) when analysed by the 2-way ANOVA.

The control cytogram in figure 4.2 (c) shows that 86.0% of cells were healthy, 0.2 % were in the early apoptotic phase, 6.0 % were in the late apoptotic phase, while 7.4 % are necrotic. In the cisplatin-treated cells, there was considerable cytotoxicity; 48.5 % of cells were healthy, 1.2 % were in the early apoptotic phase, 24.2 % were in the late apoptotic phase, while 26.1 % of the cells were in the necrotic phase. The histogram (e) represents the statistical representation of the cytograms of both the control and cisplatin-treated cells.

4.3 Establishment of IC_{50} of cisplatin-resistant MCF-7 cells

To establish the resistant phenotype which is referred to as RMCF, wild type MCF-7 cells in the logarithmic growth phase were exposed to the inhibitory concentration of cisplatin (18 µM of cisplatin for 24 hours), which resulted in about 80% cell death. Media was replaced with fresh media without cisplatin and cells were allowed to grow until they became approximately 70% confluent. The procedure was repeated (section 2.3) until a population of cells were selected as resistant, and the inhibitory concentration was determined.

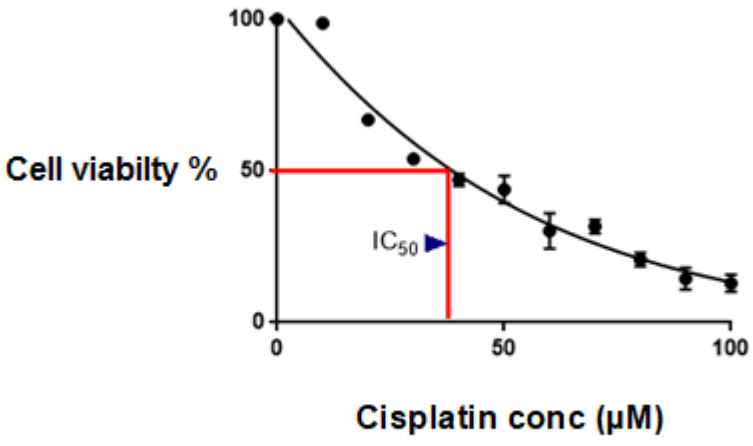


Figure 4. 3: Inhibitory concentration of cisplatin-resistant cells

Determination of the inhibitory concentration of RMCF after cisplatin treatment for 24 hours, with the CCK- 8 assay. The data were computed and presented as mean ± SEM from at least three independent experiments.

The result in figure 4.3 illustrates that the concentration of cisplatin needed to induce cell death is 35 µM. When compared with the wild type cells (fig 4.1), the resistant cells were found to have developed a two-fold increased resistance to cisplatin.

4.4 Effect of cisplatin on resistant cells' profile

Characterisation of RMCF-7 cells after cisplatin treatment was performed by flow cytometry, to investigate what percentages of the cells were still in a healthy state when compared with the wild type cells treated with cisplatin. Resistant cells were treated with 18 µM of cisplatin (inhibitory concentration of cisplatin) and incubated for 24 hours like the wild type cells. After incubation, cells were prepared for flow cytometry, as described in section 2.5.

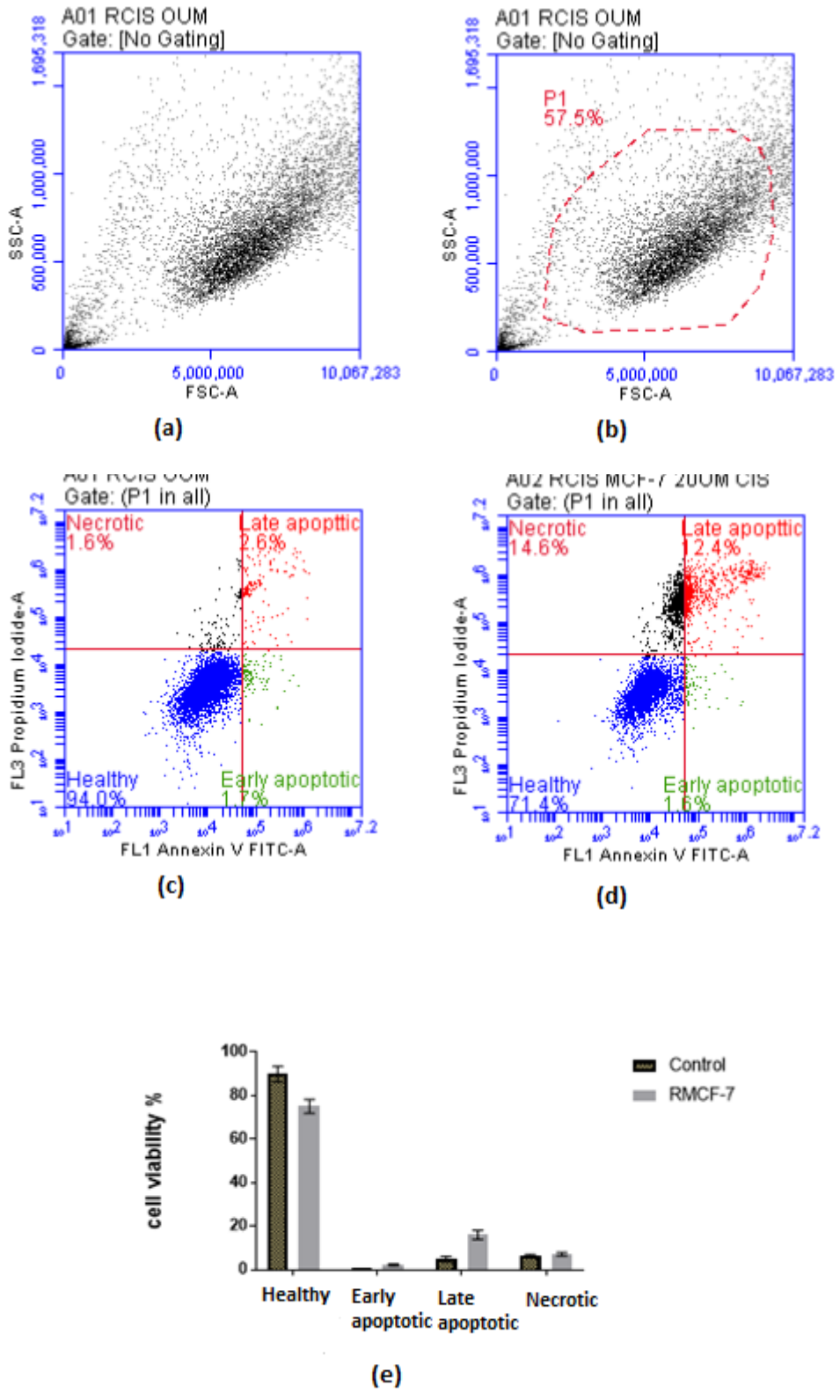


Figure 4. 4: Apoptosis analysis of cisplatin-resistant cells

Representative cytogram of resistant cells treated with cisplatin. (a) SSC-A/FSCA to collect single cells, (b) gating to exclude cell debris and doublets Representative cytogram of (c) RMCF-7 control cells and (d) RMCF cells after treatment with cisplatin showing cells in the different stages of apoptosis. The histogram (e) represents the difference between the different groups from at least three independent experiments presented as mean ± SEM and analysed with the 2 way ANOVA.

The result in figure 4.4 shows that the cells have developed resistance to cisplatin and confirms the cytotoxicity result in figure 4.3. Though there was a reduction between the RMCF control (85.2% healthy cells) and resistant cells (71.3% healthy cells), the difference was not statistically significant.

4.5 Wound-healing assay on wild type and cisplatin-resistant MCF-7 cells.

Migration of cells, also referred to as metastasis, is one of the characteristics of cancer cells, which is increased in drug-resistant cells. The assay was performed to determine the characterisation of the drug-resistant cell line developed for this research work.

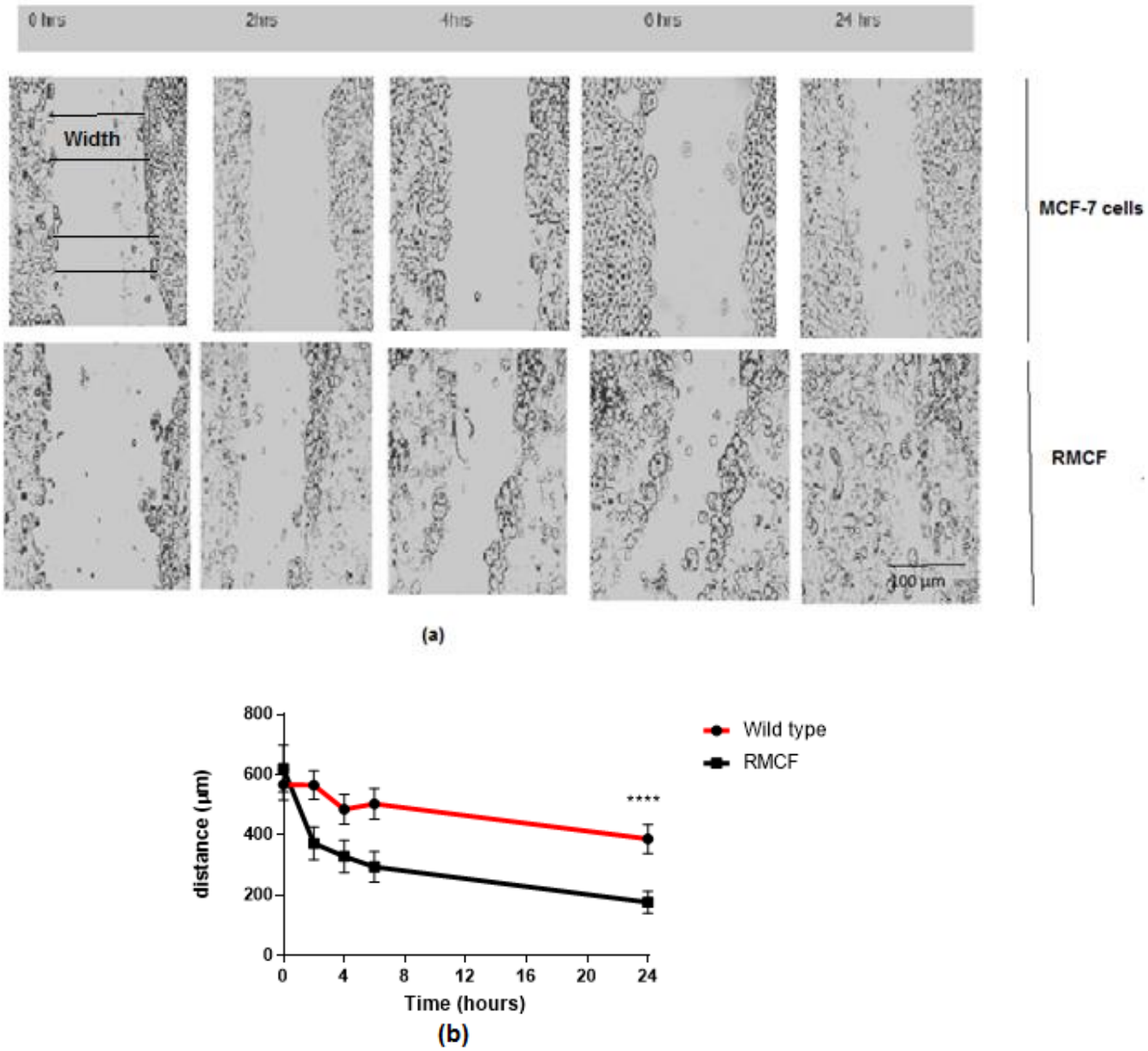


Figure 4. 5: Scratch assay on wild type and resistant MCF-7 cells

Representative pictographs (a) of wound-healing assay taken with the ZOE™ Fluorescent Cell Imager to compare time for wound healing between wild type and resistant cells. The line graph (b) compares the distance (µM) covered between the wild type and resistant cells. Data are presented as mean ± SEM from at least three independent experiments. The difference between the wild type and resistance cells was statistically significant, and **** denotes significance (<0.0001) when analysed by the 2-way ANOVA.

Figure 4.5 shows drug-resistant cells migrating after 2 hours and that the 'wound' become covered after 24 hours, whereas the 'wound' was not closed up by the wild type cells after 24 hours. The distance (µm) migrated was measured with the Image J software, and images acquired shows the resistant cells closed the "scratch" created faster than the sensitive cells. Image scale was set at 100 µm, representing the level of magnification.

4.6 Morphology of wild type and resistant cells

Images of the wild type and resistant cells were captured with the ZOE™ Fluorescent Cell Imager (Bio-Rad), and the morphologies were compared.

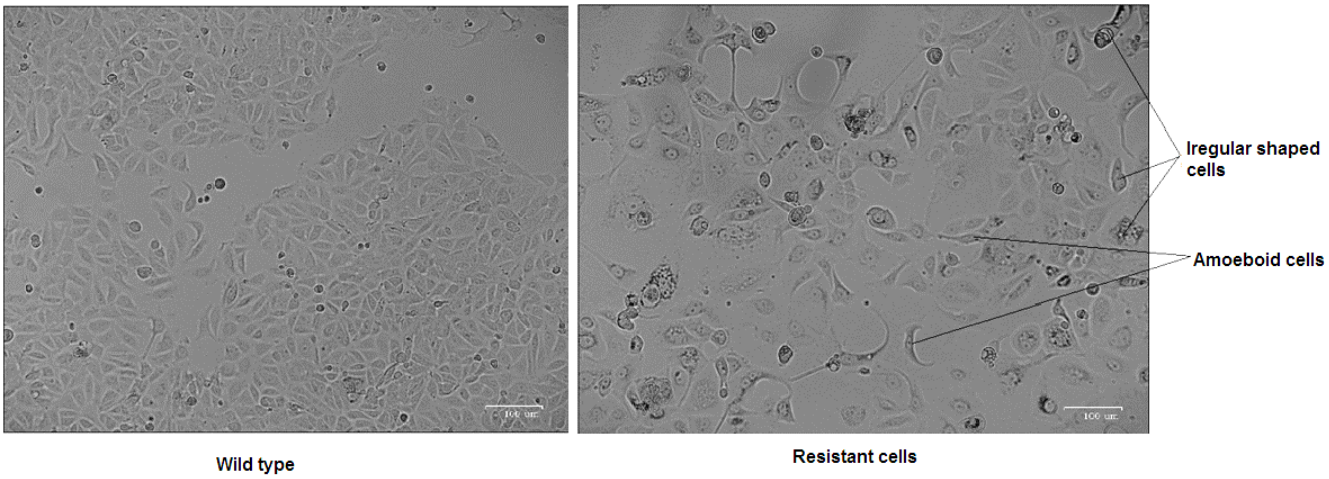


Figure 4. 6: Morphology of wild-type and resistant cells

Images of wild-type and resistant cells captured with the ZOE™ Fluorescent Cell Imager. The scale bar was set at 100 µm. Figure (a) represents the wild type cells, while (b) represents the resistant cells.

Morphologically, figure 4.6 shows the wild type cells are medium in size and quite consistent in shape while the resistant cells have a variety of irregular shapes. Some appear rounder and amoeboid exhibiting pseudopodia-like features. Dimensions of both wild type and cisplatin-resistant cells were compared by flow cytometry where the forward scatter (FSC) intensity is proportional to the diameter of the cell (see section 2.5). Smaller cells elicit a low FSC while

large cells have a high FSC (see figure 4.7). The result shows that cisplatin-resistant cells had increased FSC-A/SSC-A than the wild type cell.

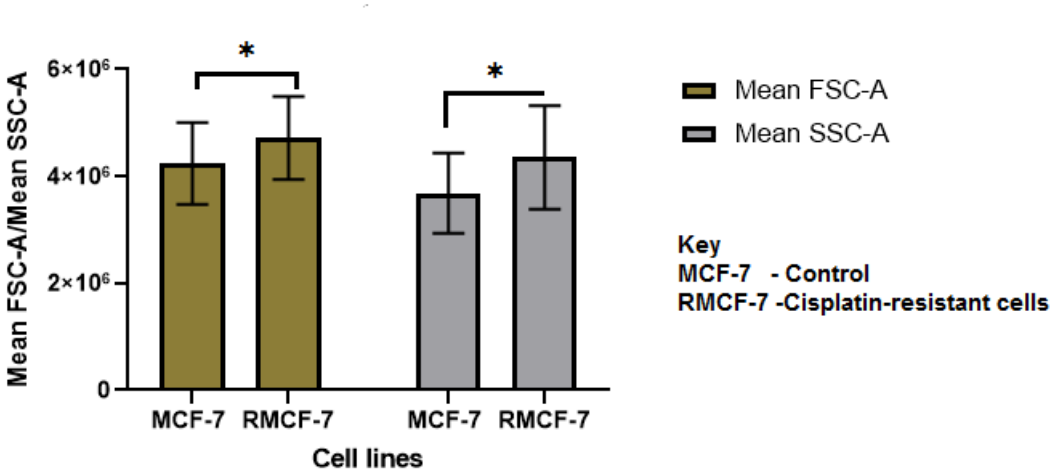


Figure 4. 7: Comparison of wild type cell and cisplatin-resistant cell sizes.

Comparison of the forward scatter values between wild type MCF-7 cells and cisplatin-resistant cells by flow cytometry. The result is presented from the mean of at least four independent experiments ± SEM and significance denoted by * (0.0332) when analysed with the 2 way ANOVA.

4.7 Determination of TG2 RNA expression levels

For the determination of RNA expression levels, cells were prepared as described in section 2.8. Cells were grown until they were about 70-80% confluent, and RNA extracted from 3 x10^6 cells from each of the wild type and resistant cell lines.

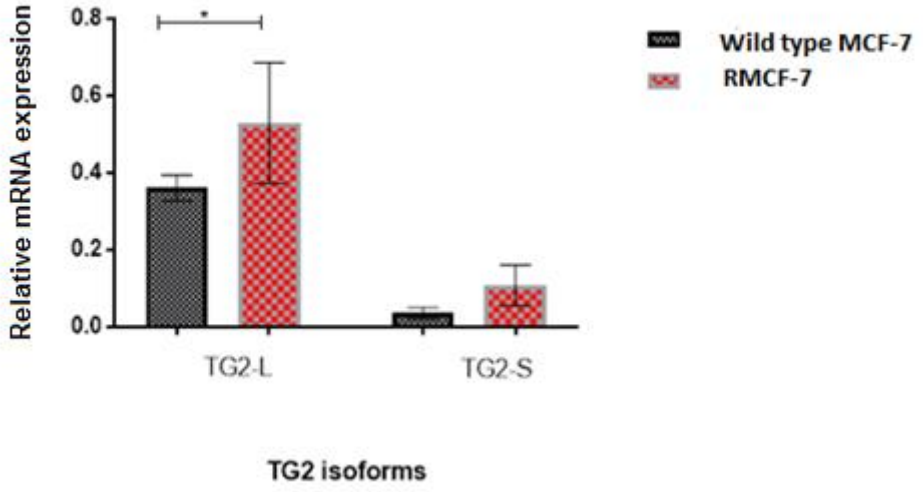


Figure 4. 8: TG2 mRNA levels in wild type and cisplatin-resistant MCF-7

Levels of mRNA were quantified using RT-PCR Roche LightCycler. Results are presented as mean ± SEM from at least three independent experiments and significance denoted by the p-value of 0.0061 when analysed by the 2-way ANOVA.

Figure 4.8 reveals higher mRNA levels of both TG2-L and TG2-S in the resistant phenotype compared to the wild type cells. The difference of TG2-L between the wild type and resistant phenotype was significant. The difference in TG2-S levels was not statistically significant though there was an increase.

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4.8 Determination of TG2 protein expression levels in wild type and drug-resistant MCF-7 cells.

Following the development of the resistant cell lines, TG2 protein expression was determined to confirm whether the levels of the enzyme are increased in the chemoresistant cell line in comparison with the wild type.

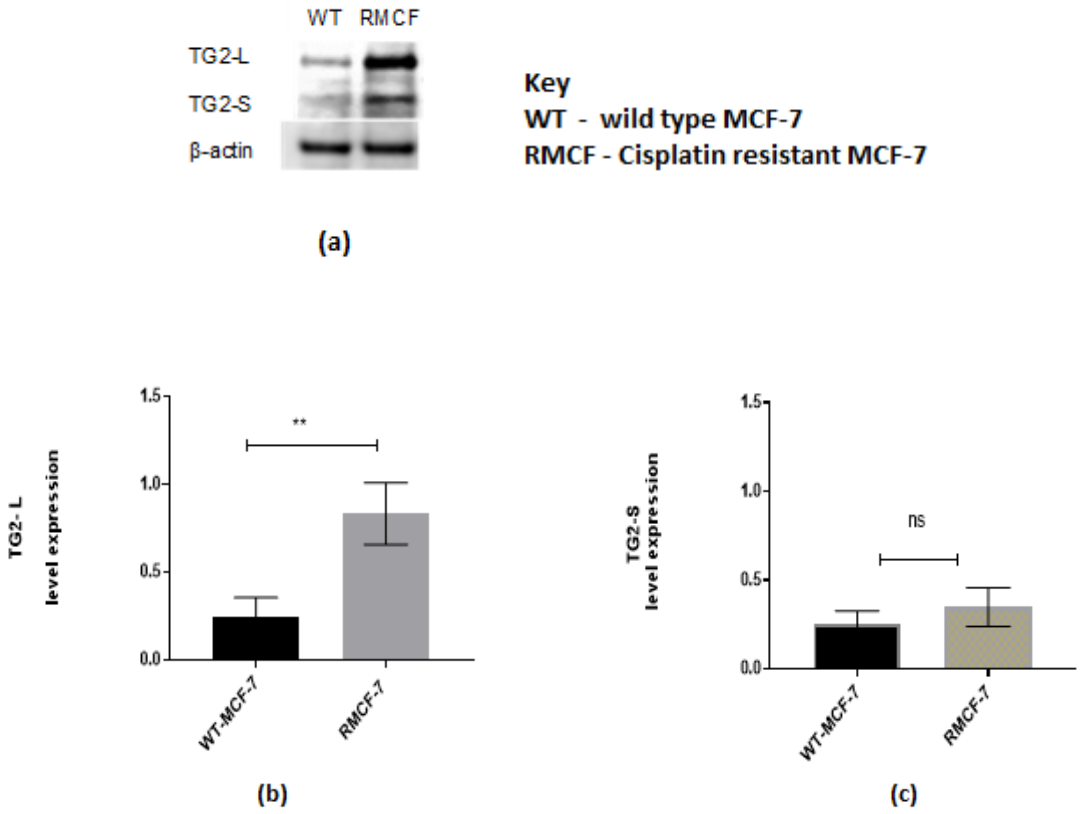


Figure 4. 9: TG2 protein expression in wild type and cisplatin-resistant cells

TG2 levels in wild type and resistant cells determined by Western blotting. (a) representative Western blot while the histograms represent band quantification for (b) the long-form and (c) the short form of the protein. The result is presented as mean ± SEM from at least three independent experiments and significance denoted by ** with a p-value of 0.0035 when analysed with the Student t-test.

From figure 4.9a, the blot shows that both TG2-L and TG2-S levels are elevated in the cisplatin-resistant cells as compared with the wild type, but the elevation of TG2-L levels is higher than the elevation of TG2-S.

4.9 Determination of TG2 enzyme activity

The activity of the enzyme was determined with the specific tissue transglutaminase microassay kit (see section 2.9.1).

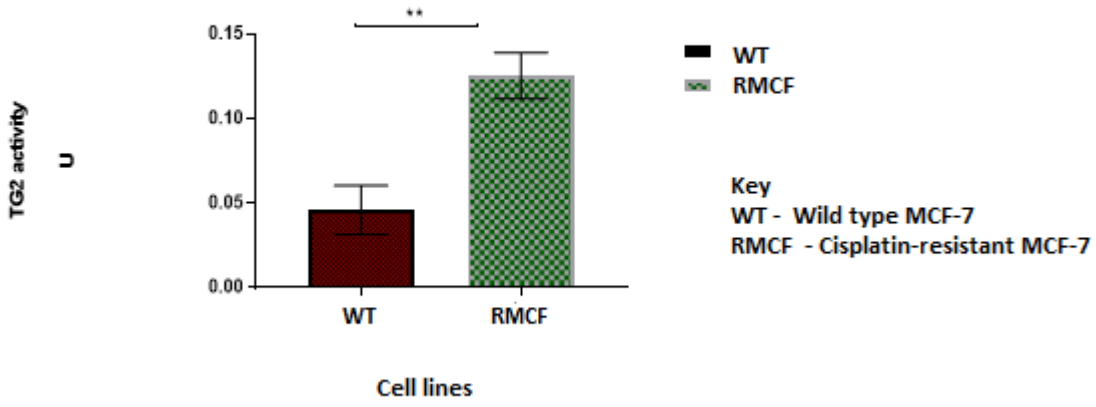


Figure 4. 10: Enzyme activity determination

Specific TG2 enzyme activity was determined with the COV-TG2 kit by spectrophotometry, in wild type MCF-7 cells and cisplatin-resistant cells. The result is presented as mean ± SEM from at least three independent experiments, and significance is denoted by the p-value of > 0.0041 (**) when analysed with the Student t-test.

Figure 4.10 reveals a higher enzyme activity in the resistant cells when compared to the wild type cells. The enzyme activity was analysed by flow cytometry to correlate the fact that the cisplatin-resistant cells have altered morphological changes when compared to the wild type cells (figure 4.7). However, the increased enzymatic activity was not due to the morphological differences (see figure 4.11).

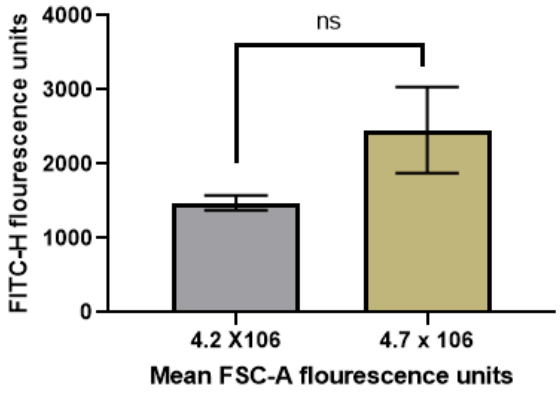


Figure 4. 11: Enzyme activity by flow cytometry

Cadaverine fluorescence (FITC-H) and forward scatter (FSC-A) between wild type MCF-7 and cisplatin-resistant cells. The results were computed from the mean of at least three independent experiments (±SEM), and the difference was not statistically significant when analysed with the Student t-test.

4.10 Discussion

Developing a drug-resistant cell model helps in studying mechanisms involved in the development of resistance to chemotherapeutic drugs and how to overcome drug resistance. A cisplatin-resistant MCF-7 cell line was developed, which had an altered phenotype from the wild type. The resistant cells' response to cisplatin was different from that of the wild type cell. Morphologically, resistant cells also had varieties of shapes conforming with previously published images (Pogribny *et al*., 2010; Wang *et al*., 2013; Li *et al*., 2013).

TG2-L mRNA expression was increased in the resistant cells, compared to wild type, while there was an elevated TG2-L protein level in the resistant cells which supports the theory that the long-form is involved in cell survival and drug resistance. The finding shows that there are Increased levels of TG2 in the resistant cells, which is consistent with findings from earlier studies that resistant cells express higher levels of the protein (Mangala et al., 2005; Herman *et al*., 2006; Eckert *et al*., 2015).

The IC_{50} on MCF-7 cells in this study, which is (18 µM), did not conform to results obtained from other studies, except that by Nikolaus *et al*.,(2018) (Table 4.1). The difference(s) evident are probably due to passage number and concentration of cisplatin used on cells. In this study, a stock concentration of 2 mM was used (Section 2.0).

Table 4.1: IC_{50} values in MCF-7 cells from other studies

Reference	IC_{50}	Method used
Suberu et al., 2014	5.25 µM	MTT
Yuwan et al., 2016	49.54 µM	MTT
Herrera et al., 2018	40 µM	MTT
H Nikolaus et al., 2018	20 µM	MTT
Hernandez-Valencia et al., 2018	4.7 µM	MTT

Results from this chapter demonstrate that the RMCF-7 cells have developed resistance to cisplatin. Differences in the biological characteristics of the resistant cells, as compared to the parental cells, such as being present in a variety of shapes foster wound healing, and increased TG2 levels are indicative of a resistant phenotype. The resistant cells also appear to lack the cell-to-cell adhesion of wild type cells; they exhibit spindle-like shapes and demonstrate the presence of pseudopodia (figure 4.8). Results from this study conform to the previous studies (Han *et al*., 2014; Wen *et al*., 2009) which suggest that morphological changes may help cisplatin-resistant cells proliferate after cisplatin therapy.

The wound-healing assay is based on observation(s) of cells on the periphery of the scratch, which move to close it up until new cell-to-cell links have established. Resistant cells are seen to exhibit increased cellular invasion and migration; these properties are seen in the cisplatin-resistant cells, while the wild type did not display such properties.

The elevated protein expression of TG2 observed in the resistant cells conforms with the hypothesis that resistant cells express high levels of the protein. Increased TG2 expression has been observed in different tumours and also from metastatic sites such as pancreatic carcinoma (Verma *et al*., 2006), breast carcinoma (Mehta *et al*.,; 2004), Mehta., 1994), ovarian cancer (Sarpathy *et al*., 2007), Liver carcinoma (Meshram et al., 2017), skin cancer (Fok *et al*., 2006).

Chapter 5: Effect of retinoic acid-induced TG2 expression on cisplatin chemoresistance

5.0 Introduction

Retinoic acid (RA), a metabolite derived from vitamin A, is involved in different cellular functions such as foetal development, cell differentiation and proliferation (Kastne *et al*.,1995; Means *et al*., 1995). It has been shown to induce TG2 expression in different cell types such as macrophages (Chiocca *et al*., 1988), rat hepatocytes (Placentinni *et al*., 1988) and human hepatocarcinoma cells (Meshram *et al*., 2017).

From the preceding chapter, it has been established that TG2 is elevated in the cisplatin-resistant cell model. The objective of this chapter is to determine the degree of involvement of TG2 in cells' resistance development. In order to establish this, both wild type and resistant cells were induced to increase expression of TG2 with retinoic acid, and the effect of the increased level of TG2 on cisplatin sensitivity was investigated.

For the experiments in this chapter, cells exposed to retinoic acid for 72 hours are denoted as retinoic acid pre-treated cells - wild type (RAPC-WT) and the cisplatin-resistant cells (RAPC-CR). The cisplatin sensitivity of both is compared to the wild type cells (untreated cells (control).

5.1 Effect of retinoic acid on cell viability

MCF-7 cells were treated with increasing series of concentrations (0-20 µM) of retinoic acid for 72 hours to generate the RAPC-WT cells. The effect of retinoic acid on the viability of the cells was determined using the MTT assay.

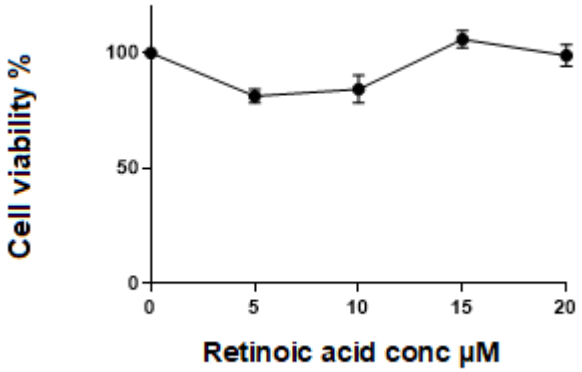


Figure 5. 1: Effect of retinoic acid on the viability of RAPC-WT cells

Cell viability of MCF-7 cells treated with retinoic acid for 72 hours determined with the MTT assay. The result is presented as means ± SEM computed from at least three independent experiments.

Figure 5.1 reveals that the concentrations of retinoic acid used were not toxic, and the RAPC-WT cells remained viable over the entire 0-20 µM concentration range.

5.2 Apoptosis analysis of RAPC-WT cells

Further confirmation of the state and viability of cells was determined by annexin V assay. In 6-well plates, 2 X 10^5 of wild type MCF-7 cells were seeded and allowed to adhere to the plates for 24 hours and media replaced with fresh media containing 10 µl and 20µl of retinoic acid. Both treated cells and the untreated cells (control) were incubated at standard culture conditions for 72 hours. After 72 hours incubation, both treated and untreated control cells were harvested and prepared for flow cytometry analysis as described in section 2.5.

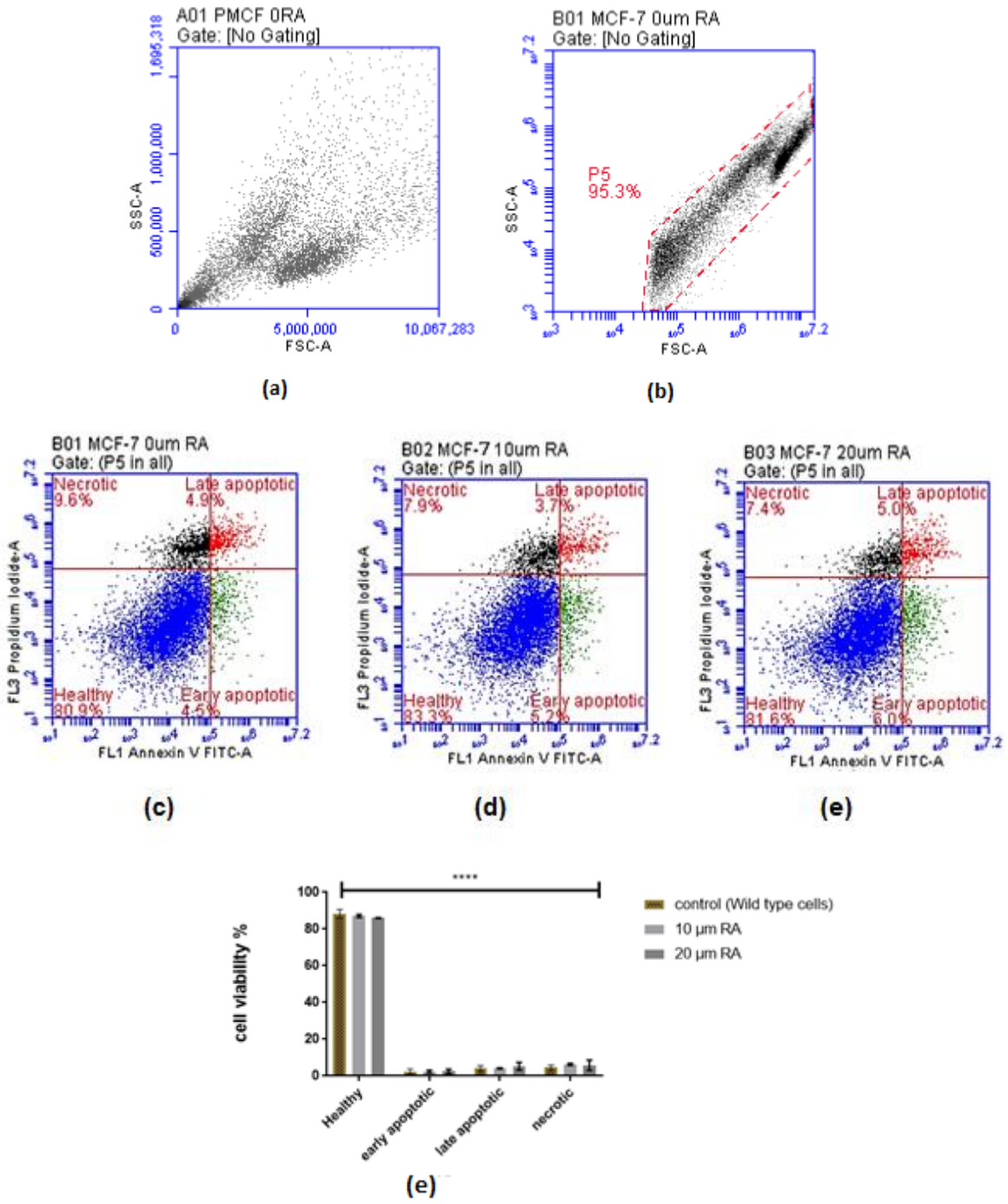


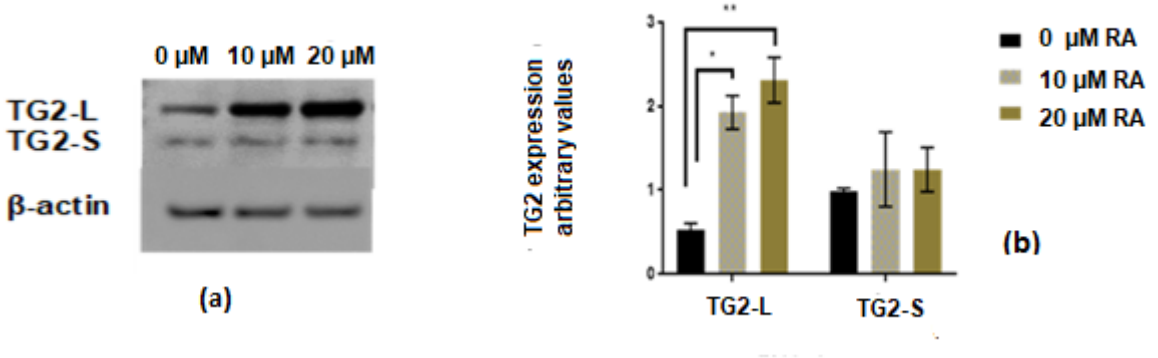
Figure 5. 2: Flow cytometry analysis of cells treated with retinoic acid for 72 hours

Apoptosis analysis of cells treated with 10µM and 20µM retinoic acid. (a) SSC-A/FSCA to collect single cells, (b) gating to exclude cell debris and doublets. Representative cytogram of RAPC-WT cells showing (c) control (untreated wild type MCF-7), (d) MCF-7 cells + 10 µM RA (e) MCF-7 cells + 20µM RA. The histogram (f) illustrates the proportions of the different cell populations expressed in percentages. The result is presented as mean ± SEM from at least three independent experiments. Statistically, the difference between control and treated was not statistically significant when analysed with the 2 way ANOVA.

Again, retinol concentration had no effect on cell viability over the 0-20 µM concentration range tested. More than 80% of cells treated with 10 and 20 µM RA concentration in (b) and (c) respectively remained healthy and comparable with the control (a). The population of cells in the other three quadrants of the cytograms are negligible, and the result shows the concentration of RA tolerable by cells, confirming the viability assay in figure 5.1.

5.3 Effect of retinoic acid concentration on TG2 expression in RAPC-WT

Following the incubation with RA for 72 hours, total protein was extracted and analysed for TG2 expression by Western blotting, to determine the pattern and level of TG2 isoform increase after incubation (see section 2.6 and 2.7).



Key-
0 µM - Untreated control
10 µM- Retinoic acid concentration
20 µM- Retinoic acid concentration

Figure 5. 3: Retinoic acid induces TG2 expression in MCF-7 cells

Representative Western blot (a) wild type cells treated with 10 µM and 20 µM retinoic acid for 72 hours. The histogram (b) quantifies both TG2-L and TG2-S levels and the result is presented as means ± SEM from at least three independent experiments and analysed with the 2 way ANOVA. Significance is denoted by *(0.0242) and **(0.0077).

The Western blot result in figure 5.3 shows that RA induced TG2 expression in the cells when the untreated control is compared to the retinoic acid-treated cells. There was a general increase in TG2 levels in both RA concentrations but a more significant induction of TG2-L

compared to TG2-S. TG2-S levels appear to be similar between the control and treated cells, and the relative increase of TG2-L to TG2-S is in a 6:1 ratio (TG2-L increase was 6x more than TG2-S). The relative ratio was calculated by dividing TG2 Level from the 20 μM RA treated cells by the untreated control.

5.4 TG2 expression over 72 hours in RAPC-WT cells.

To determine the time course of the induction of TG2 expression, cells were incubated with retinoic acid for 24, 48 and 72 hours. Briefly, cells were seeded, treated with retinoic acid and harvested over different time points. As in section 5.4, the protein was extracted, and TG2 expression determined by Western blotting (see section 2.6 and 2.7).

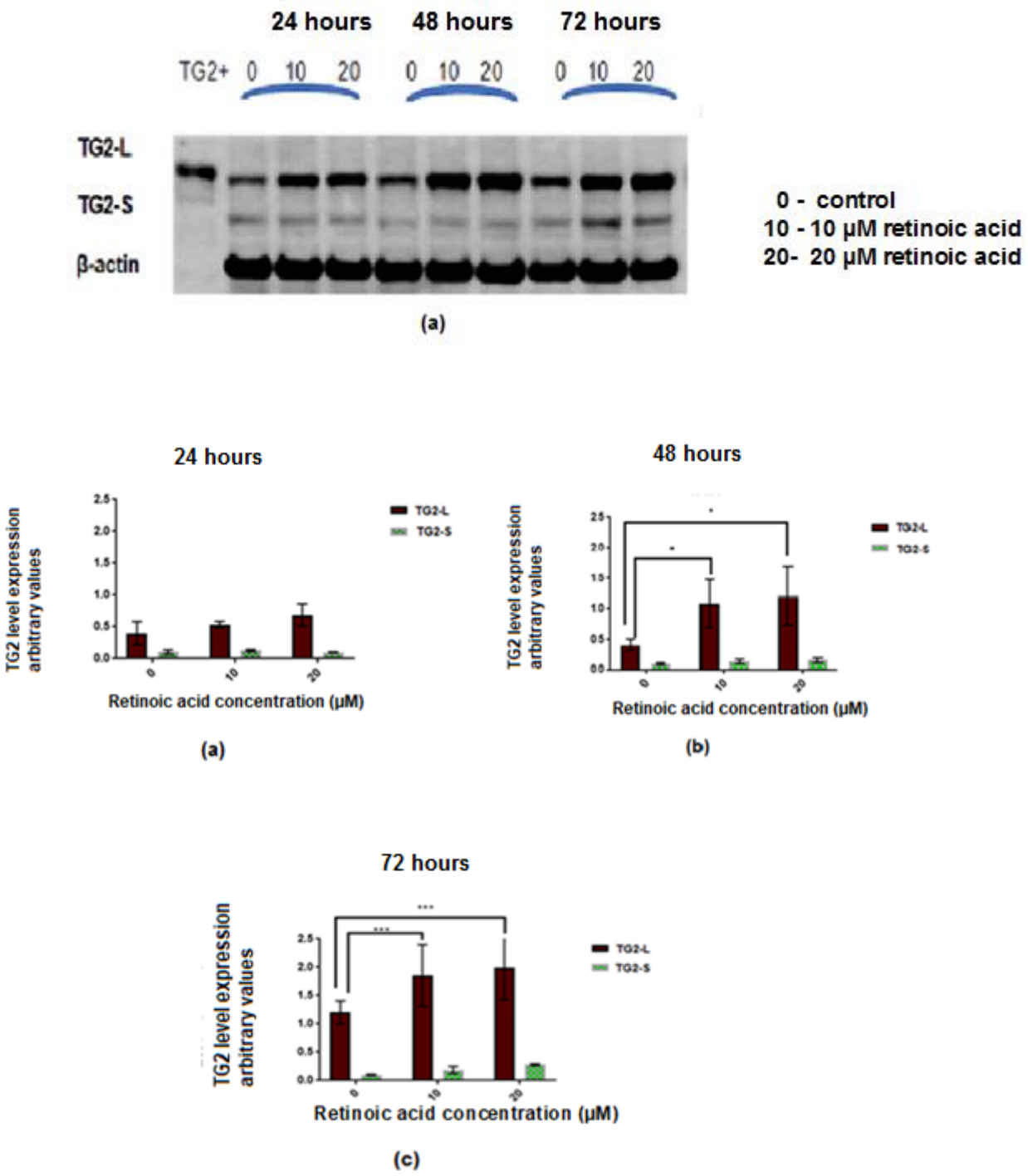


Figure 5. 4: TG2 pattern of expression in RAPC-WT over 72 hours incubation

(a) Representative Western blot showing induction of TG2 after retinoic acid treatment over 72 hours. The histograms quantify the bands after (b) 24 hours (c) 48 hours (d) 72 hours. β-actin was used as a loading control, and the difference in the levels of TG2-L was statistically significant when analysed with the 2-way ANOVA. Significance is denoted by * (0.0283) and ***(0.0003), and the results are presented as means ± SEM from at least three independent experiments.

Overall, over the 72 hours, there was no significant increase in TG2 levels in the untreated controls, but with the retinoic acid-treated cells, TG2-L increase is evident after 24 hours, with a further increase(s) after 48 hours and 72 hours. Conversely, TG2-S expression remained the same between the untreated control and treated cells after 48 hours, but an increase was observed after 72 hours.

5.5 Effect of retinoic acid on TG2 activity in RAPC-WT cells determined with fluorescent monodansylcardavarine

Briefly, 2 x10^5 cells were grown in media with 10 and 20 µM retinoic acid for 24 and 72 hours. The activity of the enzyme was determined by flow cytometry using labelled monodansylcardavarine as the TG2 substrate (section 2.9.2). TG2 incorporation of the fluorescent substrate, monodansylcardavarine into cells is detected at a wavelength of 533 nm, and the effect on TG2 activity is displayed as column graphs.

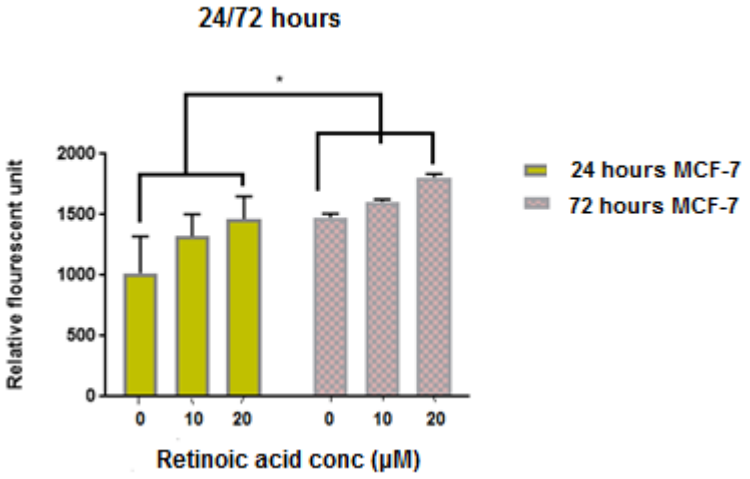


Figure 5. 5: Effect of retinoic acid treatment on TG2 enzyme activity, measured by flow cytometry

The enzymatic activity of RAPC-WT cells determined after 24 and 72 hours by flow cytometry using labelled monodansylcardavarine. The figure compares TG2 activity after 24, and 72 hours from three different experiments were statistically significant when analysed with the 2 way ANOVA, and the statistical difference is denoted by * (0.0347). The results are presented as means ± SEM from at least three independent experiments.

Overall, the histogram in fig 5.5 suggests that the activity of the enzyme is time and RA concentration-dependent and could be related to the increased TG2 levels, as seen in figures 5.3 and figure 5.4. As TG2 level increased, the activity was also seen to be increased. The limitation of this assay is the inability to determine the activity of the differential isoforms (TG2-L and TG2-S).

5.6 Effect of cisplatin toxicity on RAPC-WT cells

To assess the effect of increased TG2 levels on cell's sensitivity to cisplatin, RAPC-WT cells were treated with cisplatin. Briefly, cells were seeded in a 96-well plate, treated with retinoic acid for 72 hours, media was replaced with media containing an increasing series of concentration of cisplatin, and incubated for 24 hours. After the incubation period, the inhibitory concentration was determined with the MTT assay (section 2.2.2).

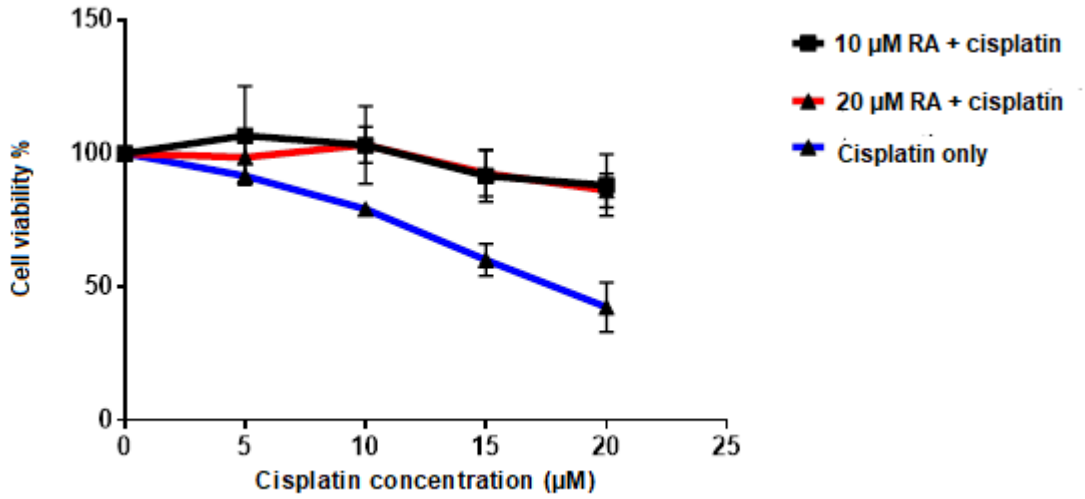


Figure 5. 6: Effect of cisplatin on retinoic acid pre-treated cells - wild type cells

Cytotoxicity of cisplatin on the viability of wild type cells after retinoic acid pre-treatment for 72 hours determined by MTT assay. The results are presented as means ± SEM from at least three independent experiments.

The viability graph in figure 5.6 shows that the treated cells (black and red curves), unlike the control, were relatively insensitive to cisplatin toxicity, which implies that treatment with RA reduced MCF-7 cells' sensitivity to cisplatin at the previous IC_{50} of 18 µM.

5.7 Apoptosis analysis of RAPC-WT cells following cisplatin treatment

RAPC-WT cells were exposed to cisplatin (18 µM) at the IC_{50}, to determine whether 50% of the cells will still be inhibited from growth. As previously described in section 2.4, cells were analysed by flow cytometry, and the apoptosis analysis (figure 5.8) confirmed the MTT assay (see figure 5.6).

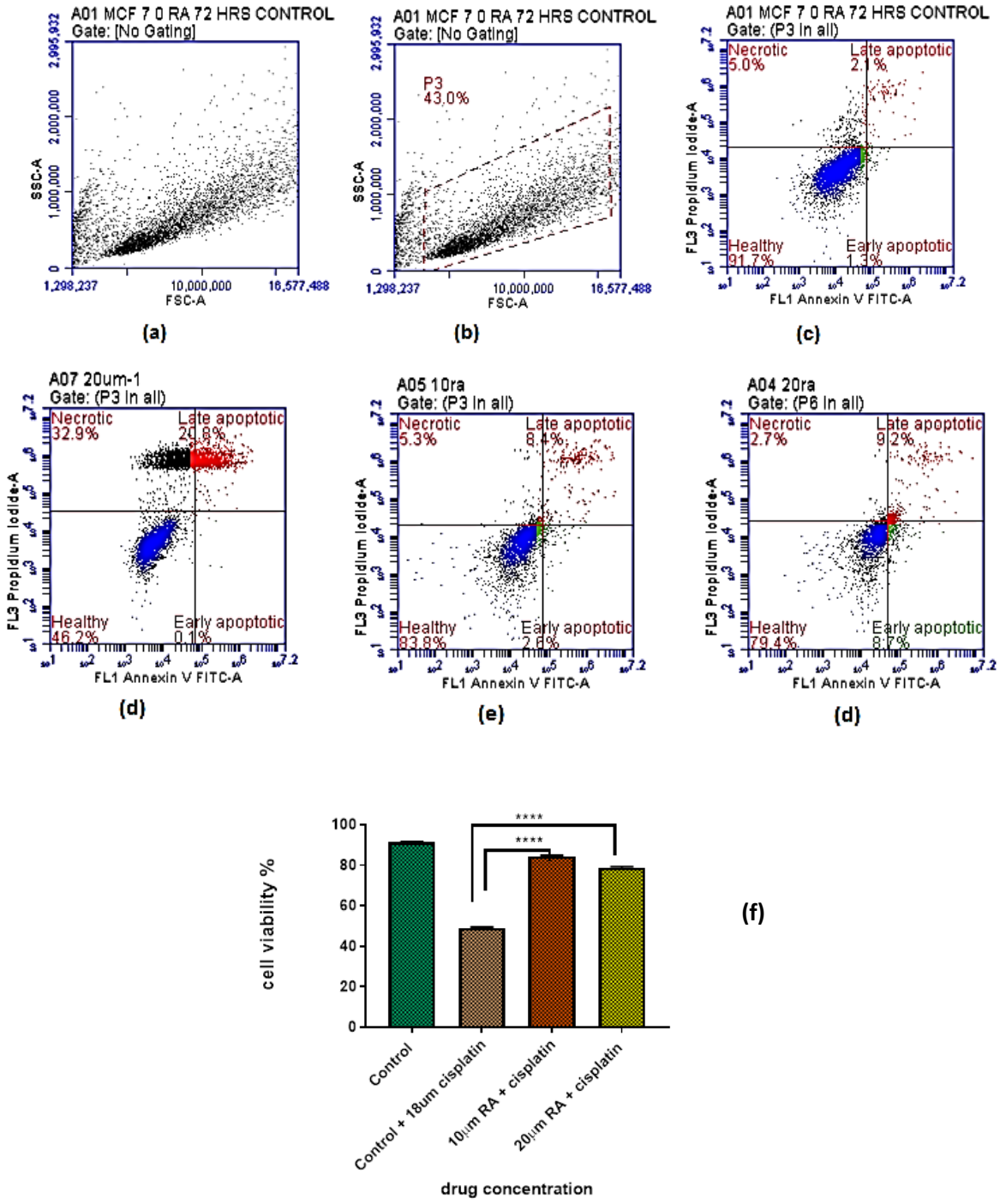


Figure 5. 7: Flow cytometry analysis of retinoic acid pre-treated wild type cells (RAPC-WT) exposed to cisplatin.

Representative cytogram of wild type cells pre-treated with retinoic acid (a) SSC-A/FSCA to collect single cells, (b) gating to exclude cell debris and doublets. Representative cytogram of RAPC-WT cells after cisplatin treatment showing the cytograms of the cells in the different treatment groups characterised into healthy, early apoptotic, late apoptotic and necrotic. (c) Untreated control, (d) cells treated with 18 µl cisplatin only (e) RTPC-WT + 10 µM RA + 18 µM cisplatin, (f) RAPC-WT + 20 µM RA + 18 µM cisplatin. The results were analysed with the 2 way ANOVA and presented as means ± SEM from at least three independent experiments. The histogram compares the mean of the different groups, and significance is denoted by **** (<0.0001).

Figure 5.7 shows that 87% of cells were healthy in the untreated control cytogram, and after RA and cisplatin, there were still over 70% healthy cells, suggesting that the retinoic acid pre-treated cells had reduced sensitivity to cisplatin. Cells pre-treated with retinoic acid had reduced sensitivity to the IC_{50} of cisplatin toxicity when compared to the untreated control (d).

5.8 Effect of cisplatin on TG2 expression in RAPC-WT cells.

The relationship between TG2 and cellular development of cisplatin resistance was investigated by exposing RAPC-WT cells to 18 µM of cisplatin (which is the IC_{50} of control cells). Cells were then incubated for a further 24 hours to determine the pattern of TG2 expression.

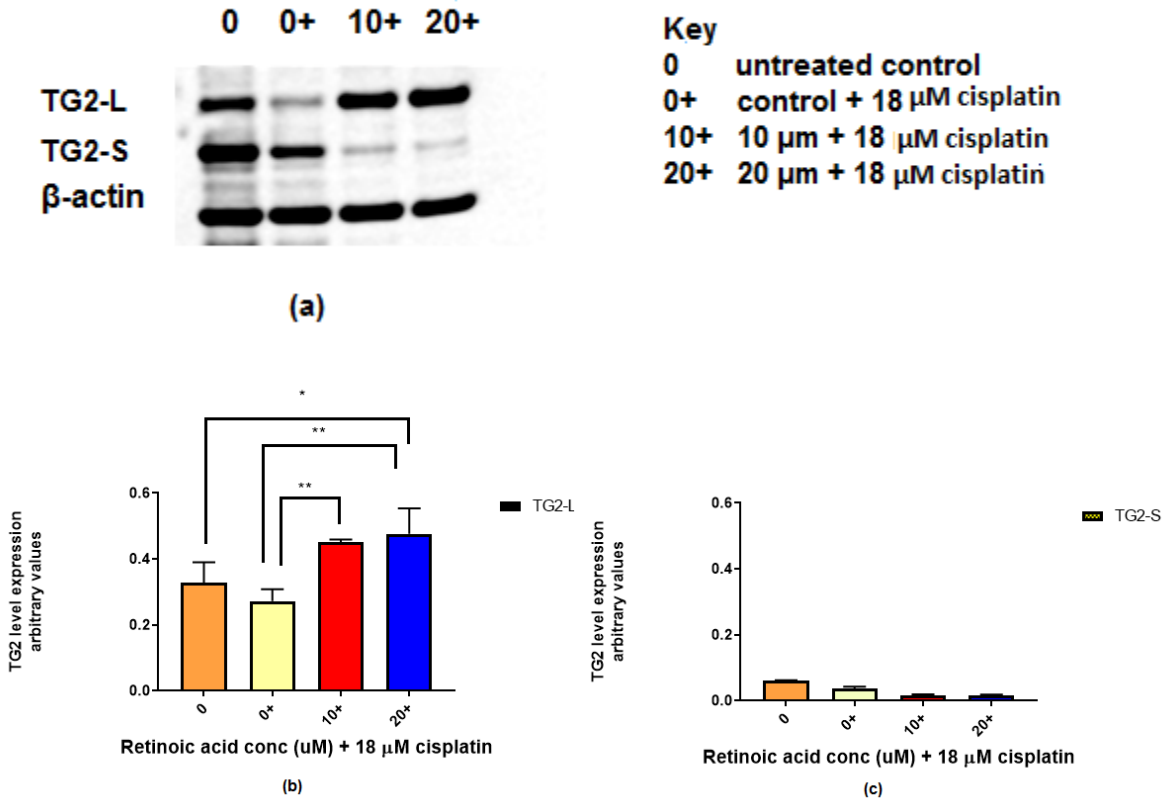


Figure 5. 8: Retinoic acid increases TG2-L expression levels in the presence of cisplatin

Western blot (a) analysis RAPC-WT cells exposed to 18 µM cisplatin with two controls: MCF-7 cells (0) and MCF-7 cells + cisplatin (0+). β-actin was used as a loading control, and the histograms quantify (b) TG2-L (c) TG2-S levels and presented as means ± SEM from at least three independent. Significance is denoted by *(0.0241), ** and (0.0086) when analysed with the 2 way ANOVA.

From the blot shown in figure 5.8a, when the untreated control (0) is compared with the other groups, cisplatin reduced TG2-L levels in the 0+ control, with a much greater effect on TG2-L than TG2-S. The opposite pattern was observed in the RAPC-WT cells, where TG2-L levels appear to be maintained, or slightly increased, at both retinoic acid concentrations, but when compared to the cisplatin control (0+), there is a significant increase of TG2-L levels. Overall, there is a decrease of TG2-S expression between the untreated control (0) and the treated cells. TG2-S levels in the cisplatin-treated control (0+) were reduced to approximately half of those in the untreated control, while in the retinoic acid-treated cells; the reduction was profound. The ratio of TG2-L to TG2-S after retinoic acid and cisplatin treatment in RAPC-WT is in a 9:1 TG2-L to TG2-S ratio.

5.9 Effect of cisplatin on RAPC-CR cells

To assess the effect of increased TG2 levels on cell's sensitivity to cisplatin, RAPC-CR cells were treated with cisplatin. Briefly, cells were seeded in a 96-well plate, treated with retinoic acid for 72 hours, media was replaced with media containing an increasing series of concentration of cisplatin, and incubated for 24 hours. After the incubation period, the inhibitory concentration was determined with the MTT assay (section 2.2.2).

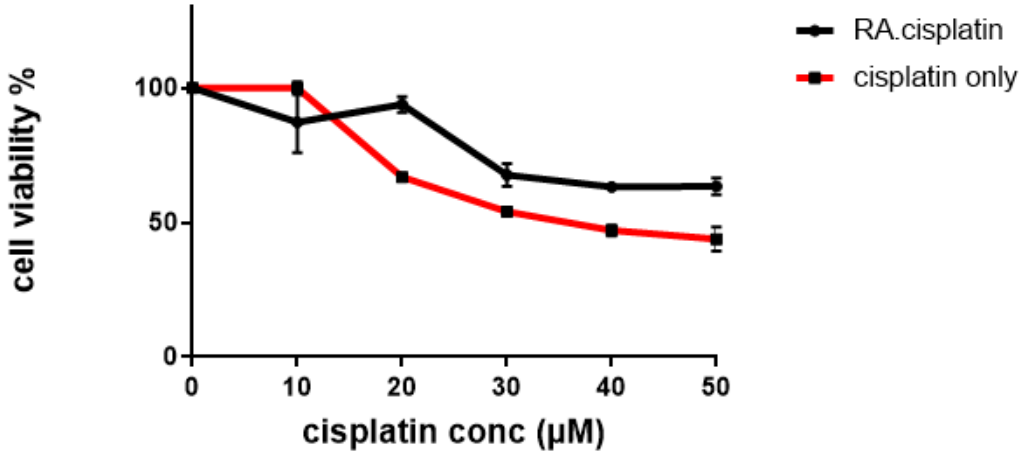


Figure 5. 9: Effect of cisplatin on RAPC-CR cells

Cytotoxicity of cisplatin on cisplatin-resistant cells after retinoic acid pre-treatment for 72 hours determined by MTT assay. The results are presented as means ± SEM from at least three independent experiments.

The viability graph in figure 5.9 shows that the retinoic acid-treated cells (black curve), unlike the control (red curve), were less sensitive to cisplatin toxicity, which implies that treatment with RA reduced RMCF-7 cells' sensitivity to cisplatin at the previous IC_{50} of 35 µM.

5.10 Determination of TG2 levels in retinoic acid pre-treated resistant (RAPC-CR) MCF-7 cells

Equal numbers (2 x 10^5) of cisplatin-resistant cells were incubated with 10 µM or 20 µM concentrations of retinoic acid, respectively and incubated for 24, 48 or 72 hours. Cells were harvested after the incubation period and total protein extracted for Western blotting as described in section 2.6 and 2.7.

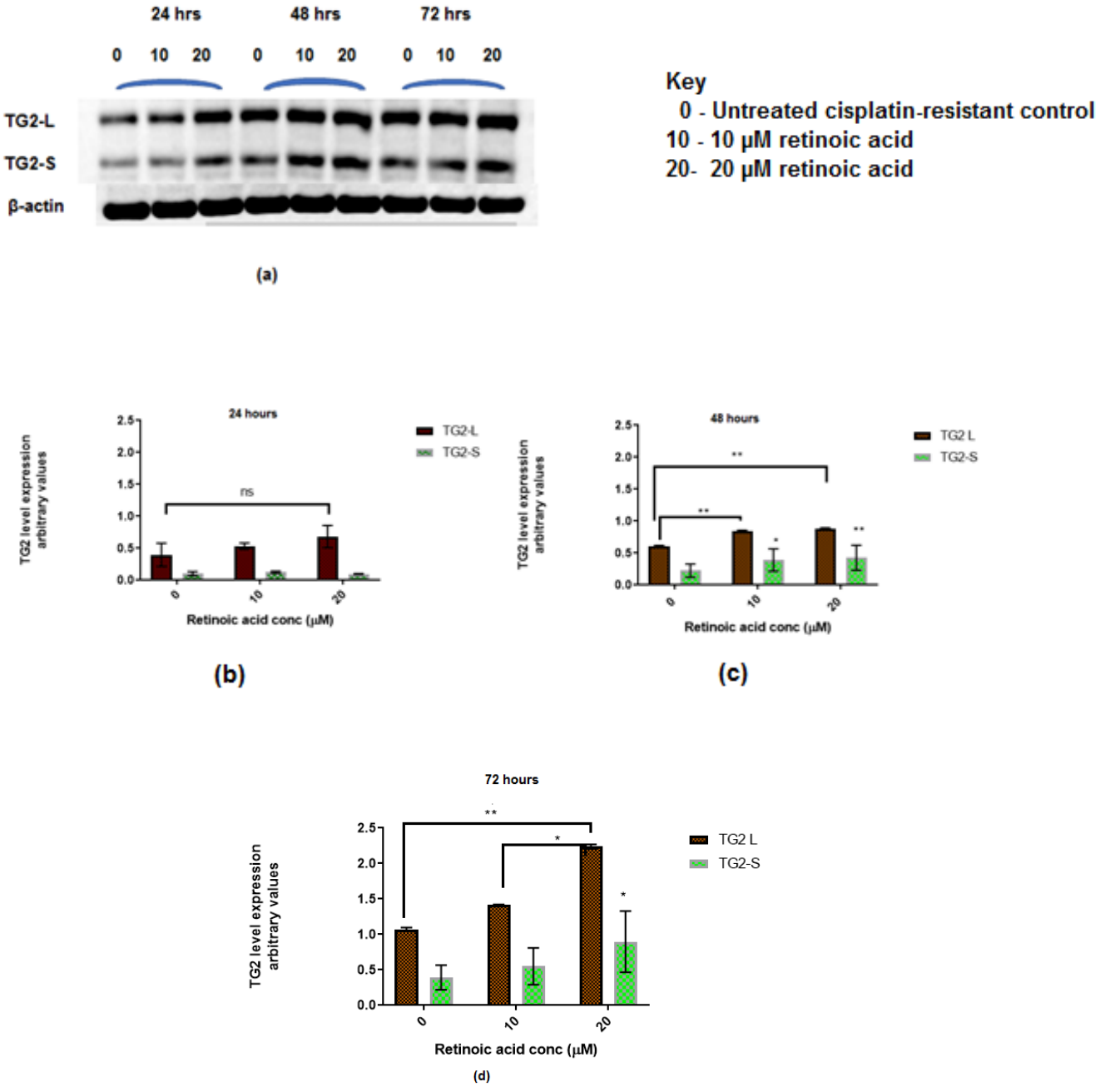


Figure 5. 10: TG2 expression is induced by retinoic acid in retinoic acid pre-treated resistant (RAPC-CR) cells

Western blot (a) of RAPC-CR cells after TG2 was induced with retinoic acid for 24, 48 and 72 hours. B-actin was used as the loading control for normalization. The histograms represent band quantifications after 24 hours (b), 48 hours (c) and 72 hours (d). All data were analysed by the 2-way ANOVA and significance is symbolised by p values * (0.0100), ** (0.0037) and *** (0.0010). The results are presented as means ± SEM from at least three independent experiments.

Generally, the blot shows that TG2 induction is concentration and time-dependent as in the wild type cells. Over the 72 hours, there was an increase in TG2 levels in the untreated control, but a more profound increase was seen in the retinoic acid-treated cells. Conversely, TG2-S expression increased after 48 hours and was maintained after 72 hours.

5.11 Effect of cisplatin on retinoic acid pre-treated resistant cells (RAPC-CR)

To determine the effect of retinoic acid on cisplatin toxicity, 5 x10^5 cisplatin-resistant cells were treated with 10 µM or 20 µM of RA in a 6-well plate for 72 hours. After incubation, media was removed and replaced with fresh media containing 35 µM of cisplatin (IC_{50} of resistant cells) and incubated for a further 24 hours. At the end of the incubation period, total protein was extracted from the cells and analysed for TG2 expression by Western blotting and β-actin was used as the loading control.

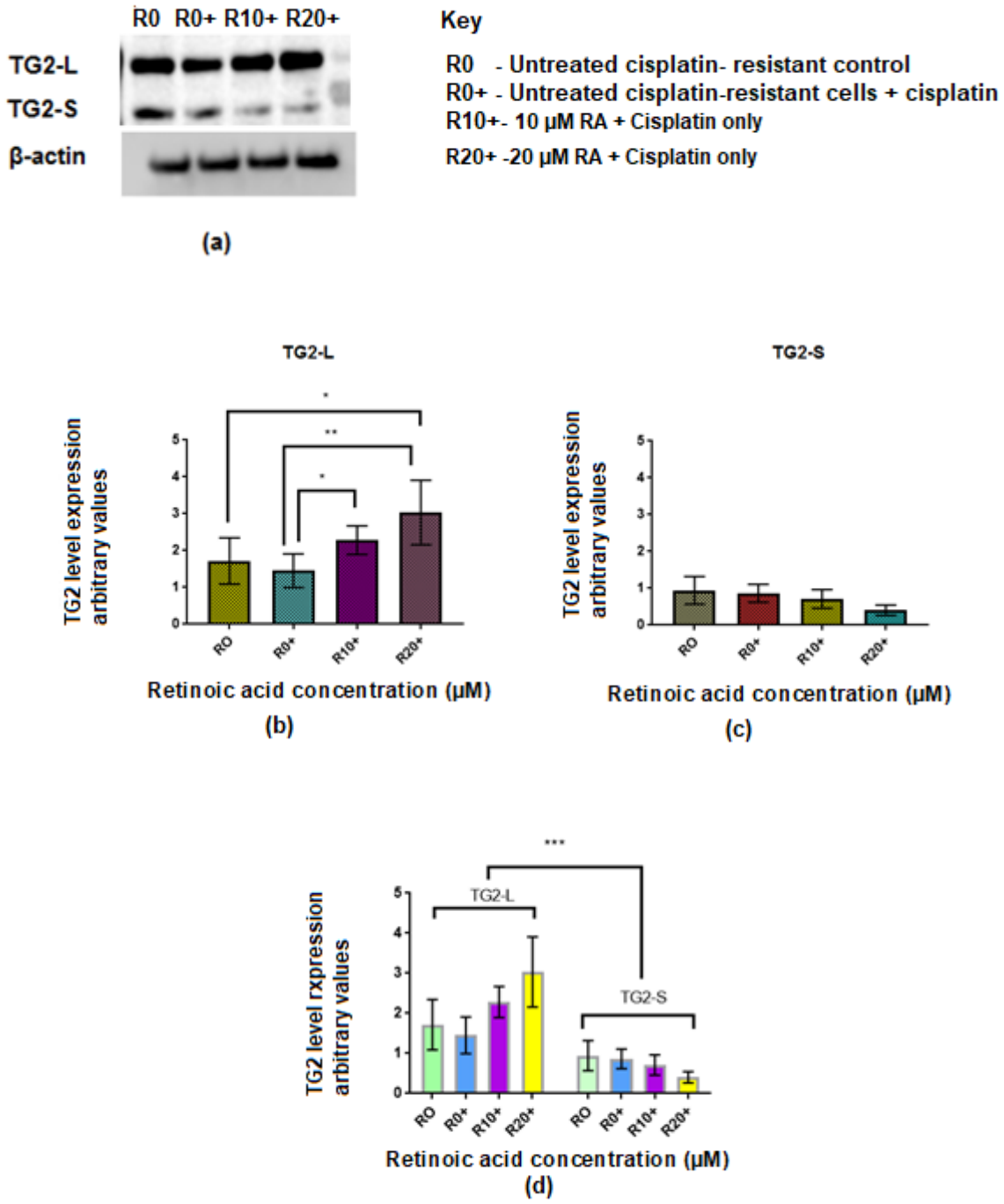


Figure 5. 11: Effect of cisplatin on TG2 expression in RAPC-CR cells

Western blot of RAPC-CR cells (a) showing the levels of both isoforms to 35 µM cisplatin treatment. The histogram represents band quantifications from three independent experiments for (b)TG2-L (c), TG2-S and (d) comparison between both isoforms. Normalisation was with β-actin, and the results are presented as means ± SEM from at least three independent experiments and analysed with the 2 way ANOVA. Statistical significance is denoted * (0.0448). ** (0.0013) and *** (0.0002).

Overall, the blot in figure 5.11 shows an increase in TG2-L levels in the untreated control that was also seen in the retinoic acid, pre-treated cells with a slight reduction in the cisplatin-resistant control cells (R0+). The opposite was observed in TG2-S levels as there was a decrease in the cisplatin-resistant control cells (R0+) with a further reduction in the retinoic acid pre-treated cells when compared to the untreated cisplatin-resistant control cells (R0). TG2-L/TG2-S ratio in the resistant cells was also similar to the wild type suggesting that the increased level of the long-form could be responsible for the reduced efficiency of cisplatin in the cells.

5.12 Comparison of TG2 activity in RAPC-WT and RAPC-CR cells with the TG2 COV assay kit

The enzymatic activity of both retinoic acid-treated cell types was determined with the TG2 COV assay kit following the protocol described in section 2.9.1 to access the difference after cisplatin treatment. Both wild and cisplatin-resistant MCF-7 cells were exposed to the IC_{50} (18 µM and 35 µM) of cisplatin for 24 hours, total protein was extracted, and the activity determined.

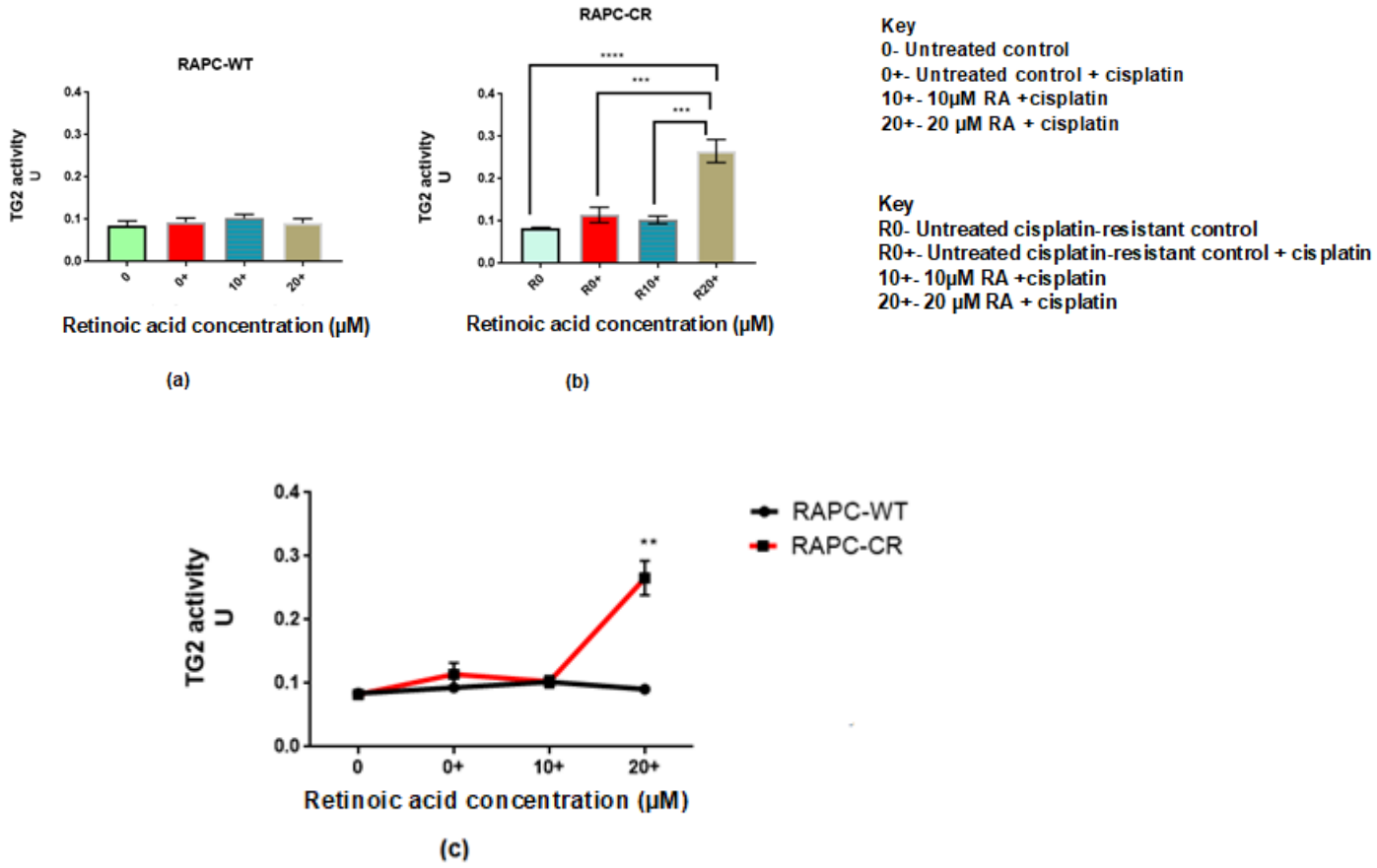


Figure 5. 12: Enzyme activity of RAPC-WT and RAPC-CR cells after cisplatin treatment

TG2 activity determined with the TG2 COV assay kit of (a) RAPC-WT and (b) cisplatin-resistant cells Comparison between wild type and resistant cells is illustrated in (c). Statistical significance is denoted by ** (0.0048), *** (0.0002).and **** (<0.0001). The results are presented as means ± SEM from at least three independent experiments and analysed with the 2-way ANOVA.

There was no significant increase in TG2 activity in the RAPC-WT cells after cisplatin treatment. Almost the same pattern is mirrored in the RAPC-CR cells except at the 20 μM concentration, where the activity doubled the wild type cells.

5.14 Discussion

In general, TG2 expression increase correlated with chemoresistance and the results from this chapter show TG2-L as the isoform most increased with an associated reduction in the sensitivity to cisplatin. Retinoic acid was an effective TG2 inducer in this study which is consistent with results from previous studies in HeLa cells (Singh et al., 1996); cervical sympathetic ganglia (Ando et al., 1995); and Chondrocytes (Lee et al., 2010).

A variation in the long and short form of the protein (control) is observed, and more studies need to be done with regards to tissue culture plate size, cell density and the effect on protein expression in in-vitro assays.

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Chapter 6: TG2 inhibition studies using cystamine

6.0 Introduction

Results from the preceding chapter show that the increased expression of TG2 and particularly TG2-L correlated with the wild type cells being less sensitive to cisplatin while the resistant cells become more chemoresistant. It also gives more credence to the hypothesis that TG2-L supports cell survival and is involved in the development of resistance. Experiments were designed to determine the effect of TG2 inhibition on the sensitivity of MCF-7 cells to cisplatin and to determine the isoform inhibited by cystamine. TG2 was inhibited in both wild and resistant cell types with cystamine and further exposed to cisplatin.

Both wild type and cisplatin-resistant cell types were exposed to cystamine for 24 hours (section 2.0) to inhibit TG2, and the TG2-inhibited cells were further exposed to cisplatin for another 24 hours. The toxicity of cystamine on the cells was first accessed to ensure a tolerable non-toxic concentration were used. Then, the effect on TG2 expression was detected by Western blotting, and the effect on cisplatin toxicity was determined. A freshly-made 2 mM cystamine stock concentration was used for each study, and results are computed from at least three independent experiments.

6.1. Effect of cystamine on cell viability

The toxicity of cystamine was determined by treating cells with an increasing series of cystamine concentrations.

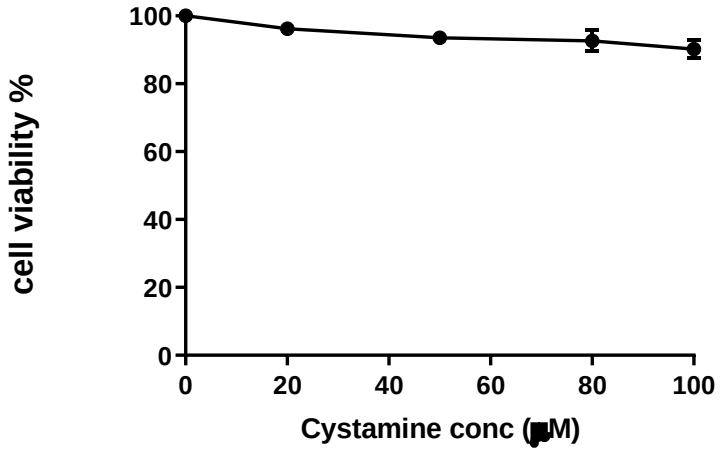

Figure 6.1: Effect of cystamine on wild type cell viability

Viability of wild type MCF-7 cells treated with cystamine for 24 hours determined with the MTT assay. The results are presented as means ± SEM from at least three independent experiments, and cells are seen to be viable at the concentrations tested.

Fig 6.1 shows a concentration range of 0 –100 µM of cystamine was not toxic to the cells, with only a 10% loss of viability at 100 µM. Choosing a tolerable concentration for cells was essential to avoid cell death due to cystamine treatment. For this study, cells were, therefore, pre-treated with a 50 µM and 100 µM cystamine to inhibit TG2 activity , before cisplatin treatment.

6.2 Determination of the viability of cystamine-treated cells with Annexin V assay

To support the MTT assay results, the viability of the cells was also determined by the Annexin V and propidium iodide analysis. This assay assesses the numbers of cells that have undergone apoptosis. Briefly,5 x 10^5 cells were treated with 50 µM or 100 µM cystamine for 24 hours. After 24 hours incubation, the cells were harvested and prepared for flow cytometry analysis, as described in section 2.5.

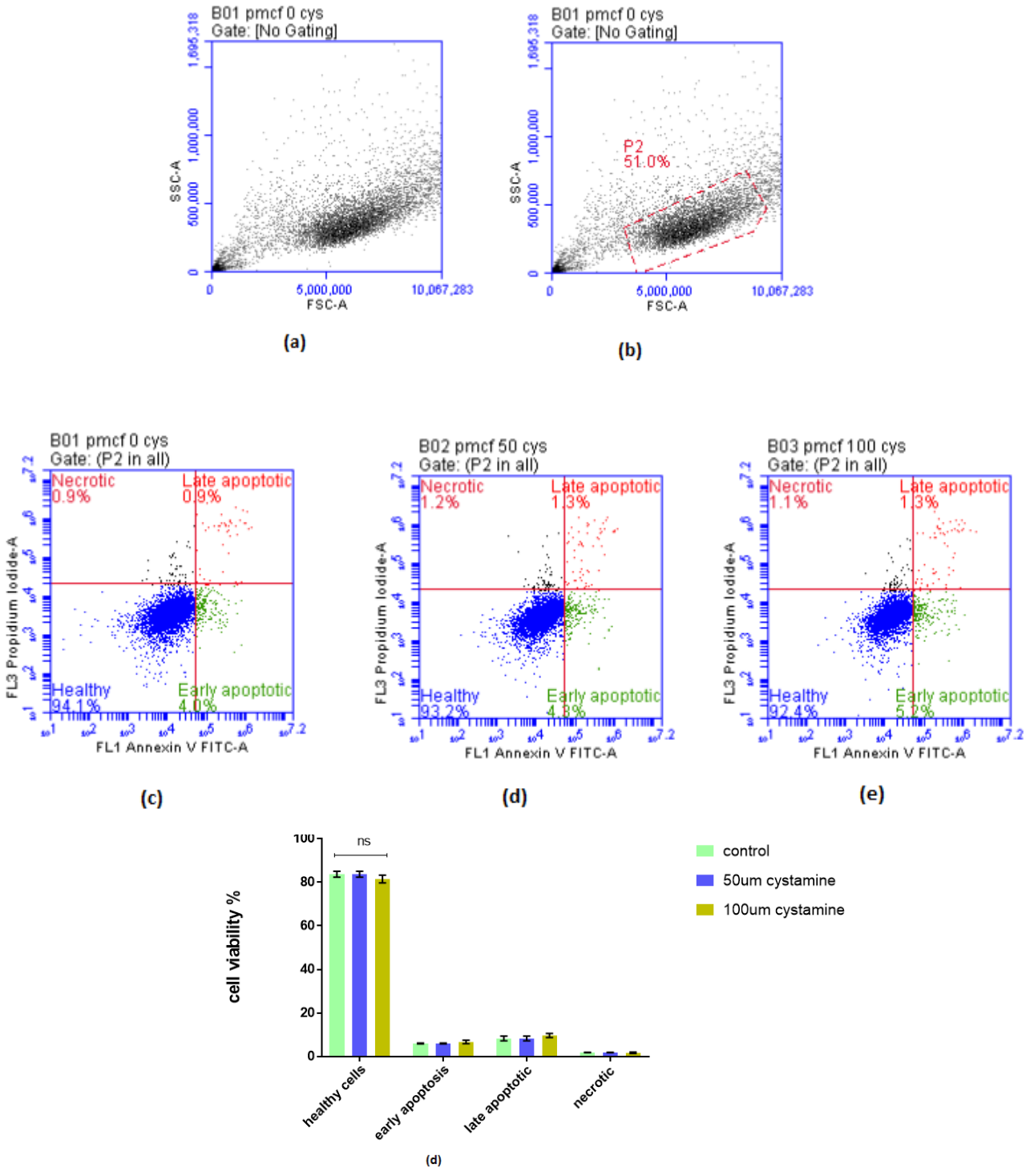

Figure 6.2: Flow cytometry Annexin V analysis of cells treated with cystamine

Representative cytogram of cystamine-treated cells analysed by flow cytometry to determine their viability. (a) SSC-A/FSCA to collect single events (b) gating to exclude cell debris and doublets represents the untreated control cells, (c) MCF-7 control cells (d) cells treated with 50 µM cystamine, (d) cells treated with 100 µM cystamine and a (d) histogram showing the viability of cells in percentages. The results are presented as means ± SEM from at least three independent experiments, and the difference was not statistically significant with the 2 way ANOVA.

The cytogram in figure 6.2 shows the population of cells at different stages of apoptosis. In the untreated control cells (a) 94.1% were healthy, and after 50 µM cystamine treatment, 93.2% were still healthy, with 92.4% of cells remaining healthy after 100 µM cystamine treatment. Overall, the results show that cystamine treatment had little effect on cell viability, supporting the result of the MTT assay shown in figure 6.1.

6.3 Effect of cystamine on TG2 levels in wild type MCF-7 cells.

Following confirmation that 0-100 µM cystamine is tolerable to the cells, the effects on TG2 expression were investigated by Western blotting. Briefly, 5 x10^5 wild type cells were seeded in 6-well plates and treated with 50 µM and 100 µM cystamine for 24 hours and protein extracted for the analysis as described, in sections 2.6 and 2.7.

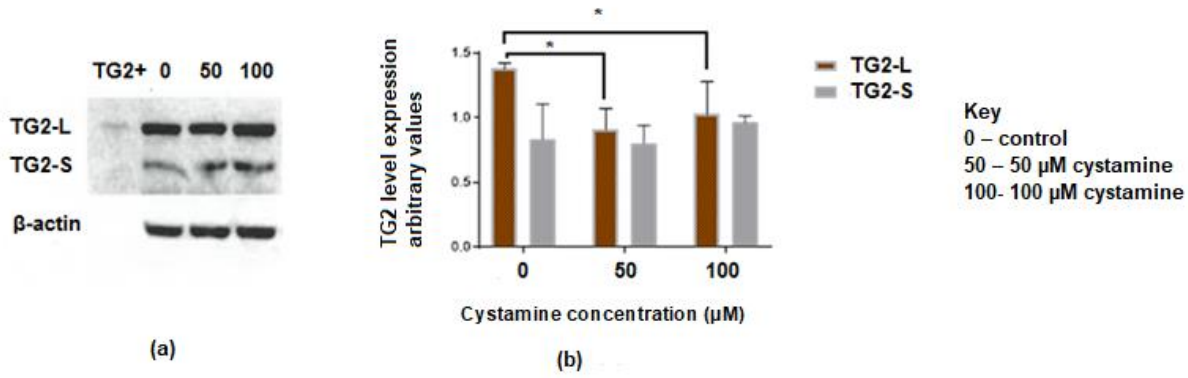

Figure 6.3: TG2 expression after treatment of wild type cells with cystamine

Representative Western blot (a) analysis of MCF-7 cells exposed to cystamine for 24 hours and the histogram (b) quantify both TG2-L and TG2-S bands and presented as means ± SEM from at least three independent experiments and analysed with the 2-way ANOVA. B-actin was used as a loading control. Statistical significance is denoted by * (0.0448).

Overall, the Western blot in figure 6.4 does not show a profound decrease in TG2 levels, but there is a decrease in TG2-L expression in the cystamine-treated cells as compared to the control. In contrast, TG2-S expression is similar in the cystamine-treated cells with the control

and Increasing the incubation time with cystamine might yield more promising results and inhibit TG2-L levels more significantly.

6.4 Effect of cisplatin on cystamine pre-treated MCF-7 cells.

The cytotoxic effect of cisplatin was investigated after TG2 inhibition with cystamine to determine if cells will be sensitised to cisplatin at lower concentrations. Briefly, approximately 5 x 10^5 cells were exposed to 50 µM and 100 µM cystamine for 24 hours, the media was replaced with fresh media containing increasing series of cisplatin concentration, and cells were re-incubated for a further 24 hours. Cell viability was determined with the MTT assay.

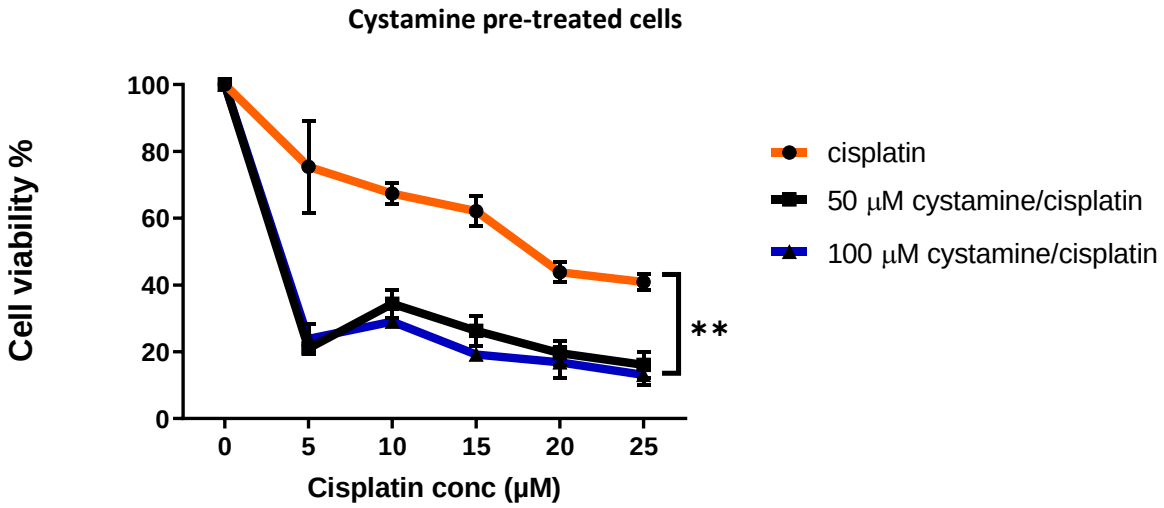

Figure 6.4: Effect of cisplatin on cystamine pre-treated wild type cells

Effect of cisplatin on cystamine-treated cells determined with the MTT assay. The line curves represent the different treatment groups, (orange line curve) cells treated with cisplatin only, cells treated with 50 µM (black line curve) and 100 µM cystamine (blue line curve) The result is presented as means ± SEM from at least three independent experiments. The difference between the cisplatin treated cells and TG2 inhibited cells was statistically significant with a p value of 0.0015 when analysed with the 2 way ANOVA.

The graph shown in figure 6.4 illustrates the viability curves of cells after TG2 inhibition suggesting that the inhibition of TG2 before cisplatin treatment sensitises the cell into apoptosis at lower cisplatin concentration. The IC_{50} of the wild type cells was 18 µM (red curve) which reduced after TG2 inhibition to 5 µM (blue and black curve). Further validation of the cytotoxicity results in figure 6.4 was confirmed using two other controls. In the first control, cells were treated with a combination of cisplatin and cystamine for 24 hours (figure 6.5), while in the second control, cells were exposed to cisplatin for 24 hours before cystamine treatment (figure 6.6). These controls were set up to confirm the effects of inhibiting TG2 activity first, before treatment with cisplatin.

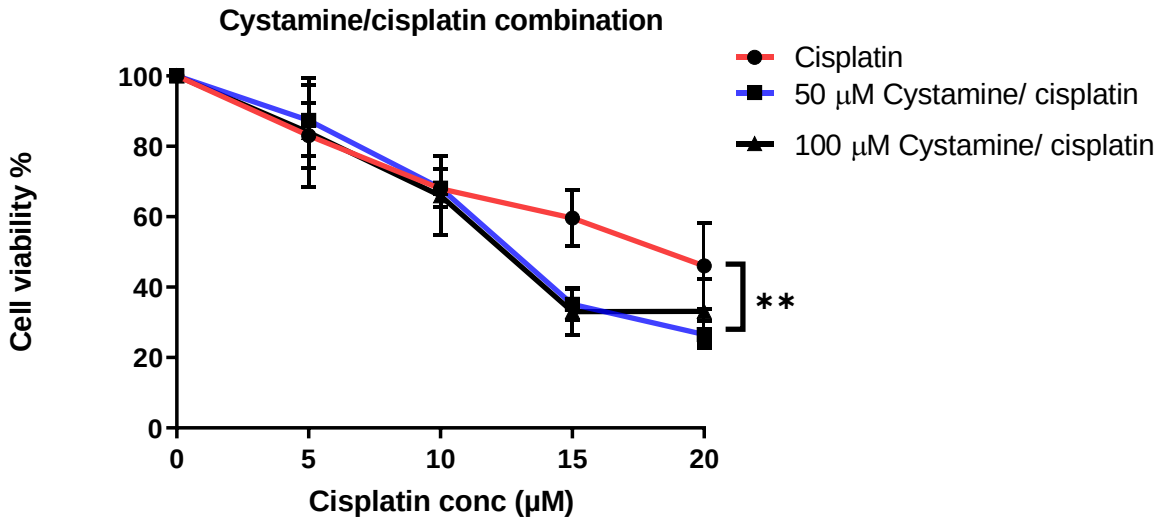

Figure 6.5: Viability of wild type cells treated with a combination of cystamine and cisplatin

Cytotoxicity of cisplatin in combination with cystamine on MCF-7 cells determined using the MTT assay. The line curves represent the different treatment groups, cells treated with cisplatin only, cells treated with 50 µM and cisplatin(black line curve) and 100 µM cystamine + cisplatin (blue line curve) The result is presented as means ± SEM from at least three independent experiments. The difference between the cisplatin treated cells and TG2 inhibited cells was statistically significant with a p value of 0.0055 when analysed with the 2 way ANOVA.

Figure 6.5 shows a reduction to 12 µM in the IC_{50} of cells that were treated with a combination of cystamine and cisplatin. In comparison, cells treated with cisplatin alone had an IC_{50} of 18 µM. The results reveal that treatment with a combination of cisplatin and cystamine does not inhibit cell viability to the same degree as pre-treatment with cystamine (figure 6.4).

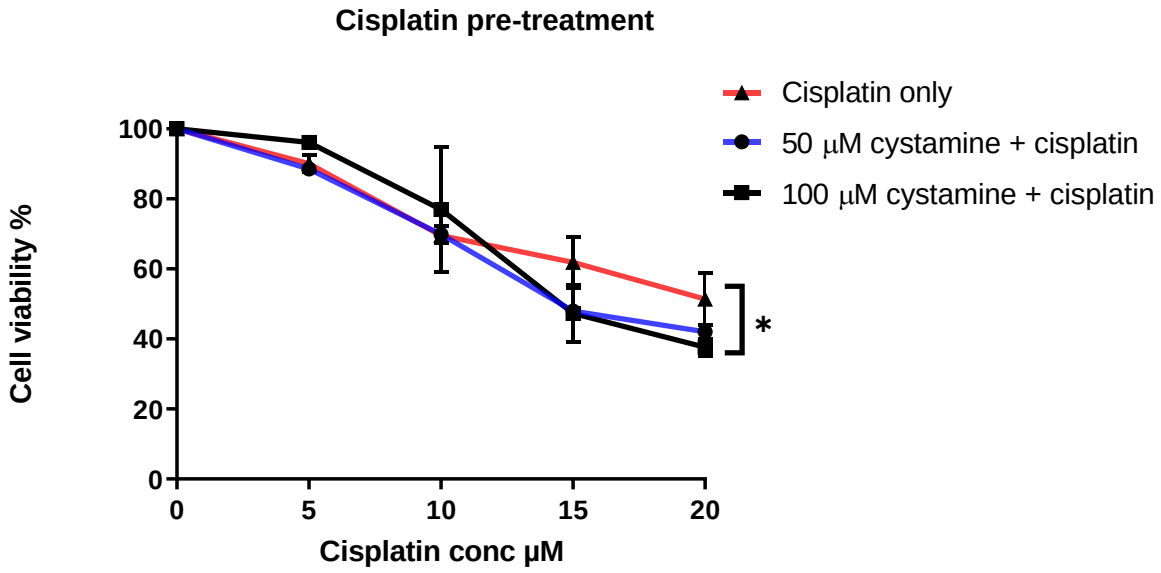

Figure 6.6: Viability of wild type cells pre-treated with cisplatin

Cell viability of cisplatin pre-treated cells before cystamine treatment determined by the MTT assay. The line curves represent the different treatment groups cells treated with cisplatin only(red curve), cells pre-treated with cisplatin and 50 μM (black line curve) and 100 μM cystamine (blue line curve) The result is presented as means ± SEM from at least three independent experiments. The difference between the cisplatin treated cells and TG2 inhibited cells was statistically significant with a p value of 0.0193 when analysed with the 2 way ANOVA.

Exposure of cells to cisplatin before TG2 inhibition reduced the IC_{50} from 18 μM to 15 μM validating the result in figure 6.4 that inhibition of TG2 before cisplatin treatment significantly sensitises cells to cisplatin at lower concentrations than cystamine/cisplatin co-treatments.

Table 6.1: Comparison of the inhibitory concentrations of the different treatments.

Type of treatment	Inhibitory concentration
Cisplatin only	18 μM
Cystamine pre-treated + cisplatin	5 μM
Cisplatin pre-treated + cystamine	15 μM
Combination of cisplatin/cystamine	12 μM

Table 6.1 summarises the inhibitory concentrations from the different cytotoxicity assays and suggests that inhibiting TG2 with cystamine sensitises cells to cisplatin.

6.5 Flow cytometric analysis of the effect of cisplatin on wild type cells after TG2 inhibition

Wild type MCF-7 cells were stained with Annexin V to determine the percentages of cells undergoing apoptosis and confirm the result in figure 6.4. Briefly, 5 x 10^5 cells were grown in 6 well plates, and TG2 was inhibited with 50 and 100 µM cystamine for 24 hours, and at the end of the incubation, the media was replaced with fresh media containing 18 µM of cisplatin (IC_{50} of cisplatin). Cells were further incubated for another 24 hours and prepared for flow cytometry.

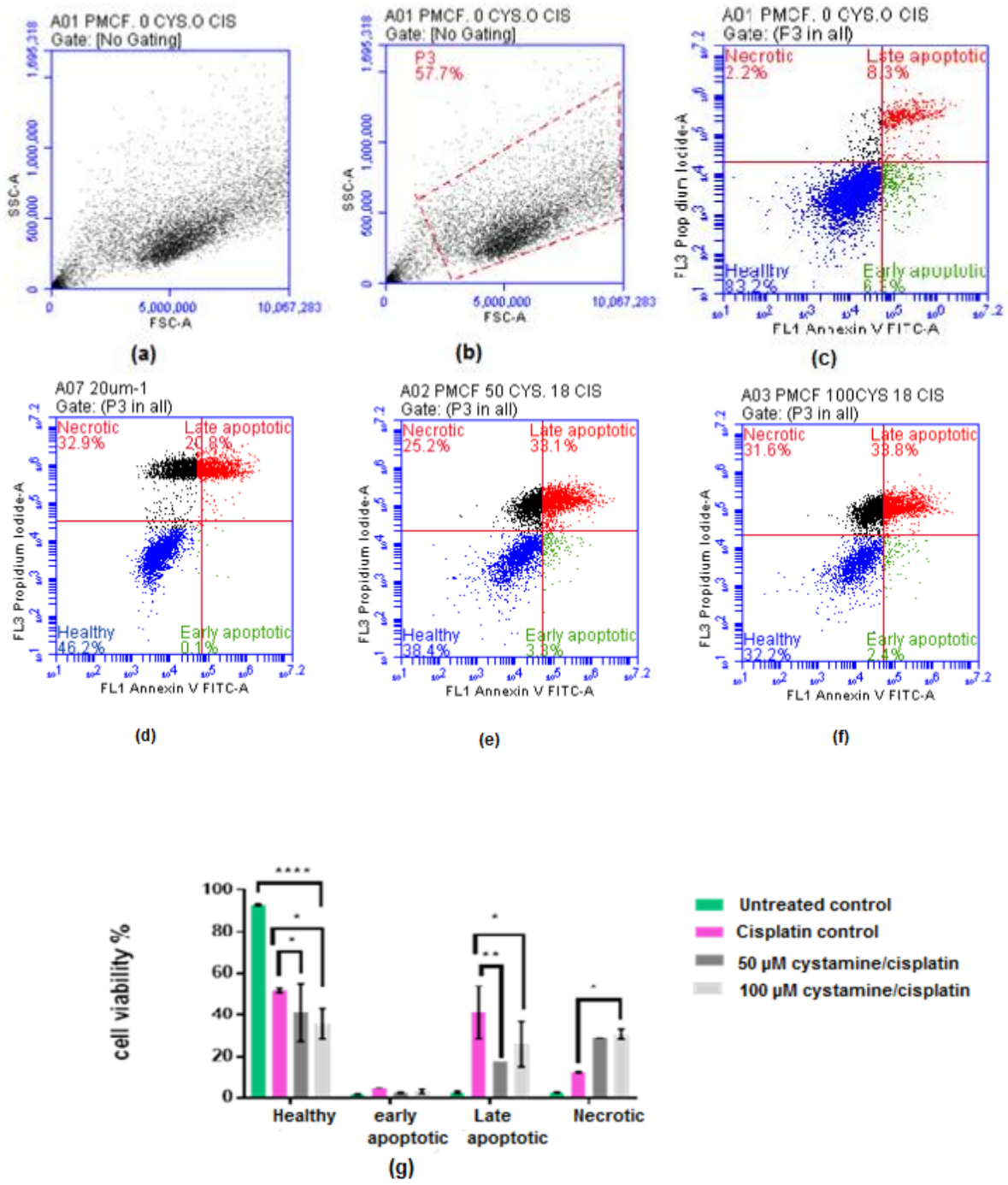

Figure 6.7: Effect of cisplatin on wild type cells after TG2 inhibition with cystamine**.**

Representative cytogram of wild type cells treated with cisplatin after TG2 inhibition with cystamine (a) SSC-A/FSCA to collect single cells, (b) gating to exclude cell debris and doublets (c) Untreated control; (d) cisplatin control(e) and (f), cystamine pre-treated cells. The histogram (g) represents the characterisation of the cells into healthy, early, late apoptotic and necrotic cells. The difference between treated and untreated are significant and symbolised by * (0.0448), ** (0.0013) and **** (<0.0001) when analysed with the 2 way ANOVA. The results are presented as means ± SEM from at least three independent experiments.

Figure 6.7 shows that the percentage of healthy cells (a) in control was 83..2%, and after cisplatin-treatment, the percentage of healthy cells was reduced to 46.2%. With TG2 inhibition, there was a further reduction of healthy cells to 38.4% with 50 µM and 32.2% with 100 µM cystamine. The result confirms that TG2-inhibited cells entered into apoptosis more than the cisplatin-treated cells, supporting the MTT assay in figure 6. 4.

6.6 Effect of cisplatin on TG2 expression in cystamine pre-treated cells.

The effect of cisplatin on TG2 levels after inhibition with cystamine was investigated. Equal numbers of cystamine pre-treated cells (5×10^5) were exposed to cisplatin for 24 hours, and total protein was extracted and analysed by Western blotting (see section 2.6 and 2.7).

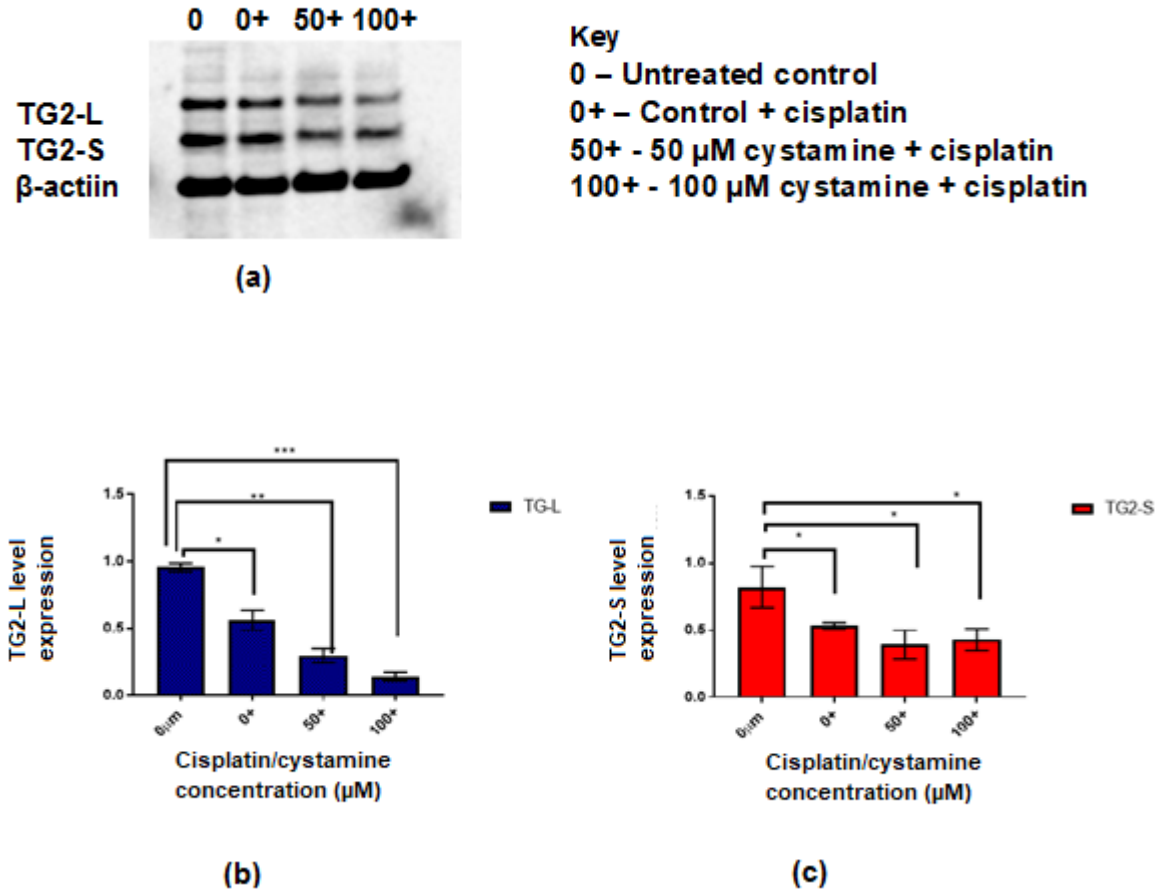

Figure 6.8: Western blot analysis of cystamine pre-treated MCF-7 cells treated with cisplatin.

Representative Western blot of (a) cystamine pre-treated cells, showing TG2 levels after treatment with cisplatin for 24 hours. The histogram (b) represents TG2-L band quantification while (c) represents TG2-S band quantification. The data is computed from at least three independent experiments and presented as means ± SEM. Statistical significance is denoted by *(0.0448), ** (0.0023) and *** (0.0007) when analysed with the 2 way ANOVA.

The Western blot in figure 6.8 overall, shows a reduction in TG2 expression between the treated cells and the untreated control. There is a significant decrease in TG2 expression in cells where TG2 was inhibited as compared to cisplatin only treated cells. TG2-L expression was observed to reduce as the concentration of cystamine increased, and cells treated with 100 µM cystamine had lower TG2-L expression than cells treated with 50 µM cystamine. There was also a reduction in TG2-S expression, but that reduction was less significant than TG2-L.

6.7 Effect of cystamine on the viability of cisplatin-resistant MCF-7 cells

Cisplatin-resistant MCF-7 cells were also pre-incubated with a series of increasing cystamine concentrations for 24 hours to compare the toxicity observed in the wild type cells.

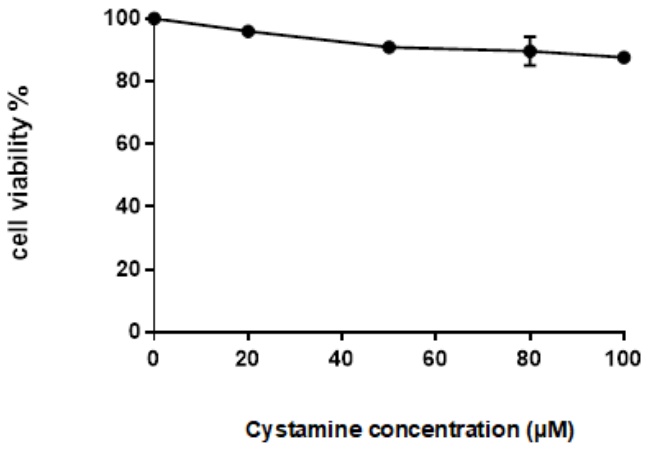

Figure 6.9: Effect of cystamine on the viability of cisplatin-resistant cells

The effect of cystamine on the viability of cisplatin-resistant cells was determined with the MTT assay. The result was computed from at least three independent experiments and presented as means ± SEM.

Compared to the wild type cells, after 24-hour incubation, the cisplatin-resistant cells were still seen to be viable. The 0-100 µM cystamine concentration range was not detrimental to the cells.

6.8 Effect of cystamine on TG2 levels in resistant cells

After confirmation that 0-100 µMcystamine is tolerable, chemoresistant cells were treated with cystamine for 24 hours to determine the expression of TG2. Equal numbers of cells (5 x 10^5) were seeded in a 6-well plate and treated with 50 µM or 100 µM of cystamine for 24 hours. After incubation, cells were harvested, and protein extracted for Western blotting (see section 2.6 and 2.7).

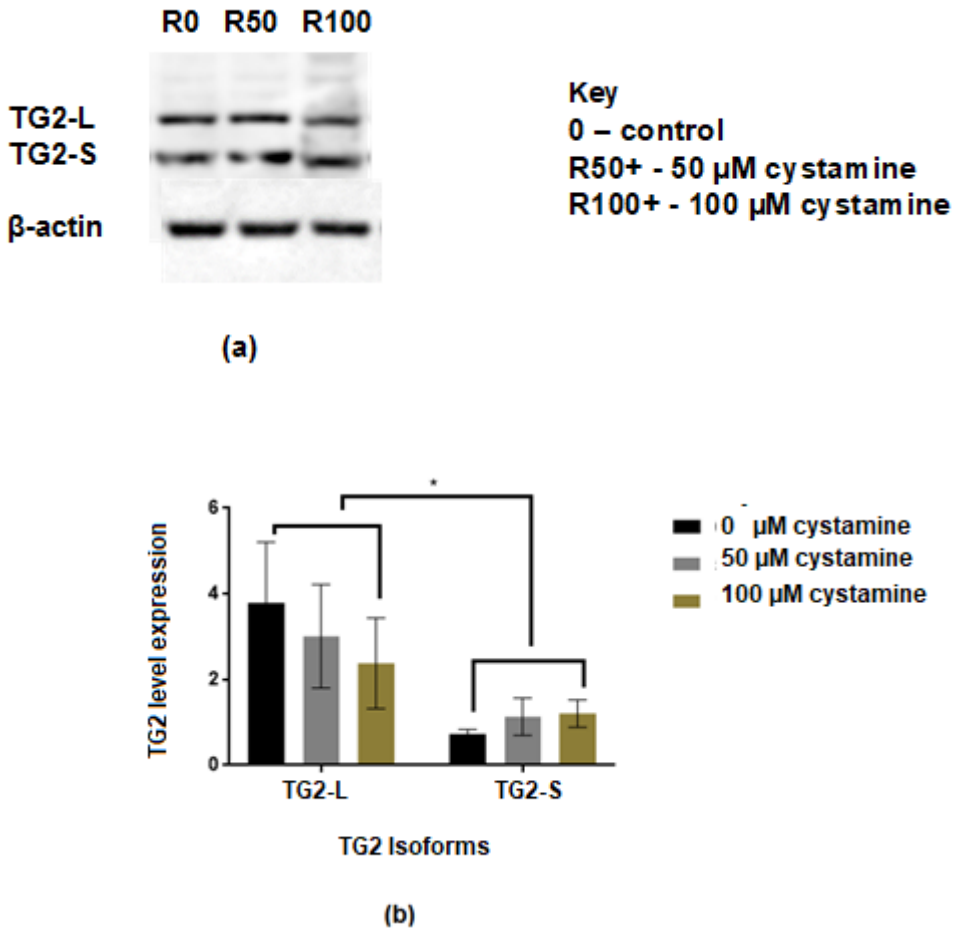

Figure 6.10: Effect of cystamine on TG2 level in cisplatin-resistant cells

Representative Western blot (a) of resistant cells after cystamine treatment for 24 hours, The histogram (b) represents total band quantification from three independent experiments, and the difference between the TG2-L and TG2-S levels was significant and denoted with * (0.0298). β-actin was used as the loading control. The data is computed from at least three independent experiments, analysed with the 2 way ANOVA and presented as means ± SEM.

The Western blot in figure 6.10 shows a general reduction of TG2 expression in the treated as compared to the control cells. This follows a similar pattern to the wild type cells (figure 6.4). With regards to the isoforms, as the levels of TG2-L is reduced in the TG2 inhibited cells, an increase is noticeable in TG2-S, especially after treatment with 100 µM cystamine. The profile of TG2 inhibition is time-dependent according to existing literature (Siegel and Khosla 2007), perhaps increasing the pre-treatment with cystamine from 24 to 48 hours or even 72 hours, might inhibit TG2-L significantly.

6.9 Cell viability of resistant cells after TG2 inhibition

To determine the effect of TG2 inhibition of TG2 expression, cisplatin-resistant cells were pre-treated with cystamine for 24 hours. Media was replaced with media containing a series of increasing concentrations of cisplatin and incubated for another 24 hours. The toxicity of cisplatin on the cells was then determined using the MTT assay (section 2.2.2).

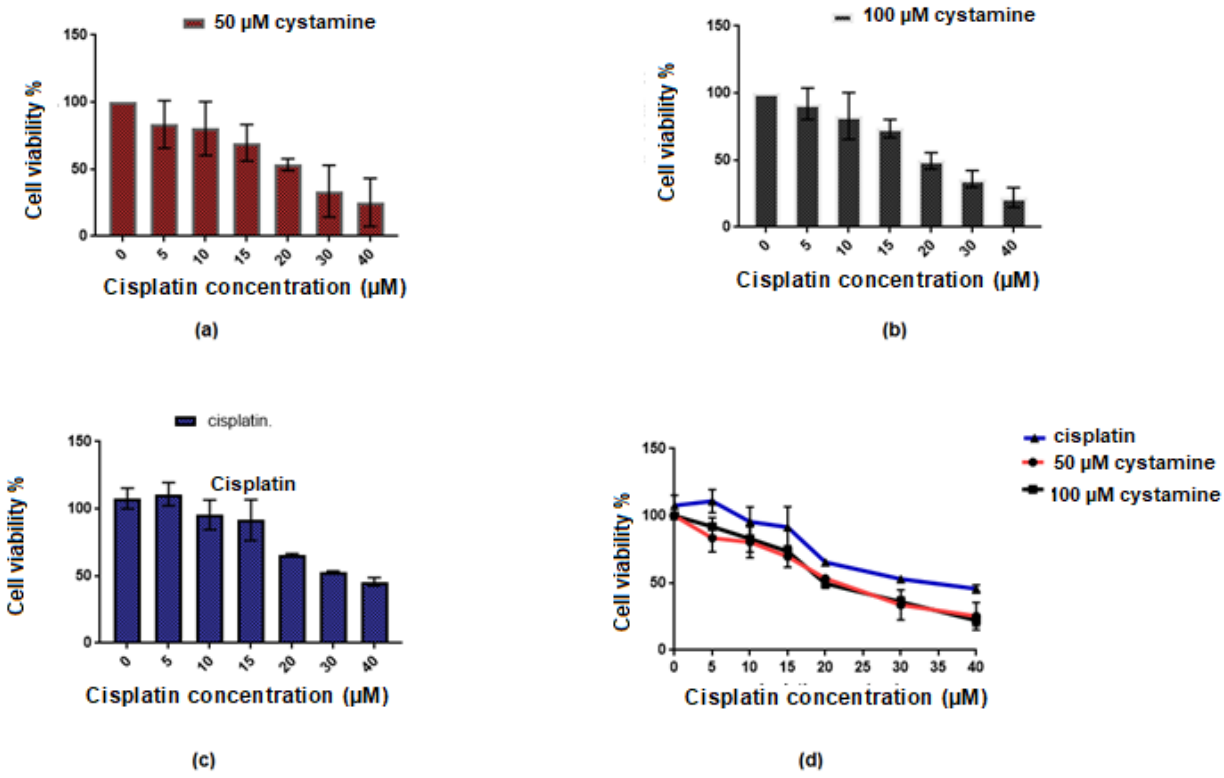

Figure 6.11: Cell viability of cystamine pre-treated chemoresistant cells exposed to cisplatin.

Viability of cisplatin-resistant cells after TG2 inhibition determined using the MTT assay. (a) displays cells pre-treated with 50 µM cystamine before cisplatin treatment; (b) cells pre-treated with 100 µM cystamine while (c) represents cells treated with cisplatin only. (d) shows combined viability from all groups. The data is computed from at least three independent experiments and presented as means ± SEM.

There is a reduction in the IC_{50} of cells in which TG2 was inhibited by cystamine as compared to the control (cells treated with cisplatin alone). Figure 6.11a shows the viability of all treated groups, and the IC_{50} of cystamine-treated cells was reduced to 20 µM from 35 µM (control).

The results suggest that TG2 inhibition in the resistant cells sensitises the cells to cisplatin at lower concentrations.

6.10 Effect of cystamine and cisplatin on TG2 level expression in resistant cells

The results in figure 6.11 show that the inhibition of TG2 with cystamine reduced the IC_{50} of cisplatin, so the effect on TG2 expression was also determined. Like the wild type, the resistant cells were grown in the presence of cystamine for 24 hours to inhibit TG2, before being treated with cisplatin at the IC_{50} (35 µM) of the resistant cells, (figure 4.4). Cells were then harvested, and protein was extracted for Western blotting analysis (see section 2.6 and 2.7).

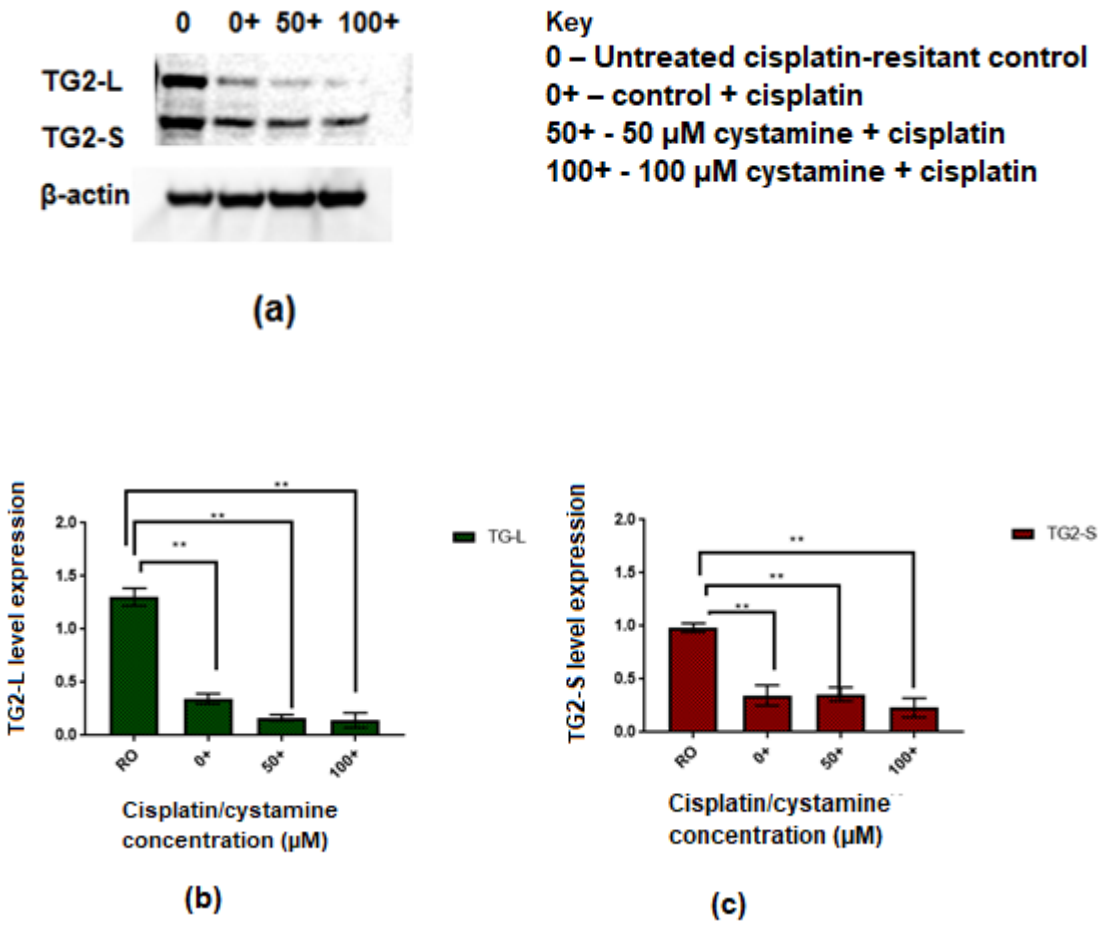

Figure 6.12: Effect of cisplatin on TG2 expression in cystamine pre-treated cisplatin-resistant cells

Representative Western blot (a) of TG2 expression in cystamine pre-treated cisplatin-resistant cells. The histogram represents the quantification of the bands from three independent experiments, (b) TG2-L and (c) TG2-S. The data is computed from at least three independent experiments and presented as means ± SEM and analysed with the 2 way ANOVA. Significance is denoted by the p-value ** (0.0023).

There is a general reduction of TG2 expression in the treated cells as compared to the control cells and decreased TG2-L levels when compared to TG2-S levels. The expression of TG2-L is seen to be further reduced in cells, where TG2 was inhibited with cystamine, and the expression decreased as the concentration of cystamine was increased. This could be responsible for the reduction of the IC_{50} of the resistant cells from 35 µM to 20 µM, as shown in figure 6.11d.

6.11 Effect of cisplatin on resistant cells after TG2 inhibition

To confirm the MTT assay (figure 6.11) and cytotoxicity of cisplatin on resistant cells after TG2 inhibition (6.12), cells were stained with Annexin V and propidium iodide analysis, which sums the numbers of cells that have undergone cell death (section 2.5). Briefly, cells were pre-treated with 50 µM or 100 µM cystamine for 24 hours; then media was replaced with fresh media containing 18 µM cisplatin. Plates were subsequently incubated for another 24 hours, and cells harvested and prepared for flow cytometry analysis as described in section 2.5.

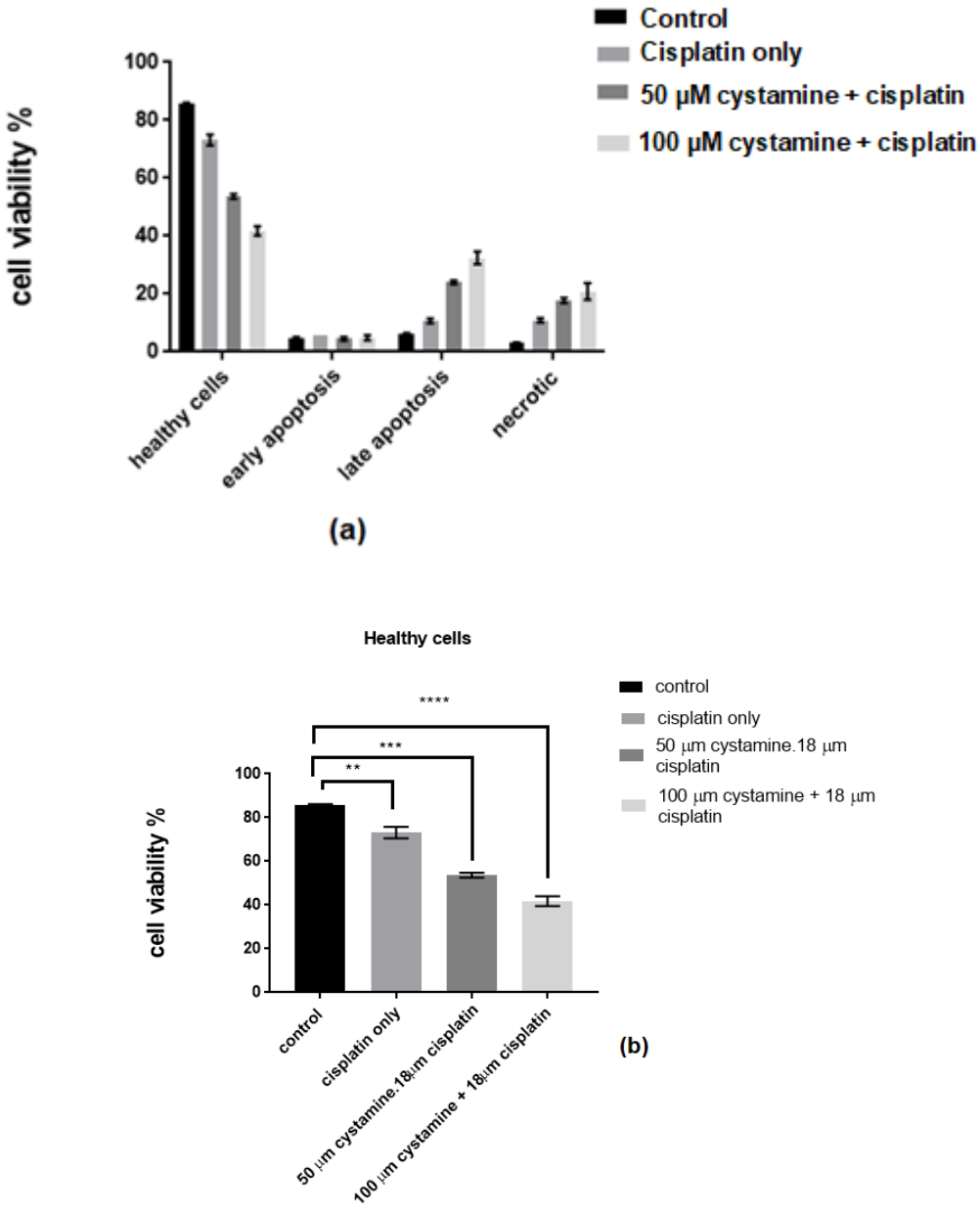

Figure 6.13: Apoptosis analysis of chemoresistant cells after TG2 inhibition by cystamine

(a) histogram of apoptosis analysis of resistant cells exposed to cisplatin after TG2 inhibition and (b) represents healthy cells amongst the groups. Significance is denoted by ** (0.0086), *** (0.0002) and **** (<0.0001), when analysed with the 2 way Anova. The results are computed from at least three independent experiments and presented as means ± SEM.

Figure 6.13 shows that TG2-inhibited cisplatin-resistant cells were more sensitive to cisplatin than the cisplatin-resistant cells without TG2 inhibition. Viability of the chemoresistant cells was 70% after cisplatin treatment, but with TG2 inhibition, viability was reduced to 52% with 50 µM cystamine and to 40% with 100 µM cystamine.

6.12 Determination of TG2 enzyme activity

The enzymatic activity following cystamine treatment in both wild type and cisplatin-resistant cell types was investigated with the TG2 COV assay kit. This was done by inhibiting TG2 with cystamine for 24 hours then media was replaced with fresh media and cells were incubated with the IC_{50} of cisplatin (18 µM for MCF-7 and 35 µM for RMCF) for a further 24 hours. Total protein was extracted, and the activity analysed, as described in section 2. 9.1.

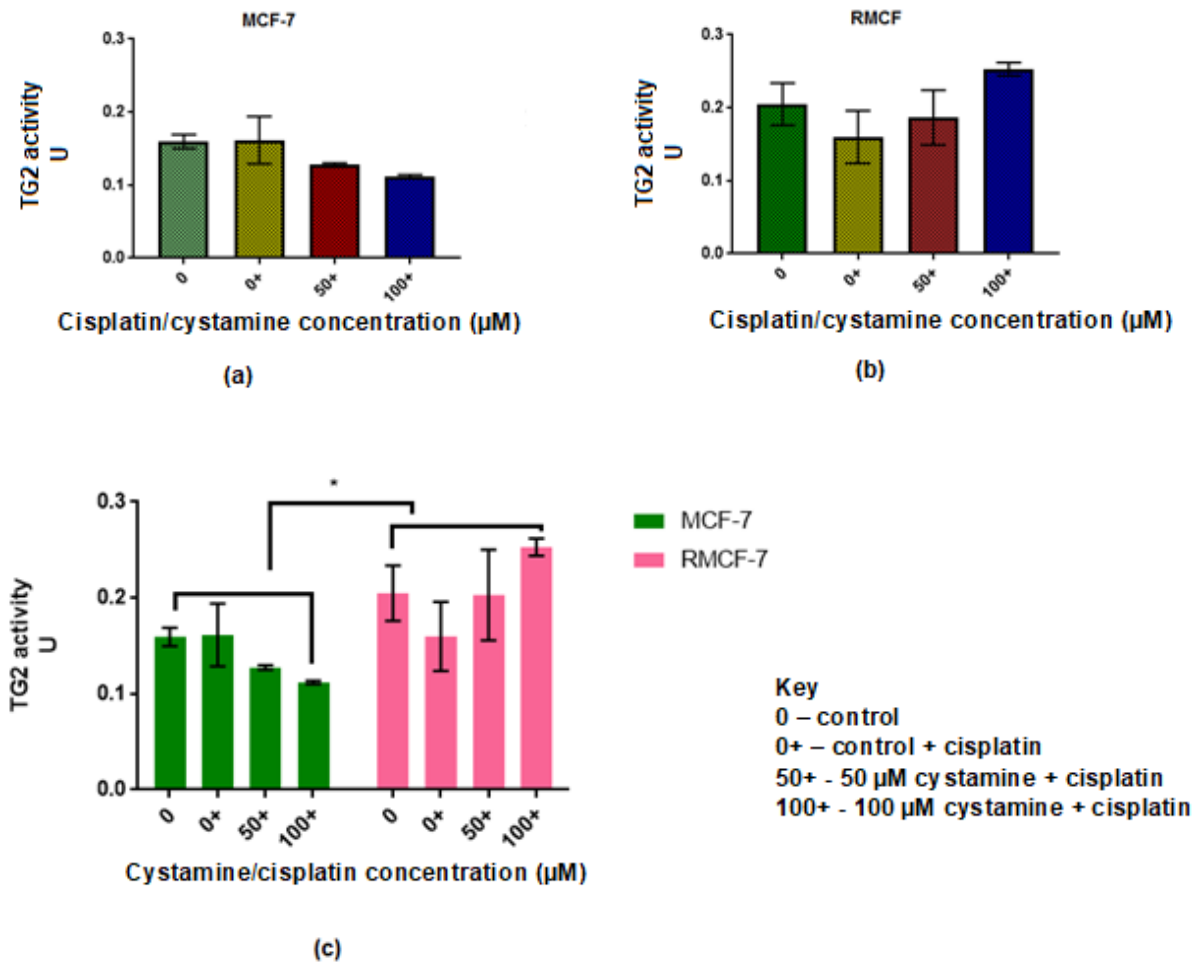

Figure 6.14: Enzyme activity of MCF-7 and RMCF after TG2 inhibition and cisplatin treatment.

TG2 enzymatic activity determined using the TG2 COV assay kit of both wild type and cisplatin-resistant cells after TG2 inhibition. (a) Represents TG2 activity in the wild type cells; (b) depicts activity in the cisplatin-resistant cells while (c) compares TG2 activity between the wild type and cisplatin-resistant cell types. Statistical significance is symbolised by * (0.0305), ** (0.0087). when analysed with the 2 way Anova. The results are computed from at least three independent experiments and presented as means ± SEM.

Generally, from fig 6.14, cystamine treatment generated a minimal reduction of the enzyme activity in the wild type. In contrast, the enzymatic activity was slightly increased (by 20%) in the cisplatin-resistant cells.

6.13 Discussion

The results generated in this chapter confirm previous reports about inhibiting TG2 activity with cystamine, and further reveal that TG2-L is more of the target isoform for inhibition than TG2-S. The inhibition of TG2 with cystamine reduced TG2-L levels substantially when compared to TG2-S (figure 6.8, figure 6.12), which led to the cells becoming sensitive to cisplatin at a reduced cisplatin dose (figures 6.6 and 6.11) than the initial inhibitory concentration of the drug. A limitation of this experiment is the inability to determine the activity of the different isomers with the assay kit.

Chapter 7: TG2 silencing: does the silencing of TG2 protein expression influence resistance reversal?

7.1 Introduction

Gene silencing is the art of 'turning off' a gene by machinery other than genetic mutation (Redberry, G.W., 2006). From the preceding chapter, inhibition with cystamine showed the effect of inhibiting the long-form of TG2 on cisplatin cytotoxicity. In this chapter, small interfering RNA (siRNA) was used to specifically knock-down TG2 to determine if knock-down produces similar results and which isoform will be silenced.

7.2 Effect of lipofectamine on cell viability

The viability of cells exposed to the transfection agent lipofectamine was tested, to rule out deleterious effects associated with using this vehicle for transfection. Cells were seeded in 96 well plates and exposed to the concentration of lipofectamine for the transfection studies (0.3 µl in 100 µl media), in the absence of siRNA.

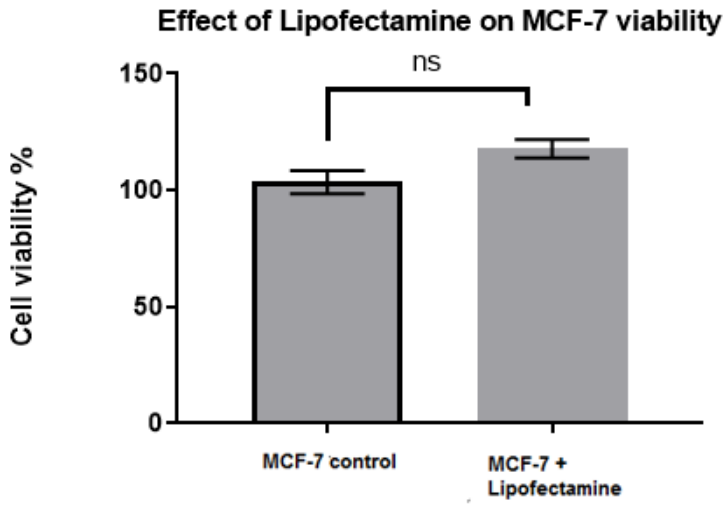

Figure 7.1: Viability of MCF-7 cells after lipofectamine treatment

Cell viability of lipofectamine-treated cells and non-treated control cells was determined with the CCK-8 assay. Results were computed from means of at least three independent experiments (±SEM), and the difference was not statistically significant when analysed with the student T-test.

Figure 7.1 shows that lipofectamine-treated cells are still fully viable at the concentration (0.3 µl in 100 µl media) used for transfection, when compared to the control cells, indicating that the concentration of lipofectamine (Table 2.3) used for transfection was not harmful to cells.

7.3 Flow cytometry analysis of cells treated with lipofectamine

The analysis of cells by flow cytometry was performed to confirm the viability assay in figure 7.1.

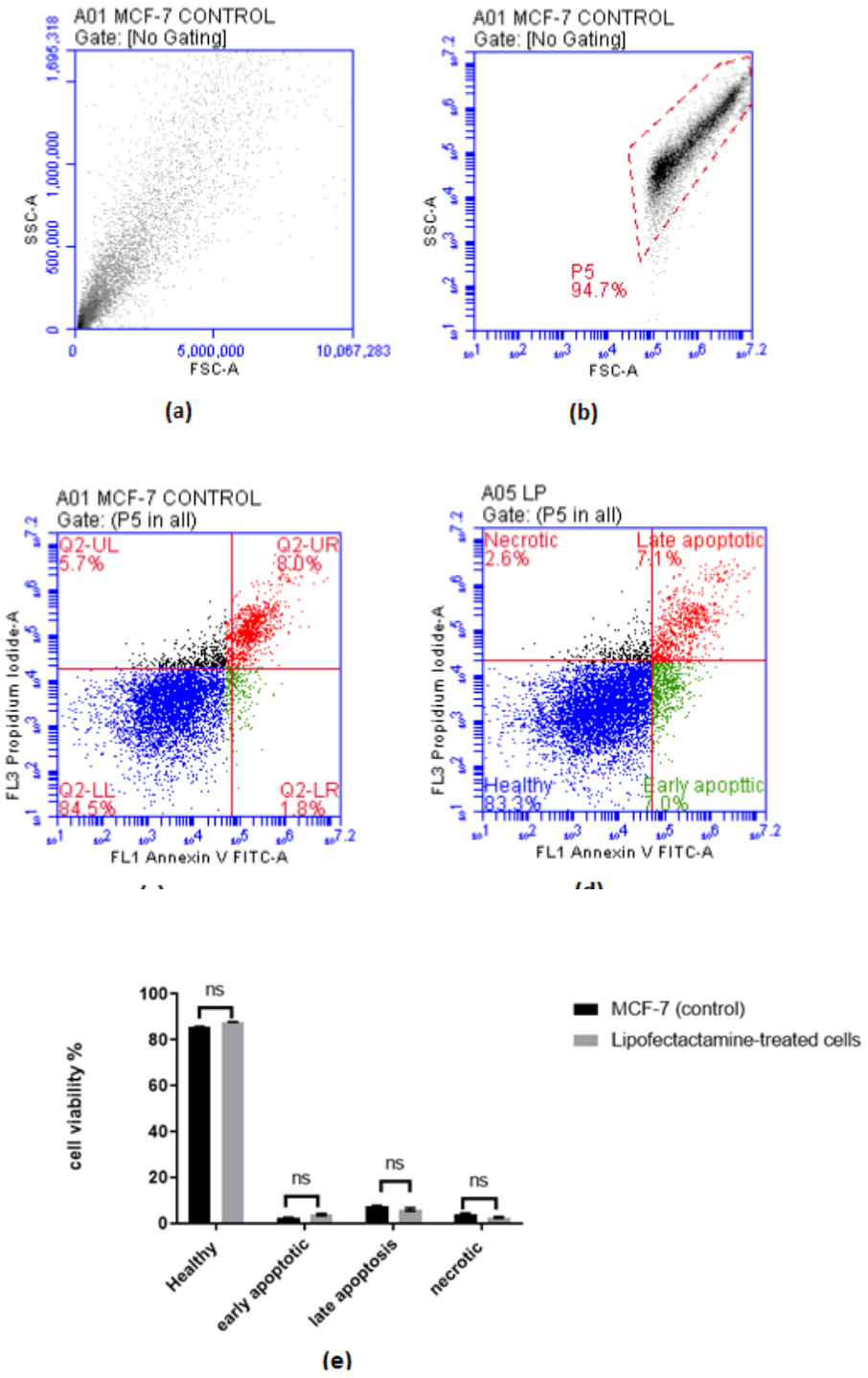

Figure 7.2: Cytometry analysis of lipofectamine-treated cells

Representative cytogram of cells treated with lipofectamine. (a) SSC-A/FSCA to collect single cells, (b) gating from excluding cell debris and doublets. Representative cytogram of control cells (c) MCF-7 control cells, (d) lipofectamine treated cells (e) the histogram illustrated the difference between both groups and were not significant by the 2 way ANOVA test. Data was computed from the means of at least three independent experiments (±SEM).

Results from figure 7.2 support the MTT assay results (fig 7.1), confirming that the concentration of the transfection agent was not harmful to the cells, as over 80% of cells were still healthy after lipofectamine treatment. After the confirmation that cells were not affected by the transfection vehicle, the process of transfecting with specific TG2 siRNA was then performed, as described in section 2.10.

7.4 Characterization of MCF-7 cells after TG2 silencing

Images of cells were taken before and after silencing to compare morphological differences between the treated and non treated cells. Cells were seeded in 6-well plates overnight to allow them to adhere and media was replaced with fresh media with the transfection reagent and TG2-specific siRNA, according to the manufacturer's protocol (see section 2.10)

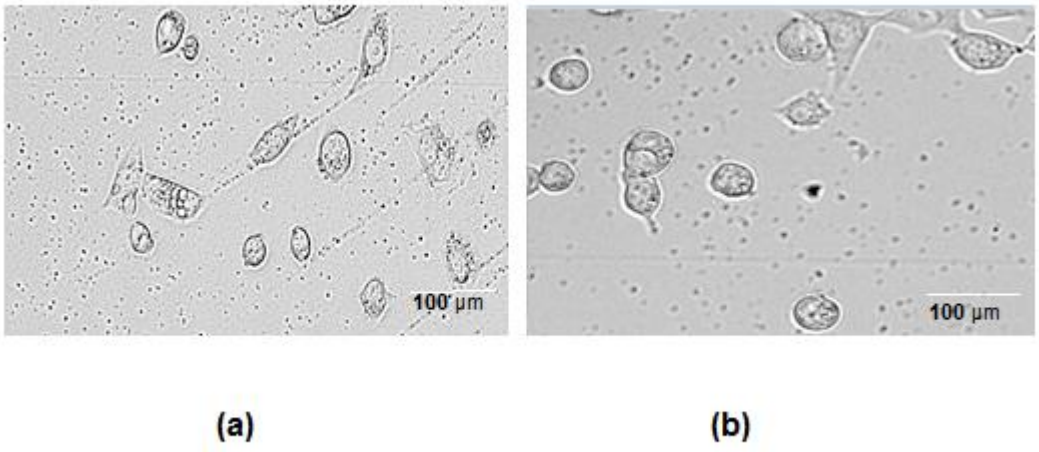

Figure 7.3: Morphology of MCF-7 cells transfected with specific TG2 siRNA

Optical pictogram of (a) MCF-7 control cells (b) TG2-silenced MCF-7 cells. The images were photographed with the ZOE™ Fluorescent Cell Imager. The scale bar was set at 100 µM.

From the image in figure 7.3, there were no significant morphological differences between the transfected cells and control.

7.5 Cell death analysis of cells after TG2 silencing by flow cytometry

After transfection, cells were harvested and prepared for flow cytometry analysis as described in section 2.5 to determine the characteristics and state of cells.

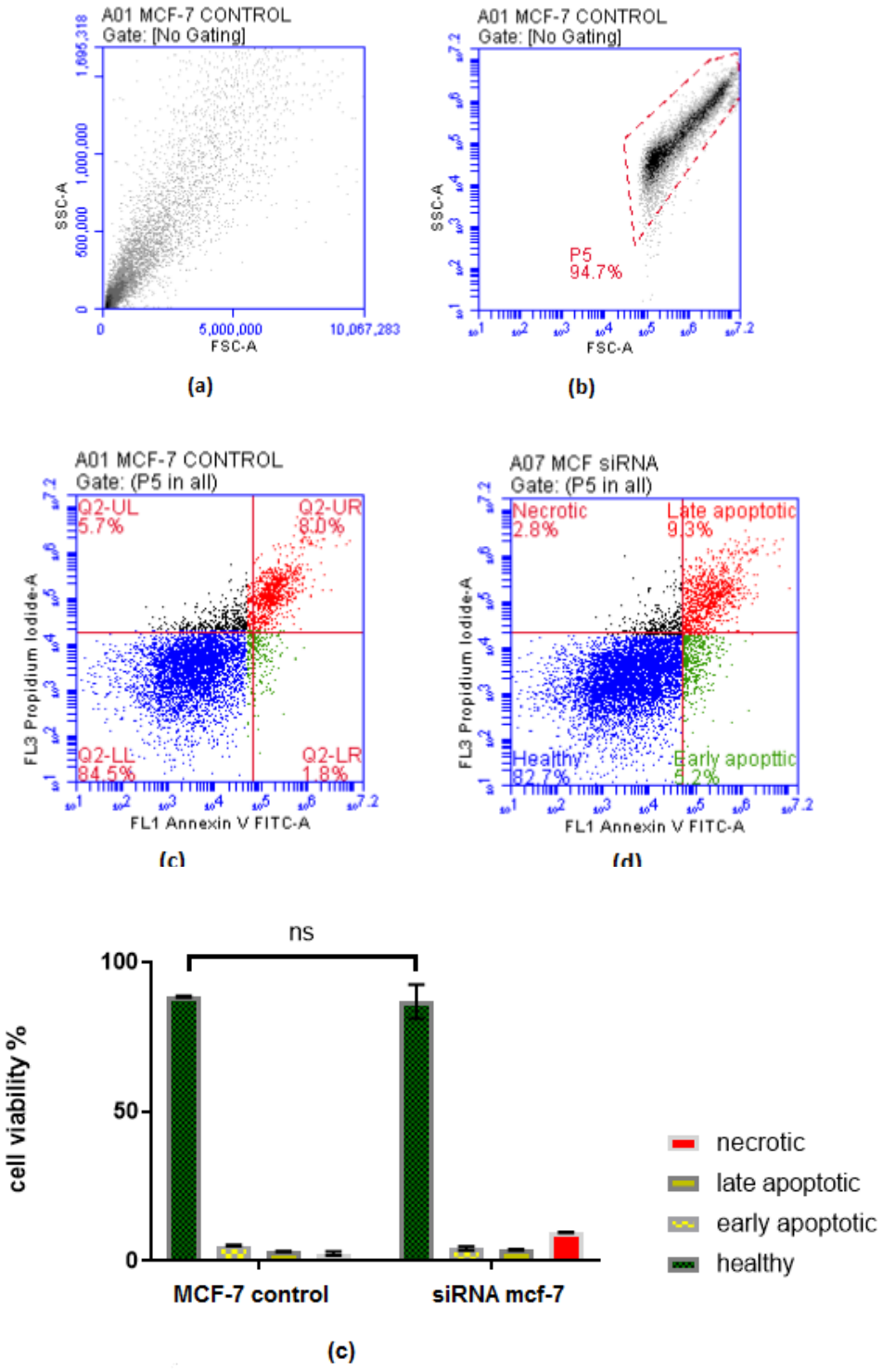

Figure 7.4: Flow cytometry analysis of MCF-7 cells after siRNA transfection

Cell death analysis of cells stained with Annexin V and propidium iodide after transfection. Cytogram of cells treated with lipofectamine. (a) SSC-A/FSC-A to collect single cells, (b) gating to exclude cell debris and doublets. Representative cytogram of control cells (c) represents the control, while figure (d) represents the transfected cells. The histogram (e) shows the populations of cells amongst the different groups. Results are computed from the means of at least three independent experiments (±SEM), and the differences were not statistically significant.

From figure 7.4, following treatment with TG2-specific siRNA, over 80% of cells in both cell groups were in the healthy state. This signifies that cells were still healthy after transfection and confirms that transfection was not detrimental to the healthy state of the cells.

7.6 Determination of TG2 expression after transfection in wild type MCF-7 cells

The expression of TG2 was determined following transfection. 1 x10^5 cells were seeded in a 24-well plate and incubated overnight so cells could attach to plates. Following the protocol (section 2.8), media was then replaced with Opti-MEM medium containing transfection agent with specific anti-TG2 siRNA and incubated for 24 hours. Media was then replaced with fresh media for another 24 hours. At the end of the period, cells were harvested, and protein extracted for Western blotting (section 2.6 and 2.7).

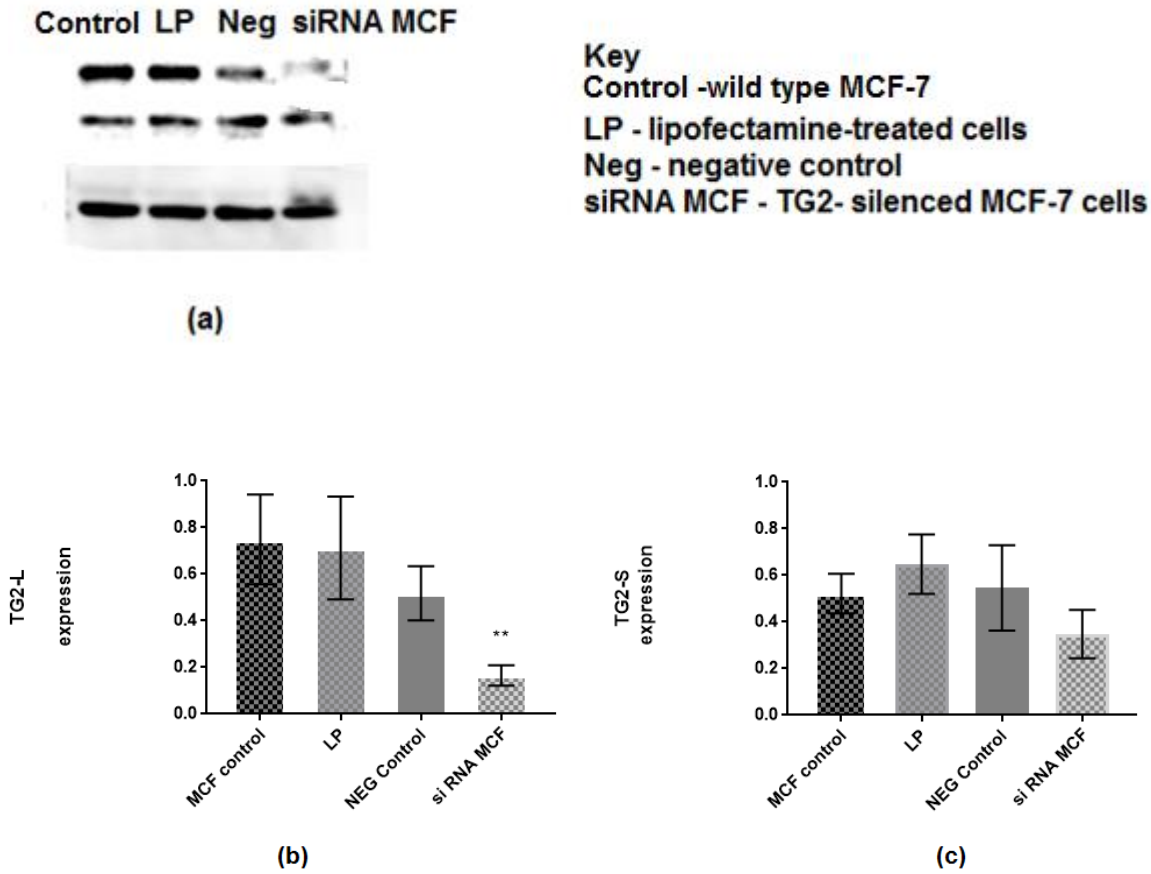

Figure 7.5: Effect of silencing on TG2 levels in wild type MCF-7 cells

Representative Western blot of TG2 levels in wild type MCF-7 cells after transfection (a), The histograms represent band quantification of TG2-L expression (b) and TG2-S expression (c). Normalisation was with β-actin, and results were computed from the mean from at least three independent experiments (±SEM), and significance is denoted by ** (0.0081) when analysed with the 2 way Anova.

Overall, the blot in figure 7.5 shows that transfection was successful. Compared to the controls (W, LP and Neg), TG2-L was silenced more substantially while TG2-S expression seemed to be maintained. The differential expression of the isoform before and after TG2-specific silencing is shown in Table 7.1. The negative control was used to validate the transfection process, but a reduction of TG2-L was noticeable.

Table 7.1: Isoform ratio in wild type MCF-7 cells

Isoforms	Ratio before silencing	Ratio after silencing
TG2-L: TG2-S	4:1	1:3

7.7 Effect of transfection on wild type cells' sensitivity to cisplatin

After silencing TG2 in figure 7.5, the IC_{50} of cisplatin was determined, to compare with the inhibitory concentration obtained in figure 4.1. For this assay, cells were seeded in 96-well plates according to the protocol for transfection in section 2.8. After cells adhered to the plate, the media was replaced with fresh media with a series of increasing concentrations of cisplatin, and the plate was incubated for 24 hours. Cell viability was determined with the CCK-8 assay (section 2.2.1).

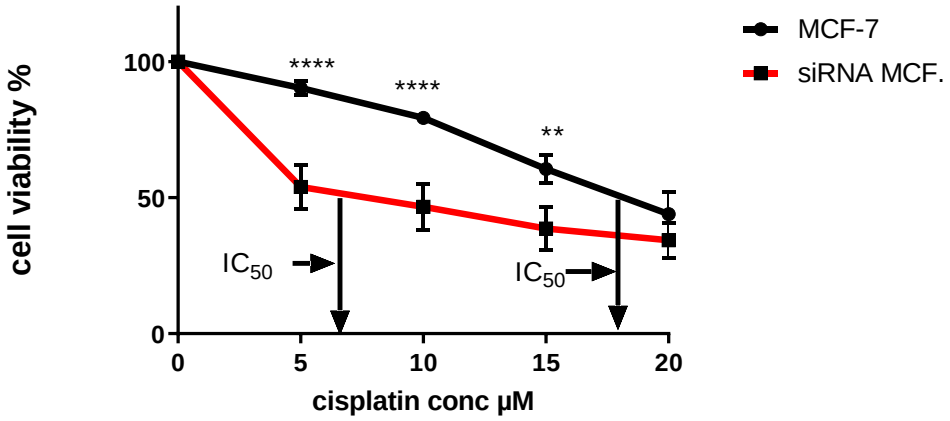

Figure 7.6: Cytotoxicity of cisplatin after transfection with ant-TG2 siRNA on wild type MCF-7 cells.

Cytotoxicity of cisplatin on transfected cells determined using the CCK-8 assay. and results were computed from the mean of at least three independent experiments (±SEM). Statistical significance is denoted by **** (<0.0001) and ** (0.0011).

Figure 7.6 shows that the inhibitory concentration of the transfected cells was lower than that of the control cells. The IC_{50} of the wild type was 18 µM, but after silencing TG2-L, it was reduced to 6 µM, suggesting a relationship between TG2-L and sensitivity of the cells to cisplatin.

7.8 Determination of TG2 activity

The activity before and after the knock-down was also determined by flow cytometry using labelled demonstrate (section 2.9.2). 2 x10^5 wild type MCF-7 cells were seeded into 24-well plates overnight to adhere to the plates. After 24 hours, media was replaced with media containing specific TG2 siRNA and incubated for a further 24 hours. Cells were prepared after transfection as described in section 2.9.2 for flow cytometry analysis. The relative fluorescent units (RFU) values were used as a measure of the activity of the enzyme.

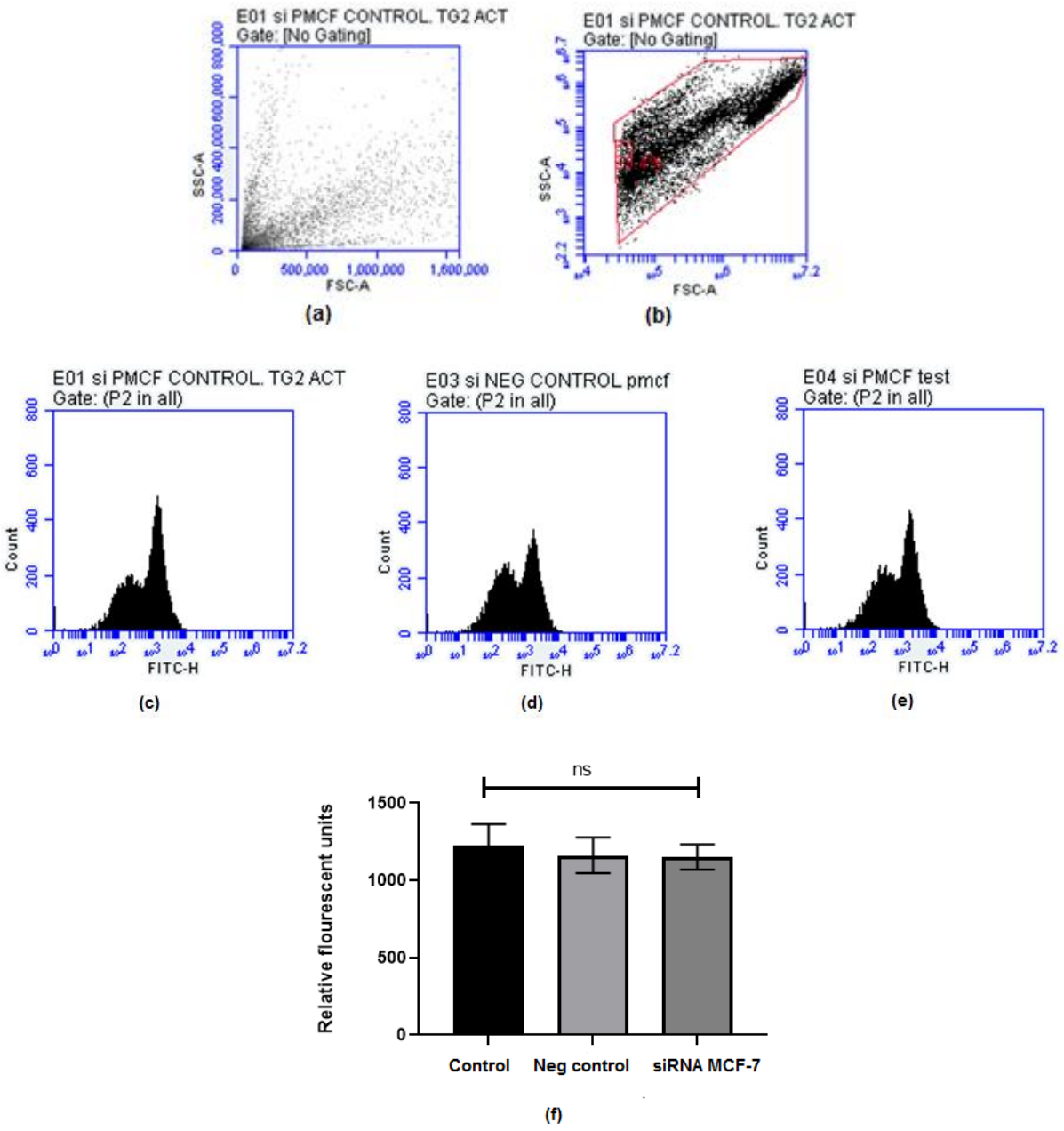

Figure 7.7: Enzyme activity of TG2 siRNA transfected wild type cells

TG2 Enzyme activity in wild type MCF-7 cells after transfection determined by flow cytometry. Cytogram (a) showing all events (b) gating to exclude cell debris and doublets (c) control MCF-7 cells (d) negative cells (e) transfected MCF-7 cells. The results were computed from the mean of at least three independent experiments (±SEM), and the difference was not statistically significant when analysed with the one-way ANOVA.

The cisplatin-resistant cells were also transfected with specific TG2 siRNA for comparison with the wild type cells.

7.9 Apoptosis analysis of cisplatin-resistant cells after TG2 silencing by flow cytometry

The state of the cells was assessed to ensure cells were healthy after transfection and cell death was due to the silencing of the TG2 gene.

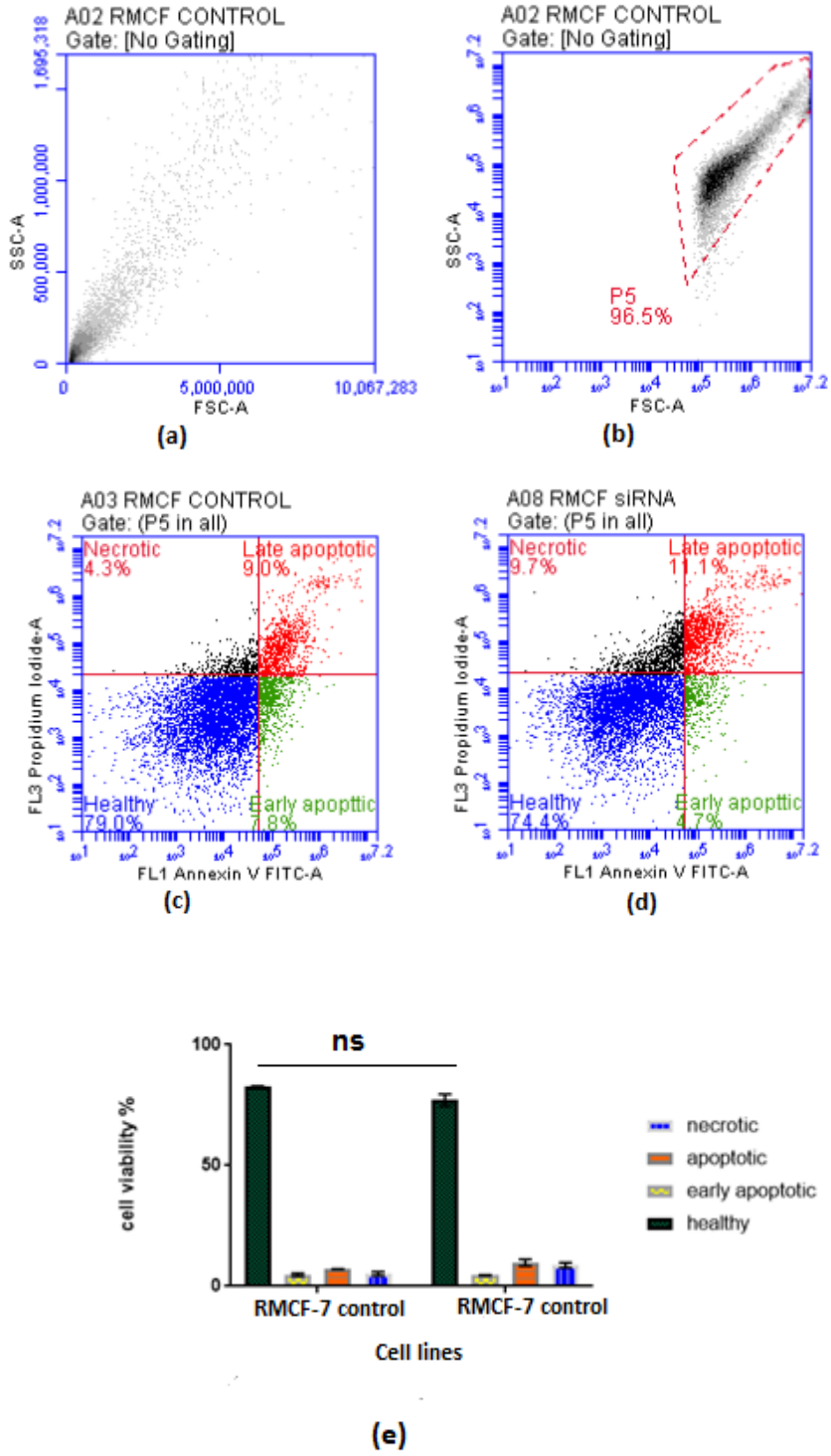

Figure 7.8: Flow cytometry analysis of cisplatin-resistant cells after transfection with TG2 specific siRNA

Cell death analysis by flow cytometry after transfection. (a) SSC-A/FSCA to collect single cells, (b) gating to exclude cell debris and doublets. (a) representative cytogram of RMCF-7 control cells and (b) RMCF-7 cells after transfection with TG2 specific siRNA. The histogram (c) shows that the populations of cells amongst the different groups was not statistically significant when analysed with the 2 way Anova. Data were computed from the mean of at least three independent experiments (±SEM).

Figure 7.8a characterises the RMCF-7 cells into the healthy, early apoptotic, late apoptotic and necrotic categories, while figure 7.8b shows the equivalent characterisation for RMCF-7 cells treated with TG2 specific siRNA. Differences observed between the transfected cells and control was not significant. This result confirms that the chemoresistant cells were still healthy following transfection with TG2 specific siRNA.

7.10 Effect of silencing TG2 on the sensitivity of the cisplatin-resistant cells to cisplatin

The sensitivity of the transfected cisplatin-resistant cells to cisplatin was also investigated. Briefly, cells were seeded in a 96-well plate, transfection was carried out (see section 2.8), and then cells were exposed to a series of increasing cisplatin concentrations (0-40 µM).

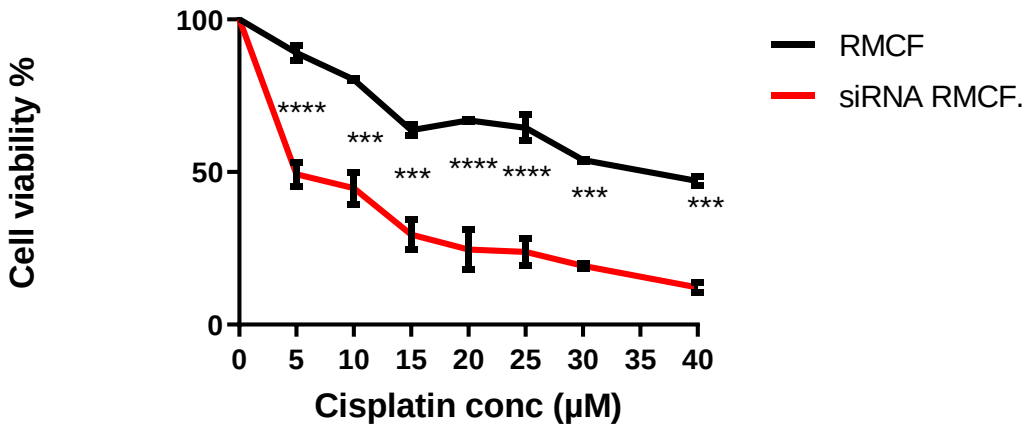

Figure 7.9: Cytotoxicity of cisplatin after transfection with TG2 specific siRNA on the resistant cells.

Cytotoxicity of cisplatin on TG2 siRNA transfected resistant cells determined using the CCK-8 assay. Data were computed from the mean of at least three independent experiments (±SEM), and **** denotes significance (<0.0001) and *** (0.0002).

The result from figure 7.9 shows the sensitivity of the chemoresistant cells to cisplatin after TG2 knockdown was significantly increased, and conforms to the pattern observed in the wild type cells (figure 7.6). The graph shows that the inhibitory concentration of the transfected cells was 5 µM, while the IC_{50} of the control was 35 µM. The same argument can be sustained

that the sensitivity of the cells to cisplatin could be due to the reduction of the TG2-L gene, which encourages cell survival.

7.11 Determination of TG2 expression following gene silencing in cisplatin-resistant MCF-7 cells

Cisplatin-resistant cells were seeded in 24-well plates and transfected with the same specific TG2 siRNA used in the wild type cells. Protein was extracted and TG2 expression determined by Western blotting.

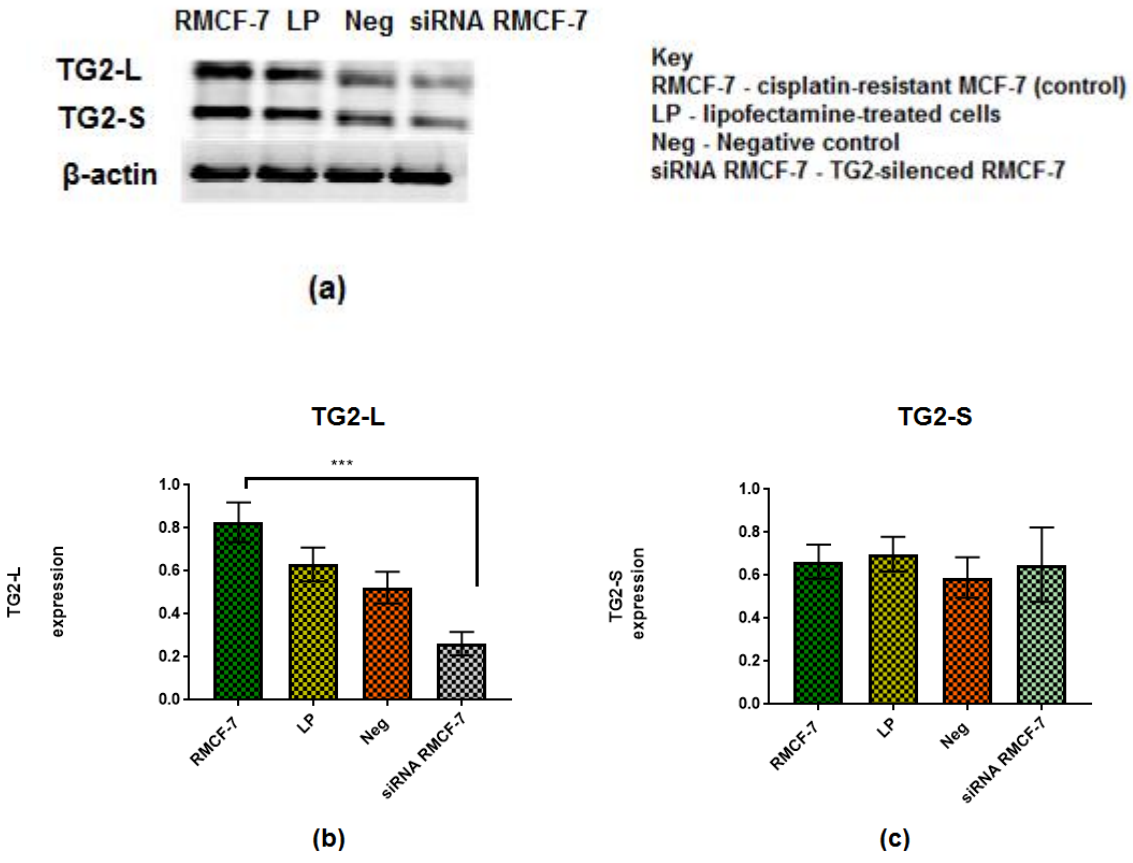

Figure 7.10: Effect of TG2 silencing on TG2 levels in cisplatin-resistant cells

Representative Western blot of cisplatin-resistant cells after TG2 silencing (a), the histograms represent band quantification of TG2-L (b) and TG2-S (c). The results were computed from the mean of at least three independent experiments (±SEM), and significance is denoted *** (0.0005) when analysed with the 2-way Anova

The Western blot in figure 7.10 shows that transfection was also successful in the chemoresistant cell. A reduction was observed in TG2-L expression in the cells silenced with specific TG2 siRNA was noticed. Isoform analysis shows a significant difference between the reduction in TG2-L levels in the RMCF-7 control and the transfected cells, while the reduction in TG2-S levels was not significant. The reduction of the isoform ratio with and without TG2 specific siRNA silencing is shown in Table 7.2. Knocking-down, a large percentage of the long-form may have allowed the short form to exert its function, stimulating cell death, as seen in figure 7.9.

Table 7.2: Isoform ratio in cisplatin-resistant MCF-7 cells

Isoforms	**Ratio without TG2 silencing**	**Ratio with TG2 silencing**
TG2-L: TG2-S	1.2:1	1:3

7.12 Determination of TG2 activity after TG2-silencing in the cisplatin-resistant cells

The enzyme activity with and without TG2 knock-down was determined by flow cytometry, using labelled dansylcadaverine (section 2.29,2). Briefly, 2 x10^5 RMCF cells were seeded into 24-well plates overnight to adhere to the plates. After 24 hours, media was replaced with media containing specific TG2-siRNA and cells were incubated for a further 24 hours. Cells were prepared after transfection as described in section 2.7.2, for flow cytometry analysis. The relative fluorescent units (RFU) values were used as a measure of the activity of the enzyme.

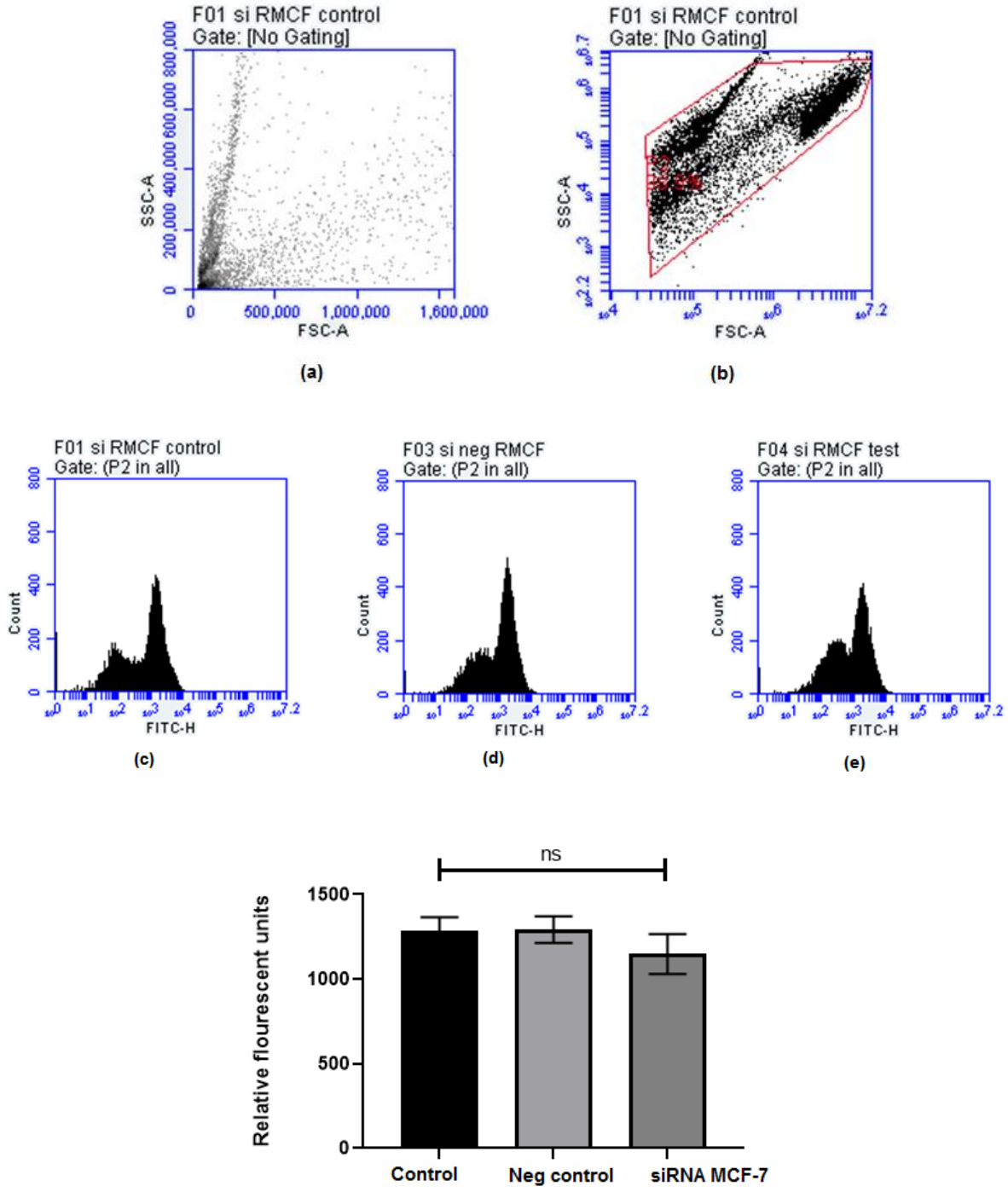

Figure 7.11: TG2 enzyme activity in RMCF

TG2 enzyme activity of transfected cells determined by flow cytometry in cisplatin-resistant cells. Cytogram (a) showing all events (b) gating to exclude cell debris and doublets (c) control RMCF-7 cells (d) negative control and (e) transfected RMCF-7 cells. Data were computed from the mean of at least three independent experiments. The results were computed from the mean of at least three independent experiments (±SEM), and the difference was not statistically significant when analysed with the one-way ANOVA.

Figure 7.11 shows a reduction of enzyme activity after transfection with TG2.specific siRNA. This method is nonspecific and does not indicate the activity of TG2-L or TG2-S separately, but the total activity of the enzyme. The reduction of activity could be due to the knockdown of the long-form, leaving the short-form active in the cells.

7.13 Conclusion

Overall after transfection, there is a marked reduction of TG2-L compared to TG2-S, which tipped the scales in favour of apoptosis and cell death. Silencing of the TG2 gene has shown TG2-L knockdown sensitises cells to cisplatin at lower concentrations. The result validates the inference that the proportion of TG2-L in the cell dictates the function of the protein.

Chapter 8:Comparison of tissue transglutaminase levels in MCF-7 and HCC 1806 cells

8.1 Introduction

The results generated in this study have shown the roles played by the different isoforms with regards to cisplatin resistance. This chapter determines and compares the levels of TG2-L and TG2-S in MCF-7 cells with those in a triple-negative breast cancer cell line (HCC 1806).

8.2 Determination of TG2 levels in MCF-7 and HCC 1806

Protein was extracted from equal number of cells (5×10^5) grown in a 6-well plate, as described in section 2.6 and 50 µg of whole-cell lysate was then analysed for TG2 expression by Western blotting (section 2.7).

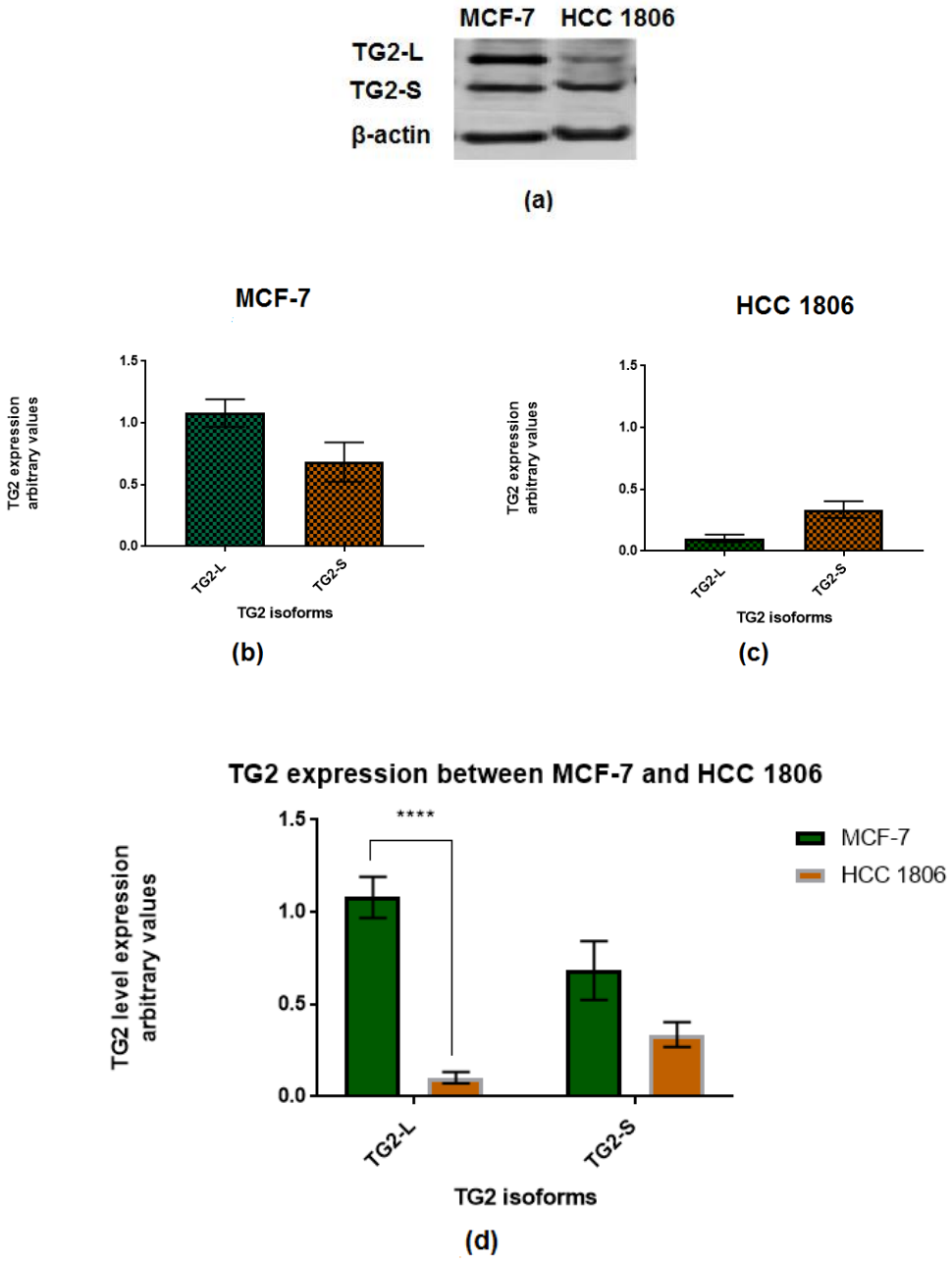

Figure 8.1: Comparison of TG2 levels in MCF-7 and HCC 1806 breast cancer cells.

Representative Western blot of TG2 expression in MCF-7 and HCC 1806 cells, β-actin was used as the loading control and for normalisation (a). The histograms represent band quantification of (b) MCF-7 cells, (c) HCC1806 cells and (d) compares TG2 expression between both cell lines. The results were computed from the mean of at least three independent experiments (±SEM), and significance is denoted by ****(<0.0001).

The Western blot in figure 8.1 generally reveals a characteristic difference in TG2 expression between the two different cell lines. The difference is observed in TG2-L levels, which are notably decreased in the HCC 1806 cells but increased in MCF-7 cells while TG2-S levels are similar in both cell lines.

8.3 Determination of enzyme activity

The activity of TG2 transamidation activity in the two cell lines was compared to determine the presence of a relationship between TG2 expression (figure 8.1) and activity. The activity was determined by flow cytometry using labelled dansylcadaverine (section 2.9.2). 5 x 10^5 cells were grown in a 6-well plate overnight to adhere to the plate after 24 hours media was replaced with fresh media containing labelled monodansylcardavarine for a further 24 hours. After incubation, cells were harvested for fluorescent detection by flow cytometry. The relative fluorescent units (RFU) value were used as a measure of the activity of the enzyme.

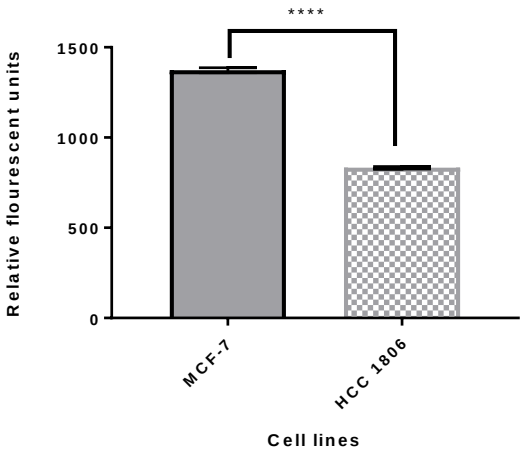

Figure 8.2: Enzymatic activity of MCF-7 and HCC 1806 cells.

TG2 enzyme activity determined with fluorescent dansylcadaverine by flow cytometry. The results were computed from the mean of at least three independent experiments (±SEM), and significance is denoted by ****(<0.0001).

The graph shows the increased activity of the enzyme in the MCF-7 cells when compared to the HCC 1806 cells which suggest that the functions of TG2-L in MCF-7 cells may be more profound than HCC 1806 cells.

8.3 Discussion

Results from this chapter have shown for the first time that the HCC 1806 cells also express TG2 and also shows the differential expression levels of isoforms. In comparison with MCF-7 cells, TG2-L levels in HCC 1806 is 50% less than that found in MCF-7 cells while TG2-S levels are higher in HCC 1805 than MCF-7 cells. Reduced TG2-L levels in HCC 1806 might make these cells more sensitive to chemotherapeutic drugs. Previous reports (Table 8.1) shows that HCC 1806 cells are sensitive to cisplatin at lower concentrations.

Table 8.1: Different responses of triple-negative breast cancer cells to cisplatin.

Triple-negative cell type	IC_{50}	Author
HCC 1806	2.5 µM	Volk-Draper,2012
HCC 1806	0.56 µM	Pendleton, 2014
HCC 70	2.3 µM	Heijink *et al*., 2019
HCC 1937	2.2 µM	Heijink *et al*., 2019
HCC 38	3.6 µM	Heijink *et al*., 2019

Chapter 9 Discussion and future work

9.1 Introduction

Cancer research scientists, in conjunction with pharmaceutical companies, have slowly progressed clinical therapies with the eventual hope of overcoming this group of specific diseases. There has been substantial progress, and many key molecular mechanisms that contribute to the underlying genesis of cancer cells have been substantially identified, leading to the development of several varieties of anti-cancer drugs that can specifically target molecular cancer biomarkers. Although these drugs have demonstrated some capacity and raised expectations of someday completely defeating cancer, the problem of chemotherapeutic resistance, however, is still a hurdle that has yet to be overcome.

Cisplatin which was discovered in 1965 and referred to as the 'penicillin of cancer', has been extensively used in many aspects of chemotherapy (section 1.18.1). It can be described as a 'first-generation' platinum drug that now falls into the category of chemotherapeutic drugs that have limited use owing to their ability to cause often rapid development of chemoresistance in patients. Apart from chemoresistance, another primary reason limiting the use of cisplatin is its toxicity, which includes: gastrotoxicity; ototoxicity; allergic reactions; and myelosuppression (Hartmann *et al*., 2003). Despite efforts over recent decades to reduce its toxicity, to date, these efforts have not been particularly successful (Manohar and Leung, 2018).

Currently, although cisplatin alone has not proved to be a particularly effective treatment for hormone-positive (ER+ve) breast cancer, it has found utility in combination with other anti-cancer drugs, particularly as adjuvant chemotherapy for the treatment of hormone negative (ER-ve) cancers (Goel *et al*., 2010; Koshy *et al*., 2010; Wahba *et al*., 2015), including metastatic breast cancer, locally advanced breast cancer and primary breast cancer (section 1.18.1). It is often combined with other drugs such as gemcitabine (Heinemann, 2002; Heinemann *et al*., 2006), docetaxel (Vassilomanolakis *et al*., 2005; Cai *et al*., 2018), or 5-Fluorouracil (Kim *et al*., 1993; Lekakis *et al*., 2012) to accomplish the maximum effect, but

chemoresistance is a perpetual challenge and remains a significant obstacle towards achieving this goal.

Thus, cisplatin is a good powerful drug with many advantages but some serious disadvantages. It is readily available, relatively inexpensive and appears that it may be a suitable drug for repurposing in order to overcome the challenge of toxicity and chemoresistance. If this could be achieved, then it might conceivably bring cisplatin back to its place as the 'penicillin of cancer' and back into general use.

9.2 Increased TG2 expression reflects chemoresistance in MCF-7 cells

It is now well established that TG2 can exert opposing effects in cells (Section 1.10.1.), modulating both cell proliferation and apoptosis functions in neuroblastoma cells (Tee et al., 2010) and fibroblast cells (Antonyak et al., 2006). Its overexpression is often associated with increased chemoresistance (Mehta, K., 1994), and this observation is consistent with the results of this study, which shows that the modulation of TG2 expression had a significant effect on ER+ve cells' response to cisplatin. For example, while the IC_{50} of parental MCF-7 cells to cisplatin was 18 µM (figure 4.1), induction of TG2 with retinoic acid resulted in cells that became more resistant to cisplatin (figures 5.6 and 5.7), similar responses being seen in the chemoresistant cells (figure 5.9) with IC_{50} levels rising higher than 35 µM(IC_{50} levels of resistant cells). Also, the inhibition of TG2 activity with cystamine reduced the IC_{50} from 18 µM to 5 µM in the wild-type cells (figures 6.4, Table 6.1) and from 35 µMto 20 µMin the cisplatin-resistant cells (figure 6.11). However, it was not self-evident from these observations whether TG2-S and TG2-L isoforms increase or decrease their expression in parallel during these processes or whether there is differential expression of isoforms that contributes towards the overall gross TG2-associated chemoresistant effect. Thus, a major aim of this project was to investigate this question.

9.3 TG2-L modulation and its effect on MCF-7 sensitivity to cisplatin

The results of this study on breast cancer cells generally reflect the pattern of TG2 induction by RA as shown in earlier studies, using diverse cancer cell lines, including liver cancer (Meshram et al., 2017), cervical cancer and prostate cancer cells (Cho *et al*., 2016). Furthermore, the data from the current study demonstrate for the first time that the specific isoform induced by retinoic acid is predominantly the long form (TG2-L) and that this is often accompanied by a reduction of expression of the short isoform (TG2-S). Also, inhibition with cystamine and silencing with TG2-specific siRNA reduced TG2-L expression significantly more than that of TG2-S, causing cells to become more sensitive to cisplatin at lower concentrations (figure 6.5, figure 7.6).

Although cisplatin therapy has not been particularly effective in the treatment of patients with hormone-positive breast cancer, the results on MCF-7 cell lines suggest that modulation of TG2 isoform levels might represent a new route to therapy. However, the picture may be somewhat more complicated than the simple scenario of reducing TG2-L levels in breast cancer cells in order to sensitise them to cisplatin therapy.

9.4 TG2 levels relationship to patient survival is dependent on the hormone status of patients

Recent results obtained within our local Cancer Research Group (CRG) that have analysed microarray biopsy samples obtained from 1,942 breast cancer patients (fig 9.1) shows that ER-ve patients expressing higher TG2 levels have a higher survival rate than ER+ patients expressing higher TG2 levels (Blows, 2019; unpublished personal communication).

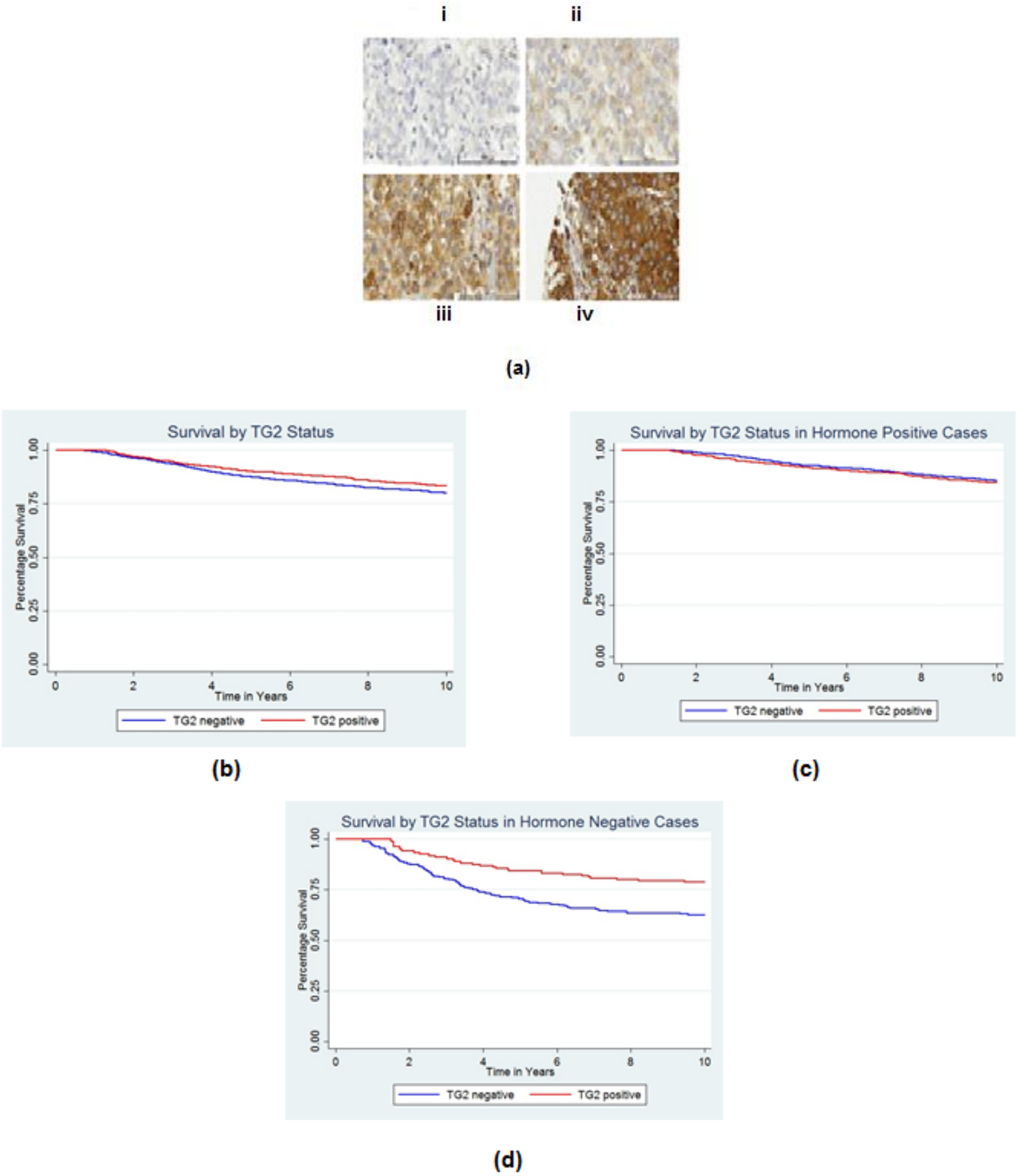

Figure 9.1: Tissue microarrays of TG2 expression in breast cancer tumours.

(a)Invasive breast tumour tissue displaying (i) control (ii) weak (iii) moderate (iv) strong TG2 expression (b) Survival curves of patients with invasive breast tumours that do and do not express TG2 (c) Survival curves of patients with ER+ive invasive breast tumours that do and do not express TG2 (d) Survival curves of patients with ER-ve invasive breast tumours that do and do not express TG2. Red lines are TG2 positive and blue lines are TG2 negative (Fiona Blows, 2019 - personal communication).

However, this histological analysis is not able to discriminate which isoform contribute to the observed effect, or why this might be relevant.

9.5 Oestrogen positive status increases chemoresistance in breast cancers

ERα, a subtype of oestrogen receptor(section 1.2.1), plays an essential role in the sensitivity of breast cancer cells to chemotherapy (Borras *et al*., 1990; Teixerra *et al*.,1995; Lee *et al*., 2005). Previous studies have shown that ERα+ve breast cancer is less sensitive to chemotherapy than ERα-ve breast cancer (Berry *et al*., 2006; Wang *et al*., 2009). This relationship between hormone positivity and chemoresistance is understandable because earlier studies have shown that oestrogen encourages proliferation and metastasis through ER*α* extranuclear signalling (Saha and Vadlamudi 2012) by promoting mitogen-activated protein kinase (MAPK), protein kinase C pathways and phosphatidylinositol 3-kinase (PI3K) in the cytosol (Chakravarty et al., 2010)

These signalling pathways mediate its oncogenic and metastasic effects in breast cancer (Saha and Vadlamudi 2012; Lipovka and Konhilas, 2016), and non-small lung cancer cells (Fan *et al*., 2017). The principal cause of death for breast cancer patients is not primarily cancer itself, but metastasis and formation of aggressive secondary cancers that are resistant to conventional cancer therapy (Fidler *et al*., 2007). Also, metastasis is associated with treatment failure that accompanies drug resistance (Qian *et al*.,2017).

Like increased oestrogen receptor expression, increased TG2 expression has been shown to encourage metastasis (Chhabra *et al*., 2009; Kumar *et al*., 2010; Cellura *et al*.,2015; Tabolacci *et al*., 2019), and also demonstrated to be involved with fibronectin and integrins, which aids adhesion (Akimov *et al*., 2000; Balklava et al., 2002). Seo *et al*. (2019) further demonstrated that increased TG2 levels induce epithelial-mesenchymal transition (EMT) by inhibiting miR-205. Particularly interesting, the TG2 gene has also been demonstrated to be regulated and induced by oestrogen and oestrogen receptors (Lally, 2010). Thus, the oestrogen/TG2 promotion of metastasis is expected to be higher in ER+ve breast cancers than in ER-ve breast cancers, although hormone-negative cancers are, broadly speaking, more aggressive and have a poorer overall survival rate than hormone-positive cancers (Li *et al*., 2017), probably owing to the multiple problems of inadequate DNA repair.

9.6 TG2 expression increases patient survival in patients with ER-ve breast cancer

By reference to the preceding section (section 9.5), the synergy between oestrogen receptor signalling and TG2 expression would be predicted to result in reduced or substantially reduced TG2 expression in hormone-negative cancers. The comparative Western blot analysis from the current study (figure 8.1) appears to confirm this and shows that the MCF-7 cell line (ER+ve) expresses higher TG2-L levels than the ER-ve HCC 1806 cell line. This observation would be consistent with lower rates of TG2-driven metastasis in hormone-negative cancers, although being a more aggressive form of cancer; other factors are expected to drive disease development. However, further explanation is needed in order to explain why increased levels of TG2 in ER-ve cancers might increase patient survival compared to low TG2/ER-ve patients.

9.7 A possible mechanism

One possible explanation of why increased TG2 levels might increase survival levels in ER-ve patients may relate to TG2's established involvement with fibronectin and integrins. The data from this study has demonstrated that the predominant isoform induced during RA-mediated TG2 induction is TG2-L. Thus, elevated TG2-L levels in ER-ve breast cancer may promote cellular adhesion, where its crosslinking activity might 'trap' cells preventing cells from migrating to other organs. Reduced metastasis in ER-ve patients, thus is advantageous with increased survival outcomes. In contrast, ER+ve breast cancer patients with elevated TG2 levels will be prone to metastasis more significantly than ER-ve breast cancer patients because the hormone oestrogen, encourages metastasis and also induces TG2 (figure 9.2). However, as TG2 also encourages adhesion, any contribution of increased TG2 adhesion to ER+ve patients cells might be reduced owing to the promotion of metastasis by oestrogen. Assuming the primary cancer were unable to break away from its primary location.

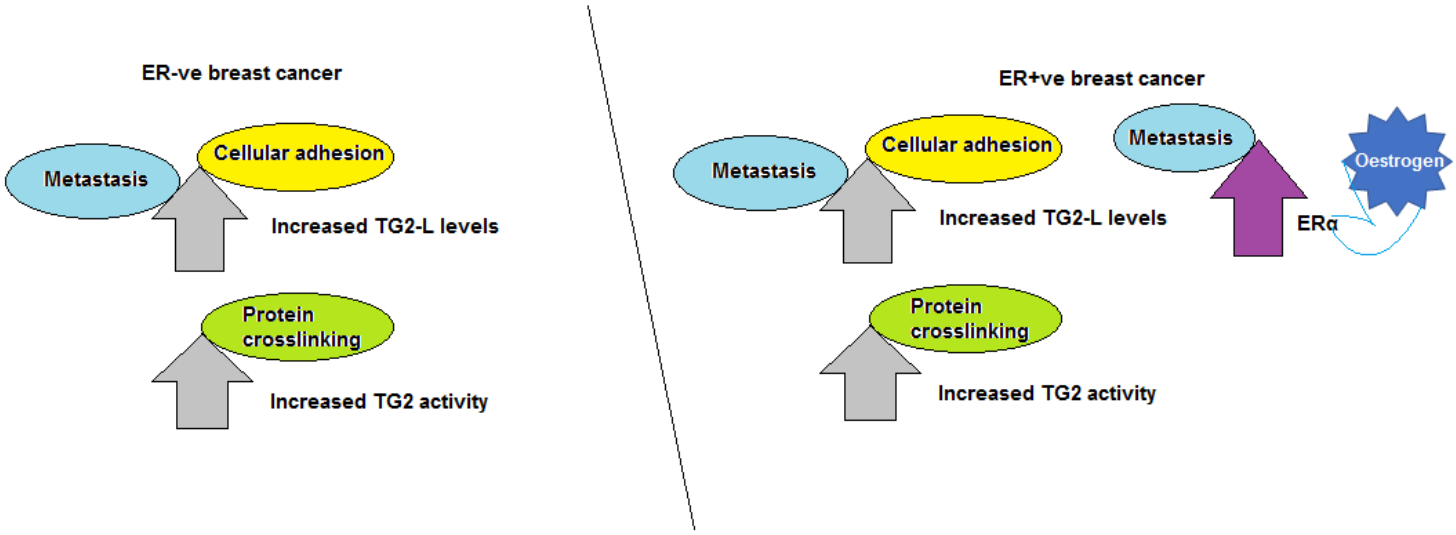

Figure 9.2: Schematic diagram comparing the promotion of metastasis in ER-ve and ER+ve breast cancer patients

TG2-L promotes both metastasis, and cellular adhesion in ER+ve and ER-ve patients, but the presence of ERα increases metastasis in ER+ve patients.

9.8 Proposed hypothesis

Collating the data generated from this study and previous reports, the hypothetical model proposed here suggests that TG2-L and oestrogen activate the PI3k/Akt signalling pathways resulting in the inhibition of Ikβα (a cellular protein that inhibits NF-kβ by keeping it in an inactive state in the cytosol). Therefore, NF-kβ becomes unrestricted and migrates into the nucleus where it activates the expression of several genes involved in inflammation and cell survival, thus promoting resistance. Also, inhibition of TG2 enzyme activity with cystamine reduces TG2-L levels in the cytosol, which then results in the translocation of the membrane-bound TG2-S into the cytoplasm to exert its apoptotic function by activating pro-apoptotic signals. This suggests a TG2-L-TG2-S equilibrium; a shift with the reduction of TG2-L may disrupt the normal equilibrium leading to the translocation of TG2-S into the cytoplasm.

Depending on its GTP-binding or transamidation activity, TG2-L can differentially promote cellular adhesion by interacting with ECM proteins (fibronectin and integrins) or promote metastasis by inducing EMT. The hypothetical model suggests that elevated TG2-L levels with both GTP-binding and transamidation activity interacts with fibronectin and integrins promoting adhesion of cells. In ER+ve breast cancer patients, the model suggests that the GTP-binding activity of TG2-L is promoted by oestrogen, and increased TG2-L levels inhibit miR-205, which elevates ZEB1 protein, inducing EMT. Thus, the induction of EMT encourages the migration of cells. The lack of oestrogen receptors and oestrogen could be suggested to be responsible for the difference between the survival rates seen in ER+ve and ER-ve breast cancer.

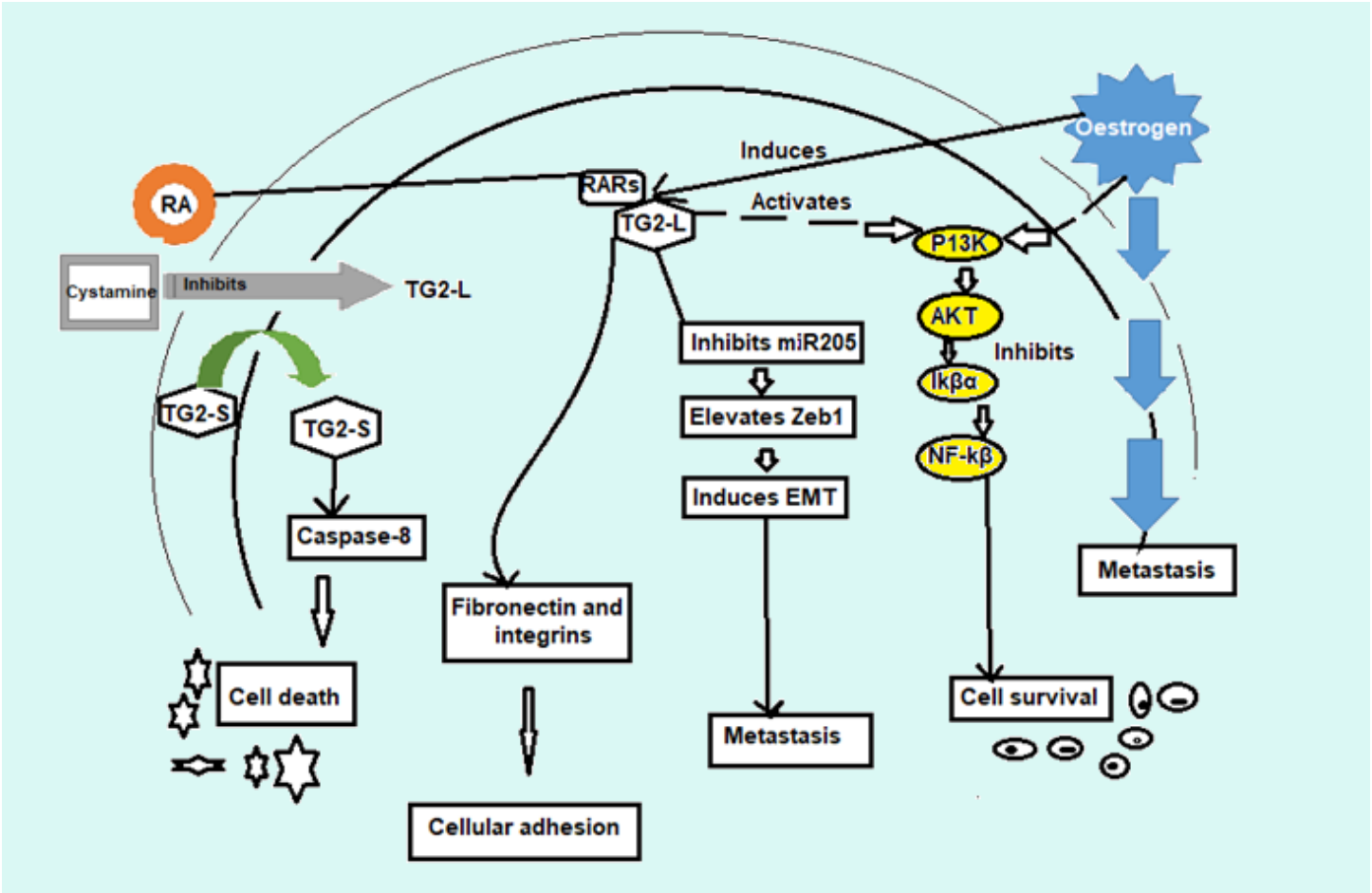

Figure 9.3: Schematic representation of the proposed role of TG2-L in mediating resistance, cellular adhesion and metastasis.

9.9 Future laboratory work:

TG2 is known to be involved in chemoresistance, and this study has provided evidence that elevated TG2-L, and not TG2-S levels, contribute to the development of chemoresistance in an MCF-7 model. Also, unpublished work from our group on the hepatocellular carcinoma HepG2 cell line suggests that the cellular location of the TG2-L and TG2-S isoforms also appears to be linked with their functions. The proportion of TG2-L and TG2-S in the cytoplasm and membrane fraction is yet to be determined in MCF-7 cells. Such knowledge will clarify the roles of the isoforms in cellular migration and adhesion.

As a part of future laboratory work,

1. Cisplatin-resistant HCC1806 (ER-ve) cell lines will be developed, and their expression patterns of the TG2 isoforms will be compared with those seen in MCF-7 cell (ER+ve) lines following retinoic acid, cystamine, and siRNA treatments, as described in this thesis.
2. With regard to the involvement of the isoforms in cellular migration and/or adhesion, the wound-healing assay (scratch assay) will be undertaken following parallel treatments described in 1. The result will provide evidence to show which TG2 isoform (s) are involved in the migration of cells.
3. The proportions of TG2-L and TG2-S in the cytosol and membrane fractions will be determined in both wild-type and cisplatin-resistant MCF-7 (ER+ve) and HCC1806 (ER-ve) cells following retinoic acid, cystamine, and siRNA treatment, as previously described in 1. The result will provide evidence of the location of the isoforms before and after the development of resistance, and whether TG2-S translocates from a membrane-associated pool into the cytoplasm in breast cancer cells after cisplatin treatment, inducing cell death by apoptosis such as is seen in the hepatocarcinoma model (Meshram Dipak, 2019 -personal communication).
4. Further histochemistry work on patient tissue biopsy samples using antibodies specific for TG-2-L and TG2-S will be undertaken. This will confirm whether one or both

isoforms of TG2 are elevated in ER-ve breast cancer patients with high survival rates. As the antibody used in the current study was not isoform-specific, new antibodies will need to be sourced for this work.

5. Further studies are also required to investigate the modulation of TG2 by oestrogen to determine its effects on TG2 isoform expression and cellular proliferation. This work will effectively require the repetition of the experiments in (1) following pre-treatment of cell lines with a biologically significant range of oestrogen concentrations.
6. The levels of expression of P13K and NF-kβ will be tested by Western blot analysis to develop the current hypothesis concerning TG2's control of cell death and metastasis in relation to chemoresistance.
7. Comparing the inhibition pattern of cystamine with other specific TG2 inhibitors which are now commercially available would improve the quality of experimental data based on cystamine inhibition of the enzyme.
8. Finally, TG2 crosslinking interaction analysis to investigate the phenomenon in both ER+ve and ER-ve cells may provide evidence on the survival outcome of breast cancer patients expressing increased TG2 levels.

9.10 Conclusion

Collectively, the results of this study demonstrate that induction of TG2-L by RA may contribute to the development of cisplatin chemoresistance, accompanied by a reduction of expression of TG2-S in an MCF-7 model system. Thus, TG2-L could potentially be a target for chemotherapeutic therapy aimed at reducing its levels in chemoresistant cells so that cisplatin may be used at lower less toxic dose,s and thus be used widely, once again as an effective and affordable anti-cancer compound.

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Appendix

TG2 substrates

Substate	Location
Acidic proline rich protein	Extracellular; enamel pellicle in oral cavity
Actin Beta tubulin Crystallin Ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 (EBP50) Filamin 1 Rho protein kinase 2 RhoA Spectrin Tau protein Troponin Vimentin Vitronectin	Cytoskeleton
Aldolase A Alpha-synuclein Bcr-Breakpoint cluster Region Betaine-homocysteine S methyltransferase Arginase I Angiocidin Annexin I Calpain Caspase-3 Cathepsin D Collagen Dihydropyrimidinase -like 2 protein	Cytosol

Dual leucine zipper bearing kinase(DLK) **Elongation factor 1α** **Elongation factor 1Ɣ** **Enolase** **Eukaryotic initiation factor 4F(eIF-4F)** **F-box only protein** **Fatty acid synthase** **Fatty acid synthase** **Fructose 1,6-bisphosphatase** **Galectin 3** **Glutathione S-transferase** **Glyceraldehyde-3-phosphate dehydrogenase** **Huntingtin protein** **Importin β1 subunit** **Myosin** **NF-kappa -B inhibitor α** **Parkin** **Phosphoglycerate dehydrogenase** **Phosphorylase kinase** **Plasminogen activator inhibitor-2** **Protein synthesis initiation factor 5A** **RAP-α-2 macroglobulin related protein** **T-complex protein 1ϵ subunit** **Thymosin beta 4** **Ubiquitin** **Valosin**	
Alpha-lactalbumin **Beta casein**	**Extracellular; Milk**
Alpha-2 macroglobulin **Receptor** **Annexin I** **C-CAM**	**Plasma membrane**

Cluster of differentiation 38 **EGF receptor** **Ephrin A**	
Adrenocorticotropic hormone **Alpha-2 plasmin inhibitor** **C1 esterase inhibitor** **Collagen** **Fibrinogen alpha chain** **Fibrinogen gamma chain** **Fibronectin** **Glucagon** **Insulin** **Lipoprotein A** **Midkine**	**Extracellular; plasma**
Alpha-2-HS-glycoprotein **Bone sialoprotein** **Osteonectin** **Osteopontin**	**Extracellular;** **mineralised compartment of bone**
Alpha-ketoglutarate **ATP synthase** **Dehydrogenase** **Heat shock protein 60** **Aconitase** **Cytochrome C** **Heat Shock Protein 7** **Heat shock protein 27** **Prohibitin**	**Mitochondrial matrix**
Amyloid beta A4 peptide	**Extracellular;** **Cerebrospinal fluid**
Androgen receptor **AT-rich interactive**	**Nucleus**

domain-containing **protein-1A** **Ataxin-1** **Deoxyribonuclease Ƴ** **Galectin 3** **Histones** **Lamin A, C** **Nuclease sensitive element-binding protein1** **Nucleophosmin** **Periphilin** **Retinoblastoma protein** **SP-1 transcription factor** **UV excision repair protein RAD23 homolog B** **Valosin** **VEGFR-2** **Vigilin**	
Band 3 anion transport **Protein** **Band 4.1 protein**	**Erythrocyte**
Beta endorphin **Calbindin** **Histatin** **Exendin 4** **Gliadoralin A** **Human clara -cell protein Ig kappa chain C region** **Plasminogen** **Substance P** **Uteroglobin** **Vasoactive intestinal peptide**	**Extracellular; secreted**
Beta-2-microglobulin	**Cell surface**
Binding immunoglobulin protein	**Endoplasmic reticulum** **lumen**

Clathrin heavy chain	**Intracellular; vesicle membrane**
Cyclic Thymosin beta 4 **Protein kinase C delta type**	**Cytoplasm**
Elafin	**Skin**
Gliadin	**Vacuole**
Insulin-like growth factor binding protein-1 **Insulin-like growth factor binding protein-3** **Inter-alpha -inhibitor**	**Extracellular fluid**