CHAPTER ONE

GENERAL INTRODUCTION

1.0.0: BACKGROUND

Beside other molecular hallmarks of cancer cells' drug resistance and metastasis, a remarkable feature among drug-resistant and metastatic cancer cells is an increased level of transglutaminase 2 (TG2) protein (Budillon *et al.* 2011). An extensive review of literature on TG2 is here presented, with emphases on its historical background, uniqueness among other members of the transglutaminase family, structural and functional elements, biochemical multi-functionality, tissue distribution and sub-cellular localization, and substrate specificity. The relationship between TG2 biochemistry and cellular physiology, with pertinence to disease pathology is also presented with particular emphasis on its role in cancer biology, drug resistance and metastasis.

1.1.0: HISTORY OF TRANSGLUTAMINASES

In 1923, Barkan and Gaspar reported the cross-linking of fibrin polymers for the first time (Barkan & Gaspar, 1923); then, in 1948, Laki and Lóránd attributed the cross-linking to a Ca^{2+} -dependent protein called 'fibrin-stabilizing serum factor' or 'Laki-Lóránd factor' (Laki & Lóránd, 1948; Lóránd, 1948; Lóránd, 1950). Subsequently, the 'serum factor' was purified by Loewy and colleagues (Loewy *et al.* 1957); and upon demonstration that haemophilia occurs as a result of its deficiency in the blood of haemophiliac patients, the enzyme was termed 'blood coagulation factor XIII' (Duckert *et al.* 1960). Lóránd *et al.* (1966), observed that the 'blood coagulation factor XIII' was an isoenzyme belonging to the transglutaminase family. However, the term transglutaminase was first used by Waelsch and colleagues, while reporting the ability of a soluble liver protein fraction (containing TG2) to incorporate labelled amines into proteins in the presence of Ca^{2+} (Waelsch *et al.* 1957). The designation,

transglutaminase was later amended by the Enzyme Commission (EC 2.3.2.13, transglutaminase = R-glutamyl-peptide, amine-γ-glutamyl transferase). Achyuthan & Greenberg (1987), demonstrated the ability of transglutaminase 2 (TG2) to bind GTP with the resultant inhibition of its activity; justifying the reason why TG2 was named a G protein with a role in signal transduction (Nakaoka *et al.* 1994).

Harding & Rogers (1971), identified gamma-glutamyl-epsilon-lysine cross-links in hair protein extracts; upon demonstration that this cross-linking enzyme was neither identical to factor XIII nor transglutaminase 2 (Chung & Folk, 1972), the enzyme was labelled an 'epidermal' or 'hair follicle' transglutaminase (TGe). Subsequently, it was observed that both membrane-bound and soluble fractions of the hair protein extract showed transglutaminase (TG) activity (Thacher & Rice, 1985), suggesting the presence of further epidermal transglutaminases. The insoluble, 'keratinocyte-specific' (corresponding to TGk) transglutaminase was detectable in in cultured keratinocytes unlike the soluble 'epidermal' TGe (Lichti *et al*. 1985). Furthermore, the demonstration of the expression of TGk, TGc (cytosolic TG), and TGe in both hair follicle and epidermal keratinocytes (Rubin & Rice, 1986) generated confusion and led to the numbering of transglutaminase isoenzymes and their corresponding genes (Parenteau *et al*. 1986; Kim *et al*. 1992). To further reduce ambiguity, transglutaminase messenger RNA is designated with 'TGM' and the gene product is denoted by 'TG', both followed by an Arabic number. Thenceforth, TGM1/TG1, TGM2/TG2, and TGM3/TG3 were respectively assigned to TGk, TGc, and TGe; with their corresponding gene products. This system of naming allowed for the classification of new TG family members that were subsequently discovered.

Other transglutaminase enzymes have been discovered either by protein isolation or through sequence homology, hence, the isolation of TGp (TG4) from prostate adenocarcinoma cells by Bures *et al*. (1980). More recently, Aeschlimann and colleagues have identified three new family members of TG: TGx (TG5), TGy (TG6), and TGz (TG7) (Aeschlimann *et al*. 1998; Grenard *et al.* 2001). A catalytically inactive erythrocyte membrane protein band 4.2 was also discovered to belong to the TG family. Though, it has over 30% similarity with other TG isoenzymes, a cysteine to alanine substitution appears to render it catalytically inactive (Korsgren *et al.* 1990). Today, a total of nine different transglutaminase isoenzymes have been identified in man.

1.2.0: THE TRANSGLUTAMINASE (TGase) FAMILY

Nine transglutaminase genes have been identified, out of which eight encode active enzymes (Grenard *et al.* 2001). Only six of the TG enzymes have been isolated and characterised at the protein level (Esposito & Caputo, 2005), and include the intracellular TG1, TG3 and TG5 isoforms, which are predominantly expressed in the epithelial tissue; the ubiquitously expressed TG2, which occur in intracellular and extracellular forms; TG4, which is expressed in the prostate gland; and factor XIII (FXIII), which is expressed in the blood. Others have been isolated at messenger RNA level, including TG6 which is expressed in the testis, lungs, and brain (Mehta, 2005; Thomas *et al.* 2013); TG7, which is ubiquitously expressed, but most prominently distributed in the testis and lungs (Mehta, 2005); and band 4.2 (table1), which is an enzymatically inactive component protein of the erythrocyte membrane that serves to maintain erythrocyte integrity (Lorand & Graham, 2003). In addition to diversity at genetic level, transglutaminases undergo a number of post-translational modifications such as phosphorylation, nitrosylation, fatty acylation and proteolytic cleavage (Aeschlimann & Paulsson, 1994; Lorand & Graham, 2003).

Table 1: Members of the transglutaminase (TGase) enzyme family, their nomenclature, tissue distribution, biological functions, and pathological involvement (Odii and Coussons, 2014)

TGase	Nomenclature	Tissue distribution, cellular and sub-cellular localization	Biological Functions	Pathology
TG1	Keratinocyte TG, particulate TG, TG1, TGK	Squamous epithelia, keratinocytes, cytosolic, membrane	Barrier function in stratified squamous epithelia, cornified envelope formation in keratinocyte differentiation	Lamellar Ichthyosis (Candi *et al* 1998)
TG2	Liver TG, tissue TG, cytosolic TG, erythrocyte TG, Ghα, endothelial TG	Ubiquitously distributed in many types of tissue, cell membrane, cytosol, nucleus, extracellular	Apoptosis, cell survival signalling, cell differentiation, matrix stabilization, endocytosis, etc	Autoimmune diseases, neurodegenerative diseases, malignancies, metabolic diseases, etc (Facchiano & Facchiano, 2006)
TG3	Epidermal TG, callus TG, hair follicle TG, bovine snout TG	Epidermis, hair follicle, brain, cytosolic	Terminal differentiation of keratinocytes, hair follicles	Human epidermis diseases
TG4	Prostate TG, TGp, androgen regulated major secretory protein, vesiculase, dorsal prostate protein 1 (DP1), type 4 TG	Prostate gland, prostatic fluids, and seminal plasma, extracellular	Reproduction and fertility, especially in rodents where it is involved in semen coagulation	Not known
TG5	TGX, type 5 TG, TG5	Ubiquitously expressed but predominant in female reproductive tissues, skeletal muscle, and foetal tissues, foreskin keratinocytes, epithelial barrier lining, cytosolic	Epidermal differentiation	Secondarily involved in hyperkeratotic phenotype in ichthyosis and in psoriasis, Overexpressed or absent in different areas of the Darier's disease lesions (Candi *et al* 2002).
TG6	TGY, type 6 TG, TG6,	Testis, lungs, and brain, cellular localization is yet to be defined	Central nervous system development, motor function, late stage cell envelope formation in the epidermis and the hair follicle	Spinocerebellar ataxias (Wang *et al.* 2010; Sailer & Houlden, 2012); polyglutamine (polyQ) diseases (Guan *et al* 2013)
TG7	TGZ, type 7 TG, TG7, transglutaminase 7	Ubiquitously expressed, prominent in testis and lungs, cellular and sub-cellular localization are unknown		Not known
FXIII A	Factor XIII A, fibrin stabilizing factor, fibrinoligase, plasma TG, Laki-Lorand factor	Chondrocytes platelets, placenta, astrocytes, macrophages, synovial fluid, heart, eye, bone, dendritic cells in the dermis	Wound healing, blood clotting, bone growth	F13A1 deficiency characterized by impaired wound healing, spontaneous abortion in women, subcutaneous and intramuscular haematomas, severe bleeding tendency
Band 4.2	Erythrocyte protein band 4.2, B4.2, ATP-binding erythrocyte membrane protein band 4.2	Surface of erythrocyte membranes, bone marrow, foetal liver, spleen, membranal	Key component of erythrocyte skeletal network, maintains erythrocyte integrity	Spherocytic elliptocytosis

Transglutaminases catalyse the calcium-dependent post-translational modification of proteins by the insertion of isopeptide bonds between or within polypeptide chains with the resultant formation of polymerised cross-linked proteins (Aeschlimann & Paulsson, 1994). This product include the formation of isopeptide linkages between the γ-carboxamide group of the protein-bound glutamine residue and the ε-amino group of lysine residue, resulting to the formation of stable, insoluble, macromolecular complexes as products. Additionally, transglutaminases catalyse a number of distinct reactions that result in the post-translational modification of a specific glutamine residue in the substrate. The transglutaminase-catalysed reaction modifies the properties of protein substrates by altering their functions (Chen & Mehta, 1999; Esposito & Caputo, 2005).

The biochemical mechanism responsible for TG activity involves 'ping pong' kinetics. The first, rate-limiting step is the formation of a thiol ester with an active cysteine site via the transamidation of γ-carboxamide group of the glutamine residue (with consequent release of ammonia) followed by the transfer of the acyl intermediate to a nucleophilic substrate, such as the ε-amino group of a peptide-bound lysine residue (Figure 1.0). Consequently, an intermolecular isopeptide ε-(γ-glutamyl)lysine cross-link is formed, whereas the monomeric protein units themselves may become internally cross-linked (Porta *et al.* 1991). In transamidation reactions, lysine can be replaced by lower molecular mass amines, especially polyamines, with the resultant formation of N-mono(γ-glutamyl)polyamine. The reaction can proceed to a covalent cross-linking between two polypeptide chains through a N,N-bis(γ-glutamyl)polyamine bridge, in the presence of a further reactive glutamine residues. In the absence of polyamines, water can act as a nucleophile and cause deamidation of protein-bound glutamine residues to convert Gln to Glu in the absence of suitable amines (Porta *et al.* 1991; Esposito & Caputo, 2005).

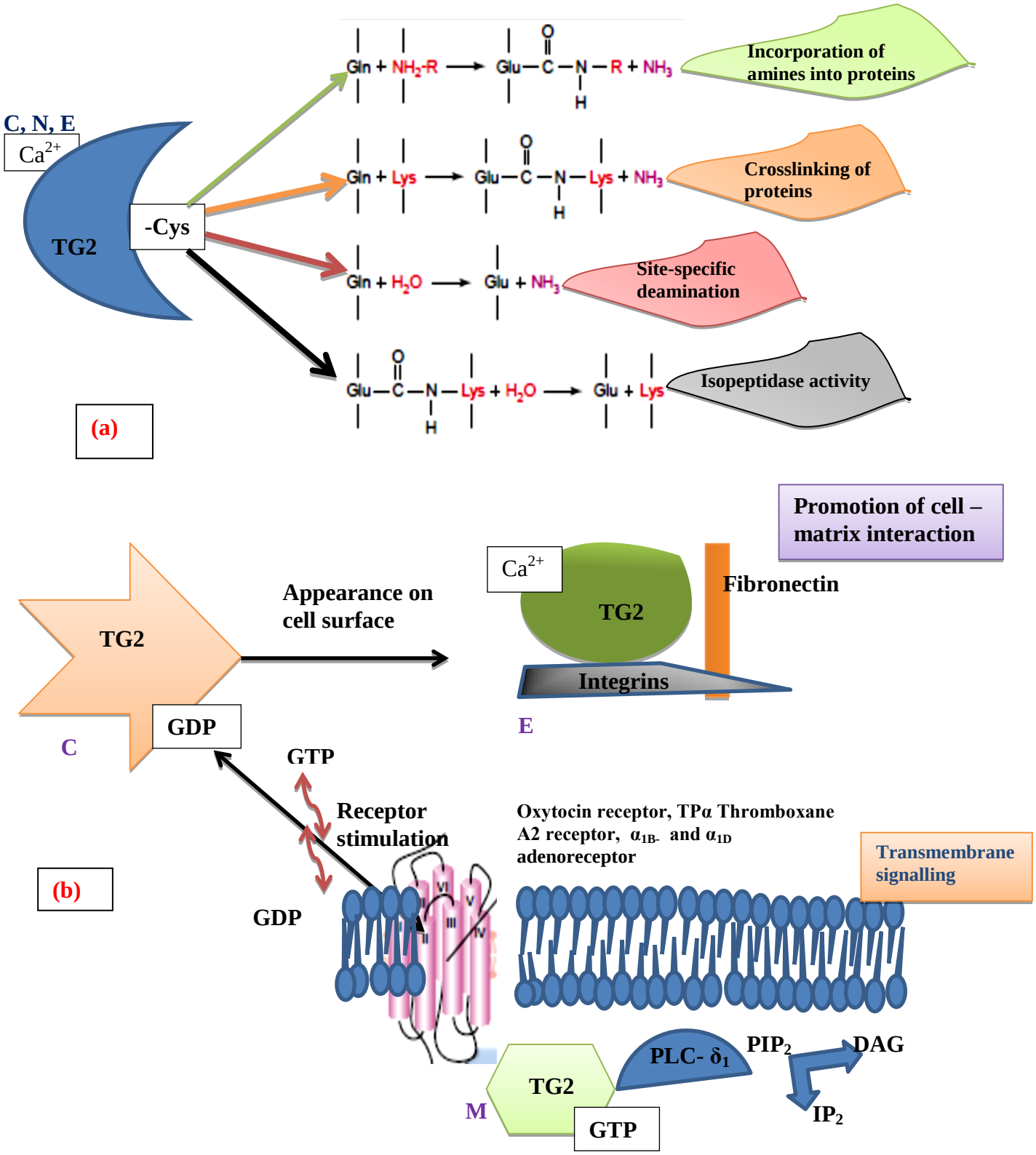


Figure 1.0: Biochemical activities of TG2 at various cellular locations; the cytosol, nucleus, cell membrane, and extracellular space are denoted with C, N, M, and E respectively. Part (a) represents Ca^{2+}-dependent activities, part (b) represents TG2's biochemical functions that occur independent of Ca^{2+} (Adapted from Fesus & Piacentini, 2002).

A high degree of sequence similarity is shared among the various transglutaminase gene products, with the sequences around the active sites predominantly conserved. Investigation of the three-dimensional structure of FXIIIA and TG2 showed a cysteine proteinase-like active site made up of the catalytic triads, cysteine, histidine and aspartic acid that is needed for transamidation (Yee *et al.* 1994; Liu *et al.* 2002). A four-sequential domain arrangement is highly conserved in TG isoforms (Lorand & Graham, 2003). It is constituted by an N-terminal β-sandwich, a catalytic core, and two C-terminal β-barrel domains. It has been proposed that glutamyl and lysyl substrates approach the enzymes from different directions, with glutamyl approaching from the direction of two β-barrels, and lysyl residues approaching the enzymes from the direction of the active site (Lorand & Graham, 2003). Although, the relative orientations of residues in the substrate-binding site region are highly conserved in TGs, there is a profound charge distribution variation surrounding the substrate binding site among the various isoenzymes. This disparity may justify the differences in substrate specificities and hence, the specialized functions of each isoenzyme (Esposito & Caputo, 2005).

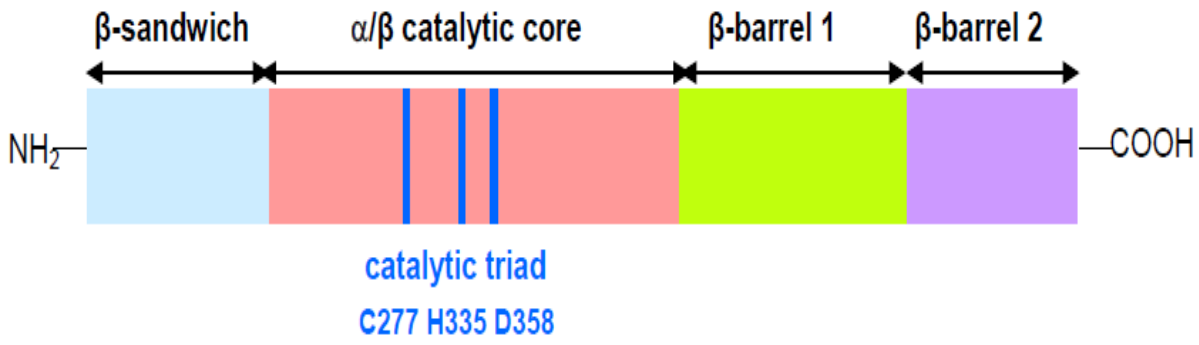


Figure1.1: A schematic representation of the four distinct domains shared by TGase: an N-terminal β-sandwich, a catalytic core (containing Cys277, His335 and Asp358), and two C-terminal β-barrel domains (adapted from Fesus & Piacentini, 2002).

1.3.0: TRANSGLUTAMINASE 2 (TG2), A UNIQUE MEMBER OF THE TRANSGLUTAMINASE FAMILY

The human TGM2 gene localises to chromosome 20q11-12 and its exons span approximately 37 kb (Gentile *et al.* 1994). The protein product, transglutaminase 2 (TG2) is made up of 687 amino acids and has a calculated molecular mass of 77.3 kDa (Fesus & Piacentini, 2002; Gentile *et al.* 1994; Fraij & Gonzales, 1997). However, following the transcription of TG2 in the free cytoplasmic space, it is N-terminally modified by the removal of the first methionine residue and the N-acetylation of the next to the last alanine residue (Ikura *et al.* 1989). Transglutaminase 2 (TG2) is also known as tissue transglutaminase (tTG), cytosolic, type II, or liver transglutaminase. It is a unique member of the TGase family of enzymes; because in addition to the general transglutaminase enzymes' primary activity of calcium-dependent posttranslational modification of proteins, it can also bind and hydrolyse GTP and may act as a small G protein (Lorand & Graham, 2003). It also has a protein disulfide isomerase activity and may function as a protein kinase (Hasegawa *et al.* 2003; Mishra & Murphy, 2004). Besides acting intra-cellularly, TG2 can play some extracellular roles by taking part in cell adhesion processes and stabilization of the extracellular matrix (Verderio *et al.* 2004). The uniqueness of TG2 as evidenced by its structural and functional elements, biochemical multifunctionality, ubiquitous tissue distribution and sub-cellular localization, and substrate specificity are discussed in the following subsections.

1.3.1: Structural and functional elements of TG2

The difference between other TGs and TG2, and the reasons for its multifunctionality are suggested by its structure, which is similar to those of other transglutaminases, but exhibits some specific features which are not characteristic of other transglutaminase gene products. Transglutaminase 2 is structurally composed of four distinct globular domains (figure 1.2): an

NH2-terminal β-sandwich which contains fibronectin and integrin binding sites, a catalytic core which contains the catalytic triads (Cys277, His335 and Asp358) for acyl-transfer reaction and a conserved tryptophan essential for this catalytic reaction (Murthy *et al.* 2002), and two COOH-terminal β-barrel domain with the second barrel domains containing a phospholipase C binding sequence (Hwang *et al.* 1995: Liu *et al.* 2002).

Unlike other transglutaminase enzymes, TG2 possesses a distinctive guanidine nucleotide-binding site, located in the cleft between the catalytic core and the first β-barrel (figure 1.3) (Liu *et al.* 2002), this sequence is coded by exon 10 of the TG2 gene, which is characterised by lower sequence homology with the same exons in other transglutaminases. Some GDP/GTP-interacting residues and those necessary for GTP hydrolysis are situated in other domains (Iismaa *et al.* 2000). In the GDP-bound form of TG2, access to the transamidation active site is blocked by two loops, and the active site cysteine is attached to a tyrosine residue by hydrogen bonding. In the latent conformation of TG2, there is a significant inter-domain interaction between the catalytic domain 2 and domains 3 and 4, which reduces the accessibility of the active centre (Liu *et al.* 2002).

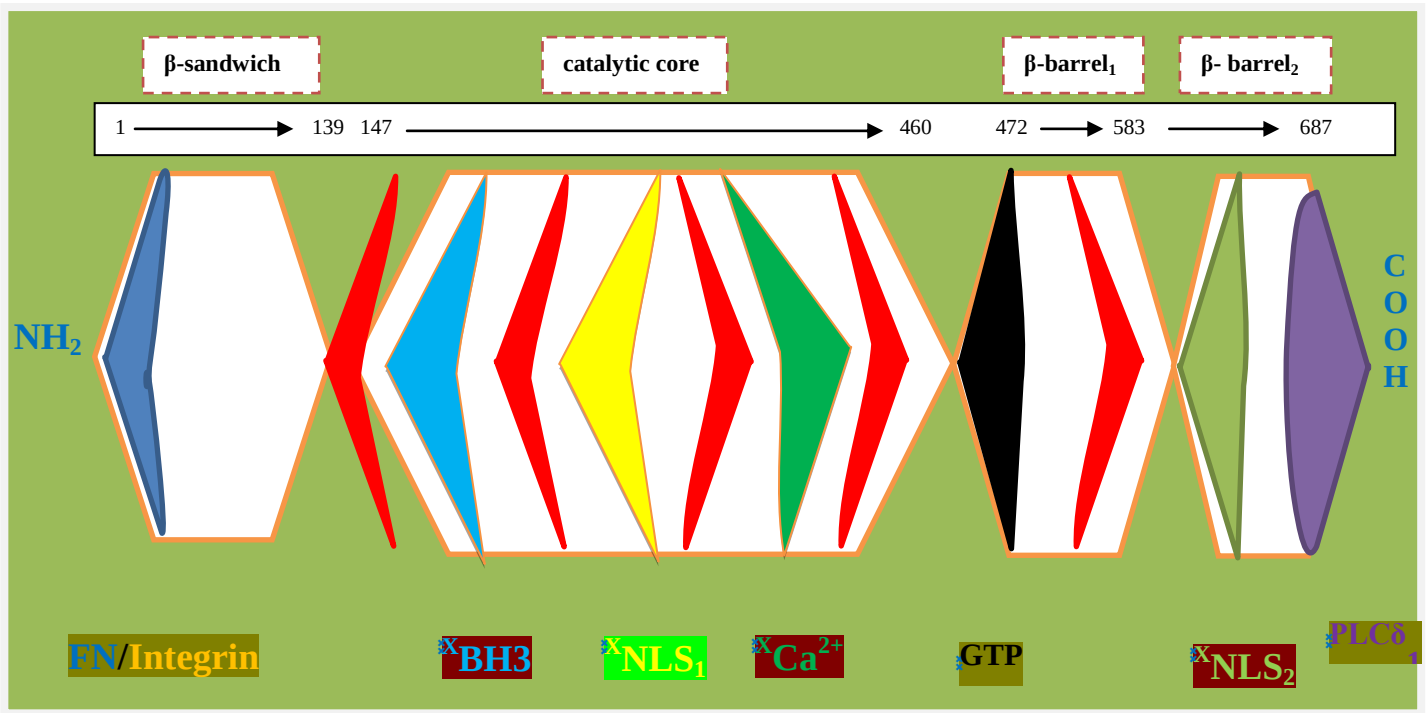


Figure 1.2: Schematic representation of the functional elements of TG2 indicating the four structural domains (arrows) and amino acid positions (top), with the different functional regions indicated: fibronectin/integrin binding site (FN/integrin), binding site for pro-apoptotic BH3-only protein, nuclear localisation sequences 1 and 2 (NLS_1 and NLS_2), calcium binding site (Ca^{2+}), GTP binding site, and phospholipase C (PLC) receptor site (adapted from Fesus & Piacentini, 2002).

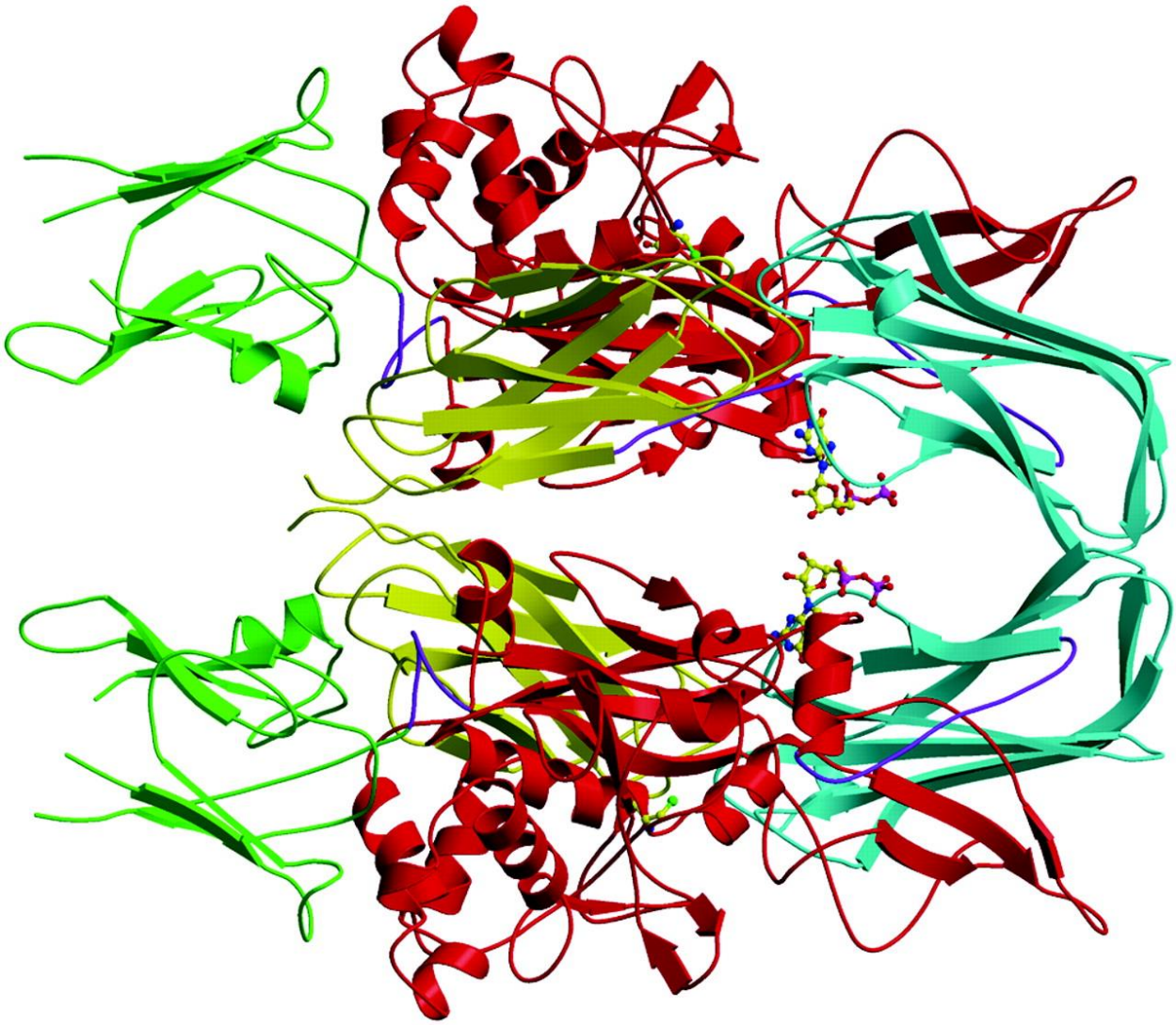


Figure 1.3: A representation of the overall structure of a human tissue transglutaminase (TG2) dimer with bound GDP. TG2 is shown in ribbon drawing with the β-sandwich domain, the catalytic core domain, and the first and second β-barrel domain shown in green, red, cyan, and light green, respectively. The loops connecting the first β-barrel domain to the catalytic core and the second β-barrel are shown in blue. GDP is shown as a ball-and-stick model between the catalytic core and the first β-barrel (Liu *et al.* 2002).

The structural conformation of TG2 in its Ca^{2+}-bound form is yet to be resolved. A putative Ca^{2+}-binding site, homologous to the one demonstrated in FXIIIA by Fox *et al* (1999), is distorted in the TG2 structure by the bound nucleotide (Liu *et al.* 2002). The binding of Ca^{2+}

to the catalytic domain of TG2 alters the conformation of proteins as domains 3 and 4 are moved further apart from the catalytic domain, thus making the active site of TG2 accessible (Mariani *et al.* 2000; Liu *et al.* 2002); the hydrogen-bonded tyrosine is also displaced in the process (Noguchi *et al.* 2001). The ability of GTP to inhibit the transamidation activity of TG2 is determined by the potential of GTP to bind and subsequently hydrolyse Ser171 and Lys173 residues of the second domain (Iismaa *et al.* 2000).

1.3.2: Tissue distribution and sub-cellular localization of TG2

The expression of TG2 is not restricted to only few tissues or certain cell types, neither is it confined to a particular location in a cell (Thomazy & Fesus, 1989). Essentially, the cellular distribution of TG2 is ubiquitous, with its expression levels highest in endothelial cells and monocyte-derived macrophages; although, it is significantly expressed in vascular smooth muscle cells, connective tissue fibroblasts, osteoblasts, neurons, hepatocytes, astrocytes, and epidermal keratinocytes (as reviewed by Fesus & Piacentini, 2002; Lorand & Graham, 2003).

Transglutaminase 2 is constitutively expressed in different types of cells, while in some other cells its expression is induced by external stimuli or as part of their differentiation/maturation (Zemskov *et al.* 2006). At the cellular level, TG2 is localized both inside the cell and on the cell surface as shown by the schematic representation in figure 1.4.

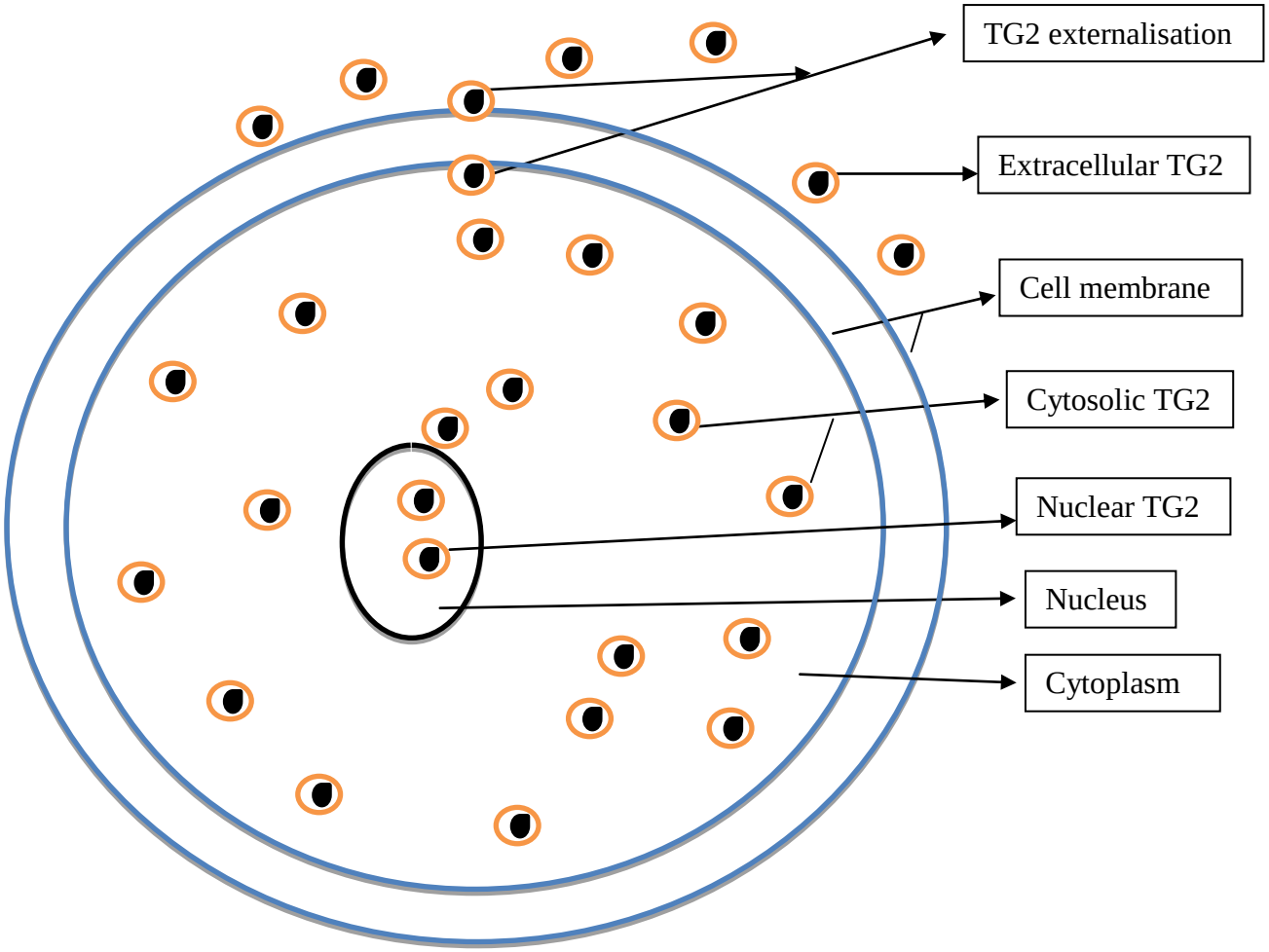


Figure 1.4: Cellular distribution of TG2 (black dot in yellow circle): TG2 is localised in the nucleus (nuclear TG2), cytoplasm (cytosolic TG2), and cell surface (extracellular TG2). It is translocated into the nucleus through the help of importin, while TG2 externalisation to the cell surface occurs through unknown mechanisms.

1.3.2.1: Intracellular transglutaminase 2

The intracellular location of TG2 is predominantly in the cytosol, however it has also been reported to be present in the nucleus and associated with the mitochondria (Telci & Griffin, 2006). It is a cytosolic protein with greater proportion of its cellular pool (70-80%) present in the cytoplasm (Griffin *et al.* 2002; Lorand & Graham, 2003). As a result of low concentration of Ca^{2+} within the cytoplasm, the transamidating activity of TG2 is thought to remain

dormant inside the cell, while the protein functions as a GTPase (Nakaoka *et al.* 1994; Fesus & Piacentini, 2002). However, cytosolic TG2 can be activated by most cellular stressors which trigger extracellular calcium ion influx or release of calcium ion from the intracellular stores (Zemskov *et al.* 2006). The nuclear localisation of TG2 has been reported to be approximately 5% or less (Lesort *et al.* 1998). Cytosolic TG2 migrates to the nucleus in response to specific stimuli (Milakovic *et al.* 2004), and importin-3 is responsible for its translocation into the nucleus (Peng *et al.* 1999); where it can either function as a G-protein (Singh *et al.* 1995) or as a transamidase activated by nuclear Ca^{2+} signals to cross-link histones (Ballestar *et al.* 2001).

1.3.2.2: Cell surface transglutaminase 2

A significant proportion of TG2 is found in association with membranes of different cell types (Aeschlimann & Thomazy, 2000). The localisation of TG2 on the surfaces of various cells types as well as in the extracellular matrix has been established (Upchurch *et al.* 1991). Irrespective of the lack of a leader sequence or transmembrane domain, which would have helped in the translocation of TG2 to the surface by the conventional endoplasmic recticulum/golgi route, the enzyme is secreted from cells in a controlled manner (Gentile *et al.* 1991; Gaudry *et* al. 1999; Di Venere, *et al.* 2000). However, the mechanism of TG2 translocation across the phospholipid bilayer and the pathway of its externalization are not well understood. Available data shows that the externalization of TG2 is determined by a number of factors, which include a fibronectin-binding site in the N-terminal β-sandwich domain of TG2 (Gaudry *et* al. 1999), and the presence of a non-proline *cis* peptide bond at Tyr^{274} as justified by the loss of both the transamidation activity and secretion of the enzyme, following the mutation of this bond (Balklava *et al.* 2002). The third criterion for TG2 externalization is the presence of a Cys^{277} intact site, necessary for the deposition of the

enzyme into the matrix (Balklava *et al.* 2002). Among these criteria, the presence of non-proline *cis* peptide bonds is a conserved feature in a number of transglutaminases (Weiss *et al*. 1998), and was first identified in Factor XIII, which has two non-proline *cis* peptide bonds (Hettasch & Greenberg, 1994).

On externalization, cell surface TG2 has been shown to facilitate cellular interactions with the surrounding extracellular matrix (ECM); which are critical physiological processes underlying many key aspects of cell behaviour, including cell adhesion, growth, migration, differentiation, programmed cell death, and ECM assembly (Zemskov *et al.* 2006). In turn, these cellular processes are vital to embryogenesis and tissue morphogenesis, wound healing and tissue repair, as well as tumour growth and metastasis. Gentile *et al.* (1992) first suggested the involvement of transglutaminase 2 in the mediation of cell-matrix adhesion. They investigated the effect of TG2 over-expression on the spreading of fibroblasts and their increased resistance to trypsinization. Subsequent convincing proofs at both cellular and molecular levels support involvement of TG2 in the mediation of cellular interactions with ECM and it has been demonstrated that TG2 serves as an adhesion receptor for fibronectin (FN) on the cell surface (Verderio *et al.* 1998; Akimov *et al.* 2000; Belkin *et al.* 2001; Kabir-Salmani *et al.* 2005).

1.3.2.2.1: Transglutaminase 2 – fibronectin interaction

Fibronectin (FN) is a high molecular weight (~540kD) dimeric modular glycoprotein present in the plasma membrane and ECM (Magnusson & Mosher, 1998). It is synthesised by most cell types, where it interacts with a variety of adhesion receptors, including one or more FN-binding integrins (α5β1, αVβ3, αVβ5, αVβ6, α4β1, α4β7, αIIbβ3, α8β1, α9β1), and other transmembrane proteins; resulting in effects on cell proliferation, migration, and

differentiation (Mould & Humphries, 2004; Humphries *et al.* 2004). Pathologically, FN is profoundly involved in wound healing, inflammation, blood clotting and thrombosis, as well as tumour growth and angiogenesis (Zemskov *et al.* 2006). FN in its polymeric form, is represented in the extracellular matrix by fibrillar matrices (Wierzbicka-Patynowski & Schwarzbauer, 2003), which not only promote cell adhesion, but as well serve as a scaffold for assembly of other ECM molecules; and provide important orientations for surrounding cells, initiating cascades of signals upon interaction with cell surface receptors (Sottile *et al.* 2000; Pereira *et al.* 2002; Sottile & Hocking, 2002).

TG2 has very high affinity for FN, to which it has been shown bind at 2:1 stoichiometry (LeMosy *et al*. 1992), independent of either Ca^{2+} or the transamidating and GTPase activities of TG2 (Turner & Lorand, 1989). The interaction of extracellular TG2 with FN has been shown to be involved in cell-matrix adhesion (Akimov *et al.* 2000) and many other adhesion-dependent phenomena, such as cell migration, matrix assembly and signalling (Akimov & Belkin, 2001; Verderio *et al.* 2003). The gelatin-binding domain (42kD) serves as the only binding site for TG2 on FN and binds TG2 with similar affinity as the whole FN (Radek *et al.* 1993). Furthermore, the adhesive function of TG2 is favoured by the fact that the 42kD gelatin-binding domain of FN contains no interaction sites for the numerous FN-binding integrins, as well as other FN-associated adhesion receptors (figure 1.5) (Hang *et al.* 2005). Therefore, TG2 and integrins can independently bind distinct domains of FN, consequently existing in collaboration rather than engaging in competition in the cell adhesion process (Zemskov *et al.* 2006). It has been established in different cell types that the binding of TG2 to the 42kD fragment of FN results in stable cell adhesion, limited spreading and formation of specialized adhesive structures at the cell-substrate interface (Belkin *et al.* 2001; Akimov & Belkin, 2001).

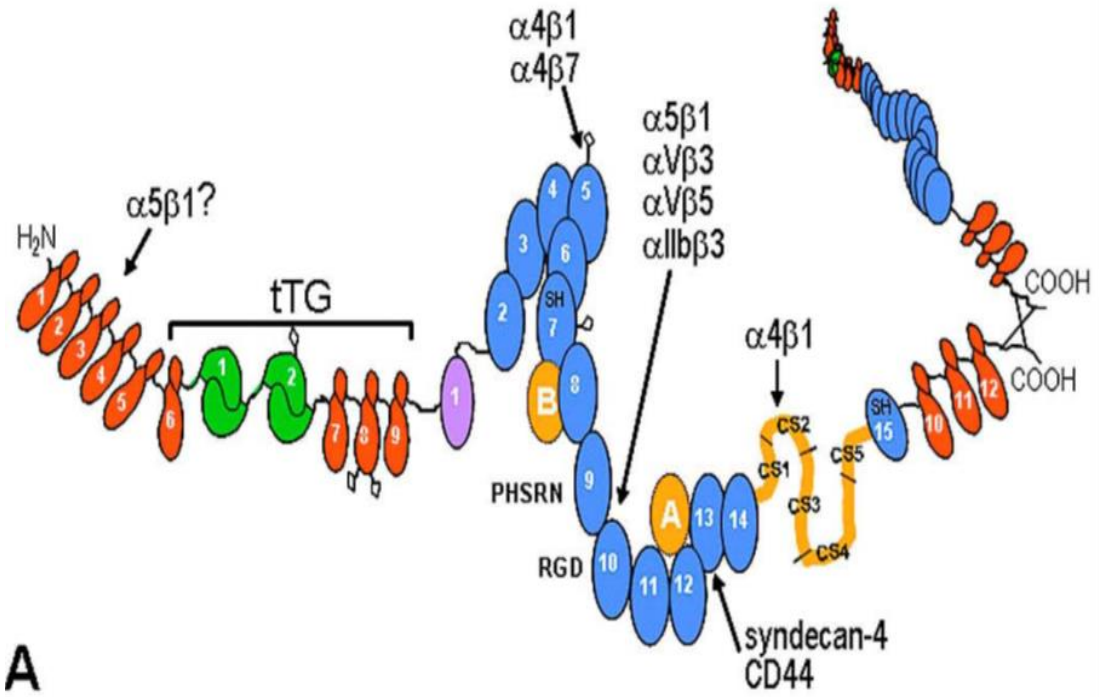


Figure 1.5: A representation of the molecular interactions of FN with tTG and other adhesion receptors showing the localization of the binding sites for tTG, integrins and other adhesion receptors on the FN molecule. Modular structure of FN is presented for one of its chains, with type I modules shown in red, type II modules in green, and type III modules in blue. Yellow coloured domains (A and B) are Pro-His-Ser-Arg-Asn (PHSRN) peptides, one of the FN cell-binding domains that activates integrins, purple domain (1) represents an extended binding site for incoming FN (an epitope for mAb L8) (Hang *et al.* 2005; Zemskov *et al.* 2006).

1.3.2.2.2: Transglutaminase 2 – integrin interaction

Integrins represent a large class of transmembrane adhesion receptors constituted by non-related α and β subunits (Hynes, 2002). In different cell types apart from red blood cells, 24 integrin heterodimers constituted by 8 β subunits and 18 α subunits have been identified,

serving as receptors for a number of ECM ligands and taking part in adhesion between cells (Hynes, 2002; Humphries *et al.* 2004). Regardless of the co-existence of TG2 and integrins at different FN-binding domians, where they streamline the cell adhesion process; TG2 also associates with integrins to maintain cell-extracellular matrix (ECM) interactions.

The role of integrins in wound healing, blood clothing and thrombosis, viral and bacterial infection, inflammation, tumour growth and angiogenesis, as well as other pathological and physiological states are testaments to the fundamental functions of integrins in cell-matrix adhesion (Zemskov *et al.* 2006). In different cell types, transglutaminase 2 has been shown to associate with many integrin receptors, by binding to the extracellular domains of the β1 and β3 integrin subunits (Akimov *et al*. 2000; Akimov & Belkin, 2001; Belkin *et al.* 2001).

The stable non-covalent TG2-integrin complexes are formed independent of the transamidating activity of TG2, and there is no evidence of integrins serving as enzymatic substrates of TG2 or other transglutaminases (Akimov *et al.* 2000). Furthermore, Akimov *et al.* (2000), in a set of biochemical experiments performed on cells that do not synthesize FN, demonstrated that the TG2-integrin interaction is not FN-mediated but direct. They further observed that integrin-TG2 complexes have 1:1 stoichiometry and all cell-surface TG2 is bound to integrin receptors; with the possibility of up to 40% of β1 integrins associating with TG2 in various cell types (Akimov *et al.* 2000; Akimov & Belkin, 2001). The ability of TG2 to form ternary adhesive complexes with integrins and FN, where all the three proteins successfully interact with each other (Zemskov *et al.* 2006), highlights the importance of TG2 effects on cell adhesion and indicates an unconventional role of TG2 as a co-receptor in cell-matrix interactions (Akimov *et al.* 2000).

1.4.0: BIOCHEMISTRY OF TG2

Transglutaminase 2 is a multifunctional protein that serves as a mediator between several distinct biochemical functions at various cellular locations (figure 1.4). The diverse physiological implications of TG2 are testament to the importance of its diverse biochemical activities in cellular functions.

The cross-linking activities of TG2 are Ca^{2+}-dependent and result from acyl-transfer reaction between γ-carboxamide group of a specific protein-bound glutamine and either the ε-amino group of a distinct protein-bound lysine residue or primary amines like polyamines and histamine (Fesus & Piacentini, 2002). The reaction primarily involves the exchange of primary amines for ammonia at the γ-carboxamide group of glutamine residues, in the presence of Ca^{2+} (Mehta & Chen, 1999). The binding of Ca^{2+} is vital to the cross-link formation because it initiates a conformational change that exposes a cysteine residue in the active site domain; the cysteine reacts with the glutamine substrate, resulting to the formation of an acyl-enzyme intermediate and release of ammonia (Iismaa *et al.* 2003). The subsequent reaction between the acyl-enzyme complex and a primary amine results to the formation of γ-glutamyl-amino cross-link, and concomitant release of the enzyme (Aeschlimann & Paulsson, 1994; Iismaa *et al*. 2003; Pinkas *et al*. 2007). Other biochemical functions of TG2 include site-specific deamidation, during which water can replace amine donor substrate, amounting to the deamidation of the recognized glutamines (Fesus & Piacentini, 2002). Furthermore, TG2, just like factor XIIIA, exhibits isopeptidase activity in the presence of Ca^{2+}, and has been shown to hydrolyse γ:ε isopeptides (Parameswaran *et al.* 1997).

At the cell membrane, TG2 plays a role in transmembrane signalling by transmitting signals from seven-transmembrane helix receptors to phospholipase C (Iismaa *et al.* 2000).

Following the stimulation of these transmembranal helix receptors, TG2 binds to and activates phospholipase C and proper regulation of the transmembrane signalling is ensured by its endogenous GTPase activity (Murthy *et al.* 1999). Transglutaminase 2 interaction with specific molecules such as sphingosylphosphocholine, could reduce the Ca^{2+} requirement for the transglutaminase activity (Lai *et al.* 1997). This activity is highly influenced by nitric oxide such that up to 15 of the 18 cysteine residues can be nitrosylated and denitrosylated in a Ca^{2+} -dependent fashion, resulting in TG2 inhibition and activation respectively (Lai *et al.* 2001).

A very striking part of TG2 function is its translocation to the nucleus under certain unknown conditions, with the help of importin-α3 (Peng *et al.* 1999); where it can crosslink histones by nuclear Ca^{2+} -dependent activation, serving as a transamidase (Ballestar *et al.* 2001) or functioning as a G protein (Singh *et al.* 1995). In a different vein, TG2 has been reported to be involved in the determination of the apoptotic fate of cells. The over-expression of TG2 primes cells for apoptosis (Fesus *et al.* 1987) and its inhibition by antisense strategy results to reduced cell death (Oliverio *et al.* 1999). Piacentini *et al.* (2002) suggested that TG2 sensitizes cells for apoptosis by interacting with mitochondria, resulting in mitochondrial shift to higher polarised state and altered redox status; which might lead to the activation of transglutaminase cross-linking activity (Lesort *et al.* 2000). During the later stage of apoptosis, membrane polarity is usually negated, resulting in a massive influx of Ca^{2+} into the cytosol. This increase in cytosolic Ca^{2+} leads to the acute activation of originally inactive TG2 to its cross-linking configuration in all sub-cellular compartments; and consequent extensive polymerization of intracellular proteins and formation of detergent-insoluble structures (Fesus *et al.* 1989). These insoluble protein scaffolds are functionally significant as they stabilize the structure of a dying cell prior to its phagocytotic clearance, hence,

preventing the release of harmful intracellular components and the concomitant inflammatory or autoimmune responses (Piredda *et al.* 1997).

1.4.1: Regulation of TG2 expression and catalytic activity

Transglutaminase 2 is involved in diverse physiological responses and as such, its expression is regulated by many factors. It can be regulated by various cytokines, hormones, and drugs (see reviews by Mehta & Chen, 1999; Lesort, *et al.* 2000; Aeschlimann & Thomázy, 2000). The pattern of TG2 regulation has been demonstrated to be cell type-specific. For instance, the intracellular polyamines, spermine and spermidine, that serve as acyl acceptor substrates for transglutaminases (Folk, 1980; Janne *et al.* 1991) are capable of modulation of TG2 expression (Mehta & Chen, 1999). However, the blockage of polyamine synthesis in different cell types was shown to differently influence TG2 expression by effecting decreased expression in one cell type and increased expression in another (McCormack *et al.* 1994; Wang *et al.* 1998).

Treatment of different cell types with natural and synthetic retinoids especially retinoic acid (RA), have been shown to induce dramatic increase in TG2 expression, at both transcriptional and translational (mRNA and protein) levels (Davies *et al.* 1985; Chiocca *et al.* 1988; Defacque *et al.* 1995; Zhang *et al.* 1995). Retinoic acid-mediated induction of TG2 has also been demonstrated in vivo; where Verma *et al.* (1992) observed a significant reduction in the level of TG2 in various tissues of a vitamin A-deficient rat, and increasing production of TG2 by the same tissues on resumption of vitamin A-containing diets.

From a catalytic perspective, TG2 could be recognized as a bi-functional enzyme, owing to its ability to catalyse the Ca^{2+} -dependent protein cross-linking and Ca^{2+} -independent GTP

and ATP hydrolysis (Mehta & Chen, 1999). In essence, the cross-linking function of TG2 is allosterically activated by Ca^{2+} -ion and reversibly inhibited by GTP, GDP, and GMP; whereas, it is not influenced by physiological concentrations of ATP or CTP (Lai *et al.* 1998; Lai *et al.* 2001). However, the GTPase and ATPase activity of TG2 occurs independent of Ca^{2+}, but depends on Mg^{2+} -ions because Mg^{2+} -GTP and Mg^{2+} -ATP are the true substrates for TG2-mediated hydrolysis reaction (Lai *et al.* 1998). Furthermore, Lai *et al* (1998) demonstrated that the binding of Mg^{2+} -GTP complex to TG2 results to a conformational change which inhibits TG2 protein cross-linking activity without affecting its ATPase activity. They further established that the Mg^{2+} -ATP interaction with TG2 induces a conformational alteration that results in the inhibition of the GTPase activity without affecting its protein cross-linking propensity. In essence, these results suggest that the concentrations of Mg^{2+} -nucleotide complexes may be of vital importance in the modulation of TG2 activities. Furthermore, a membrane lipid, sphingosylphosphocholine (lyo-SM), has been suggested to be a potent activator of TG2 cross-linking activity (Lai *et al.* 1997).

The over-expression of TG2 does not necessarily lead to increased cross-linking activity. For instance, Smethurst and Griffin (1996), while measuring TG2 activity in permeablised human endothelial cell system; showed that TG2 exists as a cryptic enzyme under normal cell physiological conditions. This finding is particularly important as it demonstrated that the presence of TG2 is not always accompanied by its protein cross-linking activity inside living cells.

1.4.2: Substrate specificity and cellular substrate proteins of TG2

Transglutaminase 2 is a multifunctional protein with over 130 substrates at various locations inside and outside the cell (Csosz *et al*. 2009). This broad range of specificity of TG2 for its

targets may account for its flexibility and multi-functionality. To achieve a particular function out of its variety of functions necessitates that the selection of specific subset of proteins related to that particular biological event must be tightly regulated by additional factors. TG2-specificity determining factors are numerous and include such factors as cell type- and tissue-dependent abundance of the enzyme and its substrate, availability of Ca^{2+}, the absence of inhibitors, the presence of modifying substances like sphyngosylphosphocholine (Lai *et al*. 1997) and nitric oxide (Lai *et al*. 2001), and the physical accessibility of modification sites on the individual molecules.

An understanding of the *in situ* TG2 substrates and its specificity to the substrates is needed in order to understand the physiological and pathological roles of TG2 (Csosz *et al.* 2008). The binding site in TG2 is organised in such a way that it permits the proper orientation of peptide-bound residues of glutamine, whilst neither free glutamine nor asparagine is used by the enzyme; even in the midst of a strong stereo-specificity towards the L-isomer (Folk, 1983). The possession of an extended active site by TG2 and its interaction with oligopeptides as proposed by Folk (1983) probably influences the catalytic efficiency of TG2 towards glutamine side chains of substrates. The role of different amino acids in TG2 substrate effectiveness was studied by Gorman & Folk (1981; 1984). They observed that the positions of the different amino acids are important factors determining TG2 substrate requirement.

Table 2: *In vivo* substrates of TG2, reactive sites, cellular localizations, and possible involvement in human physiology/diseases (as reviewed by Odii and Coussons, 2014)

TG2 substrate	Reactive site	Localization	Physiology/disease
Acetylcholine esterase	Glutamine	Intracellular	Neurological disease (Hand *et al.* 2000)
Actin	Glutamine and lysine	Intracellular	Cytoskeleton regulation (Nemes *et al.* 1997)
Aldolase	Reactive glutamine present but specific residue is unknown	Intracellular	Genetic disease, endocrine and metabolic diseases, autoimmune and inflammatory diseases (Lee *et al.* 1992)
Androgen receptor		Intracellular (nuclear receptor)	Endocrine and metabolic diseases (Mandrusiak *et al.* 2003)
Annexin I (lipocortin I)	Glutamine	Intracellular	Autoimmune and inflammatory diseases, cytoskeleton regulation (Ando *et al.* 1991)
Calgizzarin - S100C protein - MLN 70 – S100A11	Glutamine and lysine	Keratinocyte cornified envelope	Endocrine and metabolic diseases, dermatological diseases (Robinson & Eckert, 1998)
Collagen alpha 1(III)	Glutamine	Extracellular	Extracellular matrix interaction and stabilization, autoimmune and inflammatory diseases (Orban *et al.* 2004)
α - B-crystallin	Lysine	Intracellular	Cell life and death, cytoskeleton regulation, protein stabilization (Groenen *et al.* 1992)
β - A3 crystallin	Glutamine	Intracellular	Cell life and death, cytoskeleton regulation, protein stabilization (Groenen *et al.* 1994)
β - B3 crystallin	Glutamine	Intracellular	Cell life and death, cytoskeleton regulation, protein stabilization (Berbers *et al.* 1984)
β - Bp (betaB2) crystalline	Glutamine	Intracellular	Cell life and death, cytoskeleton regulation, protein stabilization (Berbers *et al.* 1984)
Cytocrome c	Glutamine	Intracellular	Cell life and death (Butler & Landon, 1981)
Fibronectin	Glutamine	Extracellular	Protein stabilization, extracellular matrix interaction and stabilization (Mehta *et al.* 2006)
Fibrinogen A alpha	Glutamine and lysine	Extracellular	Extracellular matrix interaction and stabilization, autoimmune and inflammatory diseases (Murthy *et al.* 2000)
Glutathione S-transferase	Glutamine, lysine, fluorescine	Intracellular	Extracellular matrix interaction and stabilization (van den Akker *et al.* 2011)

Gluten proteins	Glutamine	Extracellular	Celiac disease (Kim *et al.* 2002)
Glyceraldeheyde 3 phosphate dehydrogenase	Lysine	Intracellular	Neurological diseases (Orru *et al.* 2002)
H3 histone	Glutamine	Intracellular	Cell life and death (Ballestar *et al.* 1996)
H4 histone	Glutamine	Intracellular	Cell life and death (Ballestar *et al.* 1996)
H2A histone	Glutamine	Intracellular	Cell life and death (Ballestar *et al.* 1996)
H2B histone	Glutamine	Intracellular	Cell life and death (Ballestar *et al.* 1996)
Importin alpha3		Nuclear transport protein	Cell life and death (Kuo *et al.* 2011)
α - Ketoglutarate dehydrogenase	Lysine	Intracellular	endocrine and metabolic diseases (Cooper *et al.* 1997)
Latent TGF-beta binding protein-1 (LTBP-1)		Extracellular	Carcinogenesis, autoimmune and inflammatory diseases (Verderio *et al.* 1999)
α --2-Macroglobulin receptor-associated protein	Glutamine	Extracellular	autoimmune and inflammatory diseases (Rasmussen *et al.* 1999)
Microtubule-associated protein tau - isoform Tau-F (Tau-4)	Glutamine and lysine	Intracellular	Cytoskeleton regulation, neurological diseases (Murthy *et al.* 1998)
Myosin		Intracellular	Cytoskeleton regulation (Eligula *et al.* 1998)
Nidogen	Glutamine	Extracellular	Extracellular matrix interaction and stabilization (Aeschlimann *et al.* 1992)
Osteocalcin		Extracellular	Autoimmune and inflammatory diseases (Kaartinen *et al* 1997)
Osteonectin	Glutamine	Extracellular	Autoimmune and inflammatory diseases, extracellular matrix interaction and stabilization (Aeschlimann *et al* 1995)
Osteopontin	Glutamine	Extracellular	Autoimmune and inflammatory diseases, extracellular matrix interaction and stabilization (Kaartinen *et al.* 2002)
Phospholipase A2	Glutamine	Extracellular	Endocrine and metabolic diseases, Signal transduction, autoimmune and inflammatory diseases (Cordella-Miele *et al* 1990; Fesus & Piacentini, 2002)
Troponin T		Intracellular	Cytoskeleton regulation (Gorza *et al.* 1996)

TG2 substrates are widely localised within the cellular and sub-cellular spaces of the cell (table 2). The recognition and post-translational modification of extracellular TG2 substrates have been implicated in some extracellular physiological functions like the stabilisation of extracellular matrix (ECM) and cell-ECM interactions through the cross-linking of matrix proteins (Aeschlimann & Thomazy, 2000). FN, an abundant extracellular protein, is a major TG2 substrate *in vitro* and *in vivo* (Jones *et al.* 1997). Under normal cellular physiological conditions, TG2 externalised from cells becomes tightly bound to FN and forms ternary complexes with collagens that function as a cementing substance in the ECM. This mechanism is used to clean up TG2 from the circulation, hence preventing it from causing any adverse effects (Esposito and Caputo, 2005). Other TG2 substrates that are involved in the assembly, remodelling and stabilisation of the ECM are fibrinogen, fibrin (Ritchie *et al*. 2000), von Willebrand factor (Takagi *et al*. 1995), vitronectin (Skorstengaard *et al.* 1990), laminin and nidogen (Aeschlimann *et al.* 1992), liprotein(a) (Borth *et al.* 1991). TG2 stabilises the reversible interactions between molecules that form heteromeric complexes in the ECM of specific tissues, e.g. laminin-nidogen (Aeschlimann *et al.* 1992), FN-collagen (Kleman *et al.* 1995), osteonectin-vitronectin (Rosenblatt *et al.* 1997).

Intracellularly, a large number of TG2 substrates abound, especially proteins involved in the organisation of the cytoskeleton. As a result of its auto-catalytic activity, TG2 isoform in the cytoskeleton co-localises with stress fibres and cross-links myosin (Esposito and Caputo, 2005). Upon activation by Ca^{2+}, TG2 contributes to the organisation of the cytoskeleton by cross-linking various cytoskeletal proteins, such as β-tubulin, actin, microtubule protein tau, myosin, spectrin, thymosin β, troponin, and vimentin (Tucholski *et al.* 1999; Piredda *et al.* 1999; Orru *et al.* 2003). The function of this extensive polymerisation which occurs at the final step of apoptosis is thought to be involved in the stabilisation of the structures of the

dying cells, hence, preventing the release of cellular components that could cause inflammatory or autoimmune responses (Fesus & Piacentini, 2002). Furthermore, actin, retinoblastoma gene product, and nuclear proteins such as core histones, are TG2 substrates *in vivo* (Nemes *et al.* 1997; Ballestar *et al.* 1996); and the extensive polymerisation of these proteins has been established as a key signal for the initiation of apoptosis (Oliviero *et al.* 1997).

1.5.0: PHYSIOLOGY OF TG2

Physiologically, the Ca^{2+}-dependent activation of TG2 has been implicated in many biological functions as diverse as extracellular matrix stabilization during development and wound healing, hormone receptor signal transduction as G-protein, cell growth and differentiation, cell adhesion and morphology, receptor-mediated endocytosis, cornified envelope formation in the keratinocytes, apoptosis, and cancer drug resistance and metastasis (Mehta & Chen, 1999).

1.5.1: Transglutaminase 2 in apoptosis

Fesus *et al.* (1987), on observing that lead-induced hypertrophy in the liver of rats was associated with an increased expression of TG2, suggested the initial link between TG2 and apoptosis. Since then, many reports have shown the involvement of TG2 in apoptosis (see Piacentini *et al.* 2005 for a review). TG2's involvement in apoptosis could be better described as a double-edged sword as it could be pro-apoptotic or anti-apoptotic. Cells undergoing apoptosis show an increased level of TG2 expression, which primes the cell to undergo apoptosis. Its inhibition however, results in a decreased propensity for cell death (Mehta *et al.* 2006; Verma & Mehta, 2007).

TG2-mediated pro-apoptosis is underlied by its cross-linking configuration, which requires a millimolar concentration of calcium. Stressful conditions such as ultraviolet radiation, and/or chemotherapeutic agent, can generate reactive oxygen species (ROS) with resultant induction of TG2 (figure 1.6). Increase in the stressful conditions further triggers the release of Ca^{2+} from the endoplasmic reticulum (ER), resulting in the activation of TG2 and extensive cross-linking of intracellular proteins, which in turn, initiates the apoptotic process (Mangala & Mehta, 2005; Mehta *et al.* 2006). A major physiological significance of TG2's involvement in apoptotic initiation is its mediation of the crosstalk between dying and phagocytic cells, to ensure tissue and cellular integrity. The focal function of TG2 in apoptosis is to ensure that once the apoptotic process is initiated, it is completed without inflammation of tissue injury resulting from the process (Fesus & Szondy, 2005). TG2 can achieve maintenance of a cellular environment devoid of inflammation, whilst promoting apoptosis by directly promoting apoptosis in certain cell types (Oliverio *et al.* 1999; Rodolfo *et al.* 2004), or indirectly promoting the activation of TGF-β release by the macrophages, that can promote the death of various cells (Szondy *et al.* 2003; Huang & Lee, 2003), to ensure that all unwanted cells are efficiently killed preventing the occurrence of necrosis. Additionally, TG2 can promote chemo-attractant formation and the release of phosphatidylserine to respectively aid macrophage migration to the site of apoptosis and the recognition of apoptotic cells (Nishiura *et al.* 1998; Fesus & Szondy, 2005).

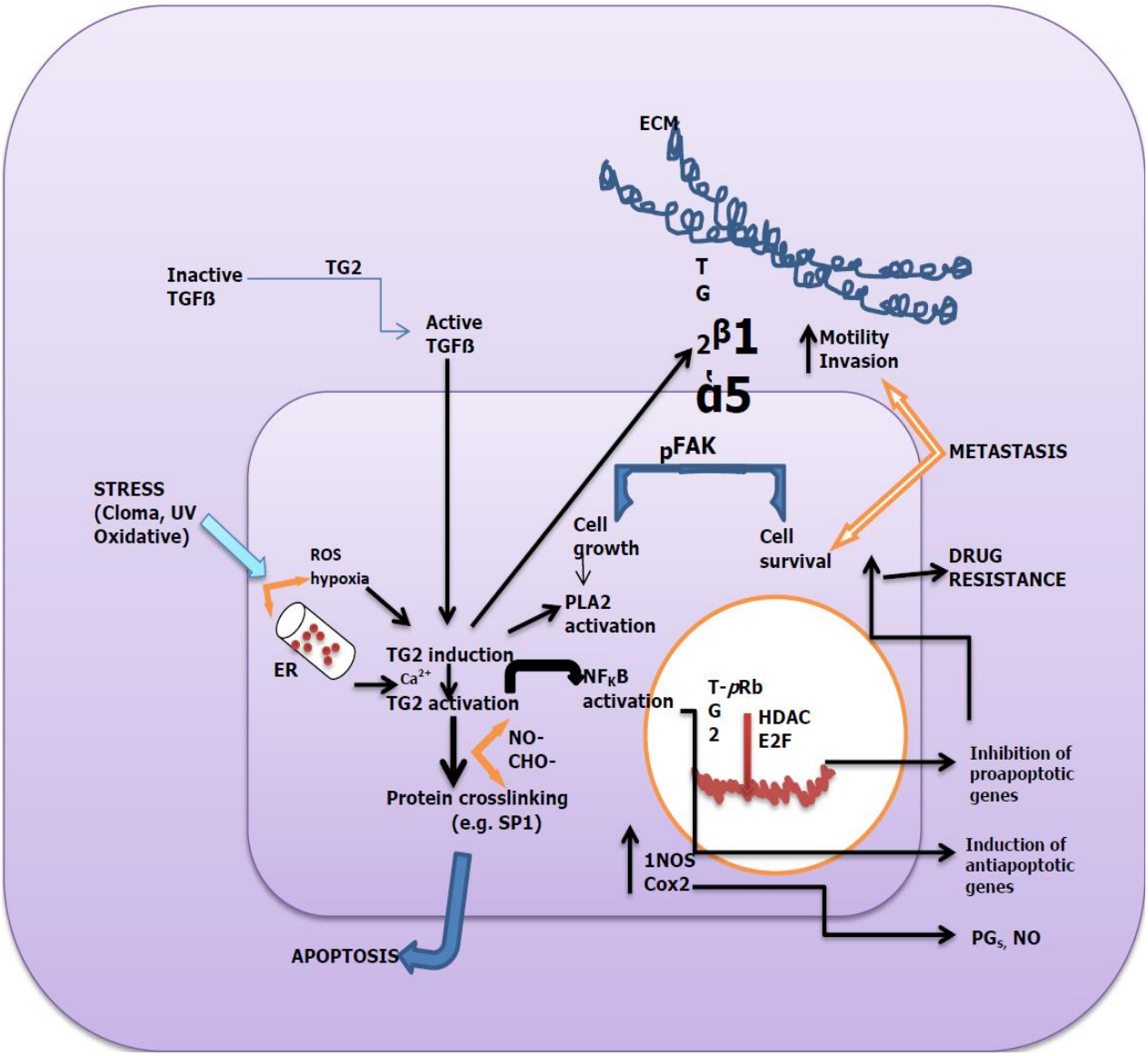


Figure 1.6: Mechanisms of TG2-mediated pro-apoptosis and anti-apoptosis. In the presence of cellular stressors such as chemotherapy or UV radiation, release of intracellular Ca^{2+} from endoplasmic reticulum (ER) results in the activation of TG2 and intracellular protein crosslinking. Consequently, apoptosis is initiated and cellular contents are prevented from spillage, hence inflammation is prevented. Conversely, the activation of TG2 can result in concomitant activation of $NF_K\beta$ and induction of anti-apoptotic genes and inhibition of pro-apoptotic genes (adapted from Mehta *et al.* 2006).

Just as TG2 could prime the cells to commit to death, so also could it protect the cells from dying. The anti-apoptotic effect of TG2 is mediated by TG2 in the nucleus and cell membrane. Nuclear TG2 protect cells from death by interacting with pRb, polymerising the alpha-inhibitory sub-unit of the transcription factor, NF-kappaB, hence, activating, transcriptional regulation of several key anti-apoptotic genes (Boehm *et al.* 2002). Similarly, TG2 can translocate to the cell membrane where it serves as a co-receptor for integrins, promoting their interaction with FN (figure 1.6). TG2-mediated interaction between integrins and FN could result to the activation of cell survival and anti-apoptotic signalling pathways, and extracellular matrix stabilisation (Mehta *et al.* 2006). Also, in the extracellular space, TG2 can maintain self-sustainability by activating latent transforming growth factor beta (TGF-β), which in turn up-regulates TG2 (Fesus & Szondy, 2005).

From the foregoing review, it is tempting to conclude that the pro-apoptotic or anti-apoptotic effect of TG2 is dependent on the activation pathways and location; with nuclear and extracellular TG2 effecting anti-apoptosis while cytosolic TG2 is pro-apoptosis in agreement with the findings of Milakovic *et al.* (2004).

1.5.2: Transglutaminase 2 in disease pathology

For the multi-functionality and ubiquitous tissue distribution of TG2, it is not surprising that its involvement in many pathological conditions has been variously demonstrated. TG2 has been shown to be involved in many chronic diseases, especially in (a) inflammatory diseases, including wound healing, tissue repair and fibrosis, and autoimmune diseases; (b) chronic degenerative diseases such as arthritis, atherosclerosis, and neurodegenerative conditions like Alzheimer's and Parkinson disease; (c) malignant diseases; and (d) metabolic diseases such as diabetes mellitus (Griffin *et al.* 2002; Facchiano *et al.* 2006). In most of these diseases, the

role of TG2 has been related to the deregulation of its functions, especially those pertinent to its interaction with, and stabilisation of cellular matrix, rather than its involvement in apoptosis.

1.5.2.1: Transglutaminase 2 in autoimmune diseases

In autoimmune diseases such as celiac disease, the presence of autoantibodies against TG2 and other substrates is an indication that TG2 may cross-link potential autoantigens to itself and to other protein substrates, in order to trigger an immunological response typical for autoimmune diseases (Sollid *et al.* 1997; Kim *et al.* 2002). TG2 function in celiac disease is related to the deamidation of the side chains of glutamine, which is abundant in gluten proteins. This deamidation reaction results to an improvement in the binding capacity of gluten to DQ2 and response of T-cell clones (Quarsten *et al.* 1999; Arentz-Hansen *et al.* 2000). Additionally, it has been reported that gluten peptides incubated with TG2 create covalent complexes through thioester bond to active site cysteine of TG2 and via isopeptide bonds to particular lysine residues of TG2 (Fleckenstein *et al.* 2004). Hence, gluten proteins and their peptide derivatives serve as substrates of various TG2-catalysed reactions (Facchiano *et al.* 2006). Recently, deamidation of gluten-derived gliadin peptides by TG2 was shown to be responsible for gliadin-induced toxicity and immune response in the small-intestinal mucosa (Rauhavirta *et al.* 2013). Consequently, Rauhavirta and colleagues suggested that the inhibition of TG2 can reduce gliadin-induced effects (Rauhavirta *et al.* 2013). In another study, Oh *et al.* (2013) reported that the initiation of allergen response in pulmonary epithelial cells requires TG2.

1.5.2.2: Transglutaminase 2 in inflammatory diseases

In inflammatory diseases, TG2 plays its role via its regulatory action on granule secretion and macrophage function, or by regulating the function of major inflammatory mediators like phospholipase A2 (Cordella-Miele *et al.* 1990). The involvement of TG2 in inflammatory diseases and related processes such as angiogenesis and wound healing has been reported (Sohn *et al.* 2003; Verderio *et al.* 2005). It is an important player in the pathogenesis of chronic inflammatory diseases like rheumatoid arthritis and osteoarthritis by transforming the latent transforming growth factor binding protein-1 into its active form, TGF-β (Nunes *et al.* 1997). Recently, TG2 has been reported to be directly involved in chronic kidney disease (CKD), where it is involved in the pathogenesis of vascular calcification through the enhancement of matrix vesicle-ECM interaction (Chen *et al.* 2013). Similarly, on analysis of TG2:creatinine ratio in relation to albumin:creatinine ratio in CKD patients, (da Silva *et al.* 2013) suggested TG2 as a potential biomarker for CKD detection and progression assessment.

1.5.2.3: Transglutaminase 2 in neurological and metabolic diseases

In vitro and/or *in vivo*, many TG2 substrates have been found in the neuronal cellular compartments, e.g. amyloid beta-A4 peptide, alphasynuclein, the microtubule-associated tau protein, synapsin I, and myelin basic protein (reviewed by Facchiano *et al.* 2006). TG2-mediated cross-linking is believed to be implicated in neurodegenerative diseases such as Huntington's, Alzheimer's and Parkinson's diseases (Kim *et al.* 2002; Bailey *et al.* 2005) and in diseases related to neurotransmitter release (Deloye *et al.* 1997). Similarly, the possible involvement of TG2 in neurotransmitter release and related pathological conditions like tetanus neurotoxin intoxication has been reported (Pastuszko *et al.* 1986; Facchiano & Luini, 1992).

1.5.2.4: Transglutaminase 2 in metabolic diseases

The covalent modification of TG2 substrates such as GAPDH, alpha-ketoglutarate dehydrogenase, phosphoglycerate dehydrogenase and fatty acid synthase (Orru *et al.* 2003) involved in energy metabolism, could account for the role of TG2 in metabolic diseases. Additionally, TG2-mediated covalent modification of hormones receptors or hormone-binding proteins is an indication that TG2-catalysed cross-linking may be involved in controlling complex metabolic responses to hormones (Sakai *et al.* 2001; Mandrusiak *et al.* 2003). The involvement of TG2 in the regulation of insulin secretion, and diabetes mellitus has also been suggested (Bernassola *et al.*2002; Bungay *et al.* 1984).

1.5.3: Transglutaminase 2 and cancer

The body of an animal is analogous to a society or an ecosystem; the constituent members are cells, which reproduce by cell division and form collaborative assemblies, i.e. tissues. However, unlike conventional human society, where survival of the fittest is the order of the day, self-sacrifice is the rule in normal cellular society. Thus, cells of a multicellular organism are subject to tightly regulated form of collaboration, apparently devoid of competition and selfishness. Consequently, each cell behaves in a socially responsible manner, and must rest, grow, divide, differentiate, or die, as needed for the good of the cellular community and the organism. The behaviours of the cells are regulated by a social control network that ensures that the cells send, receive, and interpret an elaborate set of extracellular signals- this is done via the cell cycle control system (Tlsty and Coussens, 2006; Albert *et al.* 2008). Any attempt to disobey the societal rules by a given cell or group of cells could be disastrous for the multicellular society. Most dangerously, a successful defiance of the cell cycle control system through molecular disturbances, such as mutations may result in a given cell becoming selectively advantaged, hence, growing and dividing more vigorously

and surviving more readily than its neighbours. This cell therefore, becomes the progenitor of a growing mutant clone, promoting selfishness among members of the cellular society as opposed to the original selflessness. Over time, this new wave of successive rounds of mutation, competition, and natural selection operating within the cellular population could degenerate to serious cellular conditions, characterised by over-proliferation resulting in cancer (Albert *et al.* 2008).

Cancers are heterogeneous multicellular entities constituted by cells of multiple lineages, interacting with one another, the ECM, and soluble molecules within their vicinities in dynamic fashions that favour cell proliferation, movement, differentiation, and ECM metabolism; whilst restricting cell death, stationary polarised growth and ECM stability (Tlsty and Coussens, 2006). They are cellular diseases, especially emanating from the disruption of cellular programs either intrinsically or extrinsically. For instance, genomic alterations affecting intrinsic cellular programs, such as cell cycle check-point control, apoptosis, differentiation, metabolism, and cell adhesion; or/and those affecting the extrinsic programs, such as tissue oxygenation, matrix metabolism, immune response, and vascular status (McCormick, 2004).

1.5.3.1: Roles of TG2 in definition of cancer hallmarks

Tumorigenesis in humans is a multistep process, with each step reflecting the genetic alterations that drive the progressive transformation of normal human cells into highly malignant sub-clones. Studies of human cancers and animal models have shown that the process of tumour development is analogous to Darwinian evolutionary processes, in which a succession of genetic changes, each conferring a given type of growth advantage, results to the progressive conversion of normal human cells into cancer cells (Nowell, 1976; Hanahan

& Weinberg, 2000). Hanahan & Weinberg, (2000) proposed that the vast catalogues of cancer cells' genotype are testaments of this succession of genetic alterations in cell physiology that lead to development of malignant phenotype. They classified such genetic alterations into six essential features, termed the hallmark capabilities of cancer, including sustaining proliferative signalling, insensitivity to antigrowth signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis (see figure 1.7).

TG2, as a multifunctional protein with a broad range of substrate specificity has been implicated in many genetic alterations in cellular physiology that define these hallmark capabilities in different types of cancer. The abundant distribution of TG2 in various cells of different origins and its broad substrate specificity support its involvement in definition of many important cancer cells' physiologies that encourage selfishness. TG2-related activities have been implicated in the enhancement of cell to cell interaction, ECM stabilisation, and interaction with and modification of intracellular and extracellular proteins in favour of cellular proliferation, migration, evasion of apoptosis, and insensitivity to death signals. The involvement of TG2 in the determination of these features that define the hallmark capabilities of cancer cells is discussed in the sections below.

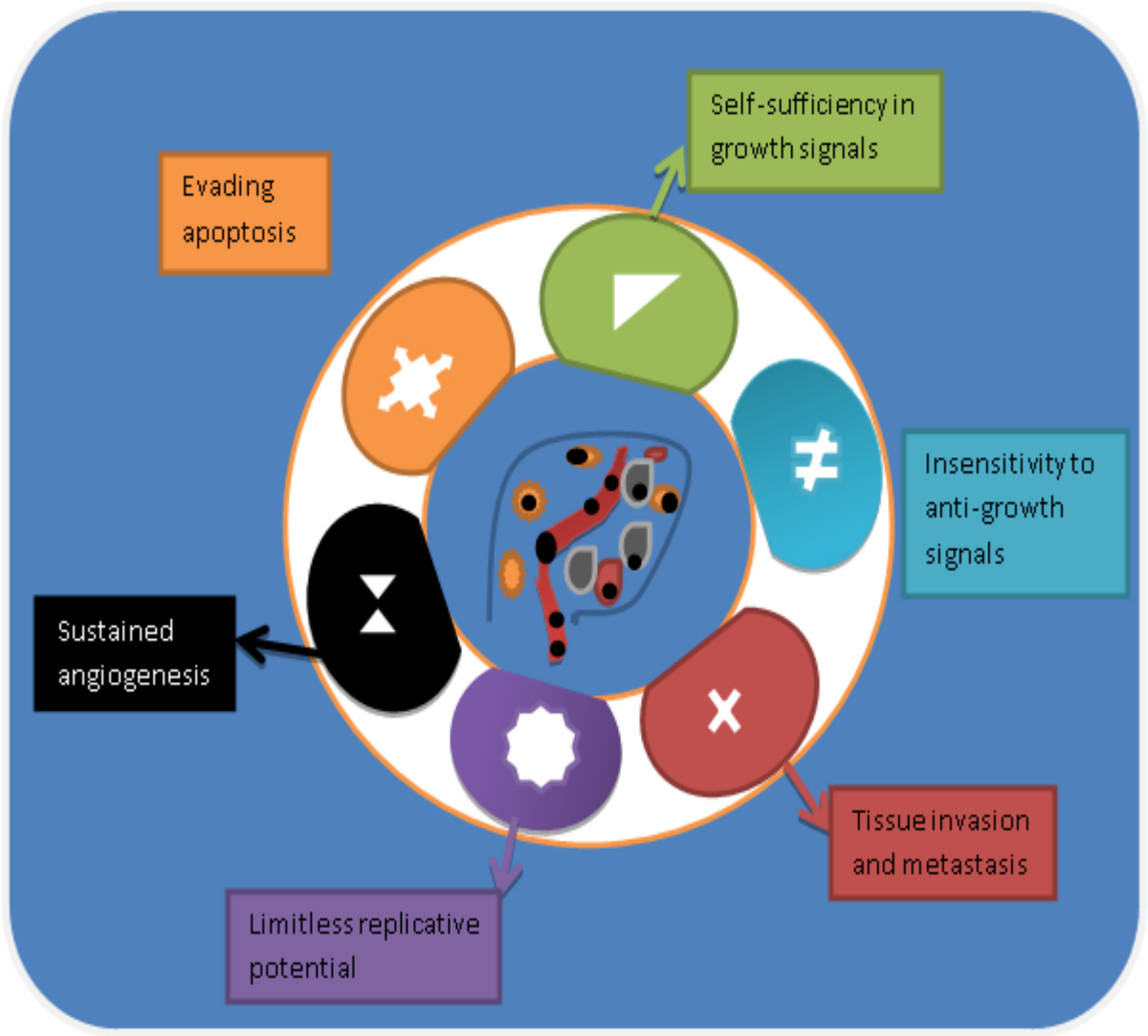


Figure 1.7: The Hallmarks of cancer as proposed by Hanahan & Weinberg, (2000), representing the acquired capabilities of cancer cells. Tumour cells defy the cell cycle control system and become insensitive to anti-growth signals, self-sufficient in growth signals, insensitive to death signals (evade apoptosis) and uncontrollably proliferative. Consequently, mutant clones accumulate in excess of the carrying-capacity of the basement membrane of the host tissue, resulting in invasion of neighbouring tissues. The need for oxygen and nutrient through blood supply triggers development of new, defective blood vessels (angiogenesis) that encourage leakage of mutant cells to distant sites (metastasis).

1.5.3.1.1: Transglutaminase 2 in cancer acquisition of self-sufficiency in growth signals

Normal cells typically move from quiescent state into active proliferative state only when there is appropriate supply of necessary mitogenic growth signals. These signals are transmitted into the cell by transmembrane receptors that interact with various classes of signalling molecules, including diffusible growth factors, ECM components, and inter-cell adhesion/interaction molecules, including TG2 (Witsch *et al.* 2010). In one hand, the role of TG2 in growth promotion and maintenance of self-sufficiency in tumour cells could be attributed to its activation of the growth factor, TGFβ leading to promotion of cell growth and survival. On the other hand, TG2 can be involved in tumour growth sufficiency through its interactions with various adhesion molecules, including integrin and FN, resulting in stabilization of extracellular matrix and activation of cell survival signalling (Mehta *et al.* 2006). The production and release of growth-promoting signals are carefully controlled in normal tissues, ensuring the homeostasis of cell number and maintenance of normal tissue architecture; whilst entering into and progressing through the cell growth and division cycles (Hanahan & Weinberg, 2011). One of the fundamental features of cancer cells is their acquired ability to sustain proliferation, as they mostly show reduced dependence on stimulation from their normal tissue microenvironment. They maintain self-sufficiency in growth signal by dysregulating the mitogenic signals to their own advantage; thus, becoming independent of exogenous signals (Hanahan & Wienberg, 2000; Witsch *et al.* 2010).

1.5.3.1.2: Transglutaminase 2 in tumour insensitivity to antigrowth signals

To maintain cellular quiescence and tissue homeostasis, myriads of anti-proliferative signals operate within a normal tissue. These antigrowth signals include both soluble growth inhibitors and immobilised inhibitors both in the ECM and on the surfaces of neighbouring cells. They are received by transmembrane cell surface receptors within the intracellular

signalling circuits; inhibiting proliferation via two discrete mechanisms. One mechanism involves forcing cells into quiescent (G_0) state, from which they could regain proliferative feature when the extracellular signals become favourable. Alternatively, cells may be compelled to infinitely relinquish their proliferative potentials by being induced into post-mitotic state (Hanahan & Wienberg, 2000; Deshpande *et al.* 2005).

Besides their acquired capability of inducing and sustaining proliferation-promoting signals, cancer cells have the tendency to evade anti-proliferative signals. Much of the circuitry that determines the ability of normal cells to respond to antigrowth signals is associated with the cell cycle clock, especially the parts governing cellular transit through the G_1 phase of its growth cycle. During this period, cellular decision to enter into proliferative or quiescent or post-mitotic state is dependent on the sensed signals from the external environment (Hanahan & Wienberg, 2000). At the molecular level, most anti-proliferative signals are funnelled through the retinoblastoma protein (pRb), which is regulated by nuclear TG2 (Kuo *et al.* 2011). In a hypophosphorylated state, pRb inhibits proliferation by altering the functions of transcription factors responsible for controlling the expression of catalogue of genes necessary for transition from G_1 to S phase of the cell cycle (Weinberg, 1995; Burkhart & Sage, 2008). Additionally, TG2 has been shown to modulate pRb, depending on its phosphorylation state, leading to cell cycle arrest (Boehm *et al.* 2002), and possible transition to quiescence.

1.5.3.1.3: Tumour cells' evasion of apoptosis: implications of TG2

Over the past two decades, the idea that programmed cell death by apoptosis naturally serves as a barrier to cancer development, has been established by functional studies (Adams and Cory, 2007, Hanahan and Weinberg, 2011, and references therein). Elucidation of the

signalling pathways of apoptosis has revealed how apoptosis is ignited in response to various physiologic stresses undergone by cancer cells in the course of tumorigenesis, or those due to anticancer therapy. Such apoptosis-inducing stresses include signalling imbalances emanating from elevated levels of oncogene signalling, and DNA damage associated with hyper-proliferation. However, other research has shown apoptosis is attenuated in those tumours that successfully progress to advanced states of malignance and resistance to therapy (Lowe *et al.* 2004; Adams and Cory, 2007).

Cancer cells can acquire the ability to resist apoptosis through various strategies. The most prominent strategy is through the loss of p53 tumour suppressor function, with the resultant removal of a key component of the DNA damage sensor capable of inducing the apoptotic cascade (Harris, 1996). Alternatively, tumours may adopt the strategy of increasing expression of anti-apoptotic regulators (Bcl-2, Bcl-x_L) or of survival signals, by down-regulating pro-apoptotic factors (Bax, Bim, Puma), or short-circuiting the extrinsic ligand-induced death route. The multiplicity of apoptosis-evading mechanisms reflects the diversity of apoptosis-inducing signals encountered by cancer cell populations during their transition to the malignant state (Hanahan and Weinberg, 2011).

TG2 has been shown to be involved in these multiple apoptosis-evading mechanisms. For instance, Boehm *et al* (2002) reported that nuclear TG2 exerts anti-apoptotic effect by up-regulating retinoblastoma protein pRb, leading to the polymerization of the alpha-inhibitory sub-unit of the transcription factor NF-kappaβ and concomitant cell protection from apoptosis with the help of other key anti-apoptotic proteins. Also, TG2 can translocate to the plasma membrane where it serves as a co-receptor for integrin, promoting its interaction with FN,

resulting in the activation of cell survival and anti-apoptotic signalling pathways (as reviewed in Odii and Coussons, 2014).

1.5.3.1.4: Acquisition of unlimited replicative potential by cancer cells: implications of TG2

For cancer cells to generate macroscopic tumours, they require unlimited replicative potential (Hanahan and Weinberg, 2011). Limitless replicative potential of cancer cells is dependent on three acquired capabilities – growth signal autonomy, insensitivity to antigrowth signals, and apoptotic resistance, all of which lead to an uncoupling of cell's growth program from the prevailing signals in its environment (Hanahan and Weinberg, 2000). The unlimited replicative capability of cancer cells remarkably contrasts the behaviour of the cells in most normal cell lineages in the body, which are only able to pass through a limited number of successive cell growth-and-division cycles (Hanahan and Weinberg, 2011). This limited replication ability exhibited by normal cells is mediated by two distinct barriers to cell proliferation: senescence, cell transition to irreversible non-proliferative but viable state, and crisis, which involves cell death (Hayflick, 1997).

When cells are propagated in culture, cellular senescence is first induced by repeated cell division cycles and subsequently, cells that are able to circumvent senescence will enter crisis phase, in which most of the cells in the population die. Rarely, cells from a population in crisis survive and assume unlimited replicative potential - immortalization, a feature possessed by most established tumour cell lines due to their ability to proliferate in culture without evidence of senescence or crisis (Hayflick, 1997; Hanahan and Weinberg, 2011). This is an indication that limitless replicative potential (immortalization) is a phenotype acquired by cancer cells *in vivo* during tumour progression and could be vital to their

development into malignant growth state (Hayflick, 1997). By implication, at some point during the course of multistep tumour progression, developing premalignant cell populations usually resort to evasion of mortality barrier, and assume unlimited replication so as to achieve tumorigenesis.

TG2 has been variously reported to enhance cancer cells' development of stem cell phenotype, through the induction of epithelial mesenchymal transition (EMT) and consequent activation of survival signalling molecules, including FAK, Akt, and NF-ƙβ (as reviewed by Mehta *et al.* 2010). Additionally, Kumar *et al* (2011, 2012) reported that TG2-expressing mammary epithelial cells showed increased tendency to form mammospheres, self-renewal ability, and plasticity (unlimited replication). Consequently, Agnihotri *et al.* (2013) suggested that sustained expression of TG2 leads to the induction of EMT and stem cell-like characteristics in breast cancer cells, contributing to development of drug-resistant and metastatic phenotypes.

1.5.3.1.5: Transglutaminase 2 in angiogenesis:

In normal tissues, oxygen and nutrients supplied by the vasculature are essential for cell survival and function; hence, it is obligatory for virtually all cells in a tissue to reside within 100μm of a capillary blood vessel (Hanahan and Weinberg, 2000). Tumour microenvironments are mostly characterised by poor vascularisation and consequent deficiency in oxygen and nutrient supplies. However, like normal tissues, tumours require sustenance in the form of oxygen and nutrients just as they need to get rid of waste metabolites and carbon dioxide (Hanahan and Weinberg, 2011). Consequently, tumours tend to abrogate these deficiencies by generating tumour-associated neo-vasculature through the process of angiogenesis.

During embryogenesis, vasculature development involves the birth and assembly of new endothelial cells into tubes, in addition to the development of new vessels from pre-existing ones. Subsequent to this morphogenesis, the normal vasculature becomes largely quiescent (Hanahan and Weinberg, 2011). As part of the physiologic processes in the adult, as in the cases of female reproductive cycling and wound healing, angiogenesis is transiently turned on. However, the process of tumour progression contrasts the transient switching in normal physiological scenario, as an angiogenic switch is almost always activated and remains on, resulting in normally quiescent vasculature to resort to sustained angiogenesis in order to keep with the needs of expanding tumour growth (Hanahan and Folkman, 1996; Baeriswyl and Chistofori, 2009).

The formation of new blood vessel is dependent on changes in the behavioural features of endothelial cells, particularly their proliferation, migration, and differentiation into tubular structures, which is influenced by changes in the ECM (Jones *et al.* 2006). TG2 is abundantly distributed in endothelial cells (Korner *et al.* 1989), and there have been many reports suggesting the importance of TG2 in the angiogenic process (Griffin *et al.* 2002). It is well known that many ECM proteins serve as TG2 substrates (Aeschlimann and Thomázy, 2000), and the crosslinking of these proteins by endothelial cells' TG2 result in the stabilisation of the basement membrane (Martinez *et al.* 1994). Recently, Wang *et al.* (2013) reported that angiogenesis is attenuated in cell culture, the aorta ring assay and *in vivo* models following the inhibition of the crosslinking activity of extracellular TG2 or down-regulation of its expression. They further posited that inhibition of the activity of extracellular TG2 in human umbilical vein endothelial cell (HUVEC) co-culture model can halt angiogenic progression, even after the commencement of tubule formation and in the presence of excess vascular endothelial growth factor (VEGF). Additionally, Wang and colleagues suggested that down-

regulation of TG2 expression by short hairpin (shRNA) inhibited HUVEC migration and tubule formation (Wang *et al.* 2013), hence, TG2-related activity has an angiogenic role.

1.5.3.1.6: Development of invasion and metastasis phenotype by cancer cells: implications of TG2

During tumour development, aggregate of primary tumours tend to amass within the confines of the basement membrane of the host tissue until the carrying-capacity of the membrane is exceeded, with resultant breakage of the membrane. Consequently, neighbouring tissues are invaded by the tumours, which thence, migrate to distant sites where they may successfully establish as new colonies – metastasis (Albert *et al.* 2008). The invasive and metastatic capabilities of cancer cells enable them to escape the primary tumour site and colonise new body areas devoid of nutrient deficiency and space limitation. Similar to the primary tumour formation, successful invasion and metastasis are dependent on other acquired hallmark capabilities (Hanahan and Weinberg, 2000). The role of TG2 in tumour invasion is reviewed in section 1.5.3.2 below.

1.5.3.2: Transglutaminase 2 in cancer drug resistance and metastasis

Exhibition of apoptotic resistance is a common characteristic of advanced cancers (Srinivasan *et al.* 1996). This feature not only gives the tumour cells the ability to metastasise but also the ability to develop a drug-resistant phenotype (Lundin *et al.* 2003). In essence, drug resistance and metastasis share many features in common. For example, tumour cells selected for drug resistance *in vitro* are more metastatic *in vivo.* Conversely, metastatic tumours generally show higher resistance to chemotherapy than their primary counterparts (Mehta *et al.* 2010). Transglutaminase 2 is involved in the modulation of apoptosis and cell fate through many crucial cellular functions (reviewed in section 1.5.1). When aberrantly regulated, TG2 is

thought to have a role in cancer cell's ability to evade apoptosis. Evidently, there seems to be direct connection of TG2 with cancer drug resistance (Mehta, 1994; Chen *et al.* 2002) and mechanism of metastatic progression (Mehta *et al.* 2004).

Many studies have demonstrated elevated TG2 expression as a hallmark of many types of cancer cells, including pancreatic carcinoma (Verma *et al.* 2006), ovarian carcinoma (Satpathy *et al.* 2007; Hwang *et al.* 2008), malignant melanoma (Fok *et al.* 2006), lung carcinoma (Park *et al.* 2010), glioblastoma (Yuan *et al.* 2007), and breast carcinoma (Mehta *et al.* 2004). For instance, Iacobuzio-Donahue *et al* (2003) on analysing the genes from tumour samples observed that out of over 30,000 genes analysed, TG2 was among those that recorded the highest expression in pancreatic carcinoma. Similarly, Jiang *et al* (2003), while attempting to identify metastasis-associated proteins through proteomic analysis, observed that TG2 was one of the eleven proteins that were constitutively elevated in metastatic human lung carcinoma. In another development, Antonyak *et al* (2004) showed that cancer cells treated with epidermal growth factor (EGF) expressed high levels of TG2 and were consequently, protected cells from doxorubicin-induced apoptosis. These observations are strong reflectors of the implications of aberrant TG2 expression in the conferment of apoptotic resistance and consequent drug resistance and metastatic potentials of cancer cells.

Park *et al* (2009) reported that TG2-specific cross-linking activity resulted in the polymerization and inhibition of nucleophosmin, and concomitant increase in drug resistance potential of cancer cells. Recent evidence shows that aberrant expression of TG2 in mammary epithelial cells bestows stem cell characteristics on the cells (Kumar *et al.* 2011). Similarly, Kumar and colleagues reported that high basal expression of TG2 in breast cancer cells promotes the development of stem cell features, but did not encourage their terminal

differentiation (Kumar *et al.* 2011). Additionally, Caffarel *et al* (2013) observed that the activation of TG2:integrin-α5ß1 interactions through the stimulation of oncostatin M receptor in cervical squamous cell carcinoma, induced pro-malignant changes.

Clinically, TG2 has been reported to serve as a predictive indicator of anticancer therapeutic efficacy. For instance, Jeong *et al* (2013) suggested that TG2 expression is a promising indicator of the effectiveness of epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) therapy in patients suffering from non-small cell lung cancer. Similarly, Assi *et al.* (2013) reported that the accumulation of TG2 in tumour stroma can serve as an independent risk factor for the identification of invasive ductal carcinomas (IDCs) of breast, and can establish breast cancer patients at high risk of recurrence. They also observed that overexpression of TG2 can serve as an indicator of poor prognosis for IDC of the breast. Agnihotri *et al.* (2013) proposed that inflammation-induced progression of breast cancer and acquisition of survival and invasive capabilities by breast cancer cells are mediated by TG2. In acute myeloid leukemia, Pierce *et al* (2013) demonstrated that increased expression of TG2 characterized a more advanced state of the disease in relapse patients. They further established that increased TG2 expression correlates with the expression of proteins involved in apoptosis, motility and extracellular matrix association; processes that have been linked with leukemia development and progression.

Metastatic tumours from patients with breast carcinoma (Mehta *et al.* 2004), malignant melanoma (Fok *et al.* 2006), and ovarian carcinoma (Satpathy *et al.* 2007; Hwang *et al.* 2008) have been shown to express higher level TG2 relative to their primary counterparts. Conversely, TG2 down-regulation or inhibition by small interfering RNA (siRNA), antisense RNA, ribozyme, or small molecule inhibitors have been shown to increase the susceptibility

of various cancer cell types to chemotherapy-induced cell death, and to inhibit invasion, both *in vitro* and *in vivo* (Verma & Mehta, 2007; Hwang *et al.* 2008; Verma *et al.* 2008). Satpathy *et al.* (2007) observed that increased TG2 expression promoted the adhesion of ovarian cancer cells to FN and facilitated directional cell migration, while TG2 down-regulation in similar cells decreased tumour dissemination on the peritoneal surface and in mesentery in an intra-peritoneal ovarian xenograft mouse model. Collectively, these observations strongly support that overexpression of TG2 may confer resistance to chemotherapeutic drugs and promotes the invasive potential of malignant cells.

1.6: CISPLATIN AND 5-FLUOUROURACIL (5-FU) IN LIVER CANCER THERAPY

1.6.1: Hepatocellular carcinoma

Worldwide, human hepatocellular carcinoma (HCC) is the most frequent type of malignant liver tumour, rated as the third leading cause of cancer-related death in adults; with over 600,000 deaths annually (Anderson *et al.* 1992; Parkin *et al.* 2005). It accounts for up to 90% of all primary liver tumours, with its incidence predominating in Southeast Asia and sub-Saharan Africa (Kumar *et al.* 2011). For most HCC patients, the disease is usually accompanied by liver cirrhosis, which is a major risk factor for hepatic cancer and is correlated to viral infection due to hepatitis B or C virus. However, non-viral cirrhosis such as alcoholic, heamochromatosis and primary biliary cirrhosis are also associated with increased risk of liver tumour (as reviewed in Tomuleasa *et al.* 2010). Due to its close relationship to the growing incidence of liver cirrhosis, HCC incidence is increasing globally and over the next two decades, mortality and incidence are expected to double (Marin *et al.* 2008; Rampone *et al.* 2009).

1.6.2: Cisplatin and 5-FU therapy

Clinically, a platinum compound, cisplatin, and an antimetabolite, 5-fluourouracil are the most commonly used and most successful combined treatment regimen for advanced hepatocellular carcinoma patients (Nagai & Sumino, 2008). Various doses of cisplatin and 5-FU combination regimen have been reported to be successfully administered in patients with advanced HCC, either as long-term, low dosage or short-term high dosage, through repetitive hepatic arterial infusion chemotherapy (HAIC) (Ando *et al.* 2002; Park *et al.* 2007). On a long-term, low dose serial courses of HAIC, Ando *et al.* (2002) reported a response rate of 48% after four courses, using a treatment regimen consisted of daily cisplatin (7 mg/m^2 for 1 hour on days 1-5) followed by 5-FU (170 mg/m^2 for 5 hours on days 1-5) per course. However, similar response rate of 48% was recorded by Park *et al.* (2007) using repetitive 3 days short course of HAIC with high dose 5-FU (500 mg/m^2 on days 1-3) and cisplatin (60 mg/m^2 on day 2). Thus, hepatic arterial infusion chemotherapy using cisplatin and 5-FU is an effective treatment option for patients with advanced HCC.

Cisplatin (cis-diamminedichloroplatinum (CDDP) has been used as a chemotherapeutic agent in many cancers, especially in testicular cancer and epidermal carcinomas of many organs, for which treatment is very successful (Wang & Lippard, 2005). Treatment of human hepatocellular carcinoma with cisplatin has shown more effectiveness than any other anti-neoplastic agents, and when combined with 5-FU, cisplatin has been shown to induce additive and synergistic results (Tanioka *et al.* 2003; Okamura *et al.* 2004). 5-fluorouracil (5-FU), a pyrimidine antimetabolite, is one of the first-line treatment options for gastrointestinal tumours and represents the most widely used chemotherapeutic agent in the management of hepatocarcinoma (Li *et al*. 2004). Regardless of the direct independent anti-tumour abilities of cisplatin and 5-FU, cisplatin synergistically acts as a modulator of 5-FU through the

inhibition of neutral amino acid uptake into the cells with concomitant enhancement of the antineoplastic activity of 5-FU (Scanlon *et al* 1989; Shirasaka *et al.* 1993). Consequently, the combined use of cisplatin and 5-FU provides room for the use of lower doses to achieve optimum effectiveness with fewer side effects (Nagai & Sumino, 2008).

1.6.3: Mechanism of action of cisplatin

Chemically, the molecular structure of cisplatin (figure 1.8) is made up of a central platinum atom surrounded by two chlorine atoms and two ammonia groups in a *cis* configuration (Page *et al.* 1985). Core platinum compound and a *cis* configuration are common denominators between cisplatin and other platinum-derived drugs; however, they are differentiated by the variation in their leaving groups (as reviewed by Barabas *et al.* 2008). Cisplatin was first described in 1845 by Michel Peyrone (when it was referred to as Peyrone's salt); and its structure was established in 1893 by Alfred Werner. However, the antineoplastic capability of cisplatin was discovered in the 1960s following the observations by Rosenberg and colleagues, that it has the capacity to inhibit bacterial fission (Rosenberg *et al*., 1965) and the growth of sarcomas transplanted in mice (Sancho-Martínez *et al.*, 2012). Hill and colleagues demonstrated its efficacy against several human malignancies (Hill *et al*., 1975), and it was first approved for clinical use in 1978 (Hill and Speer, 1982).

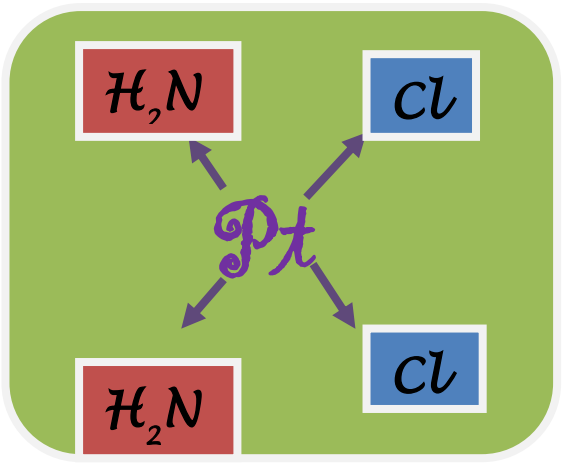


Figure 1.8: A representation of the molecular structure of cisplatin, showing central platinum (Pt) atom surrounded by two chlorine (Cl) atoms and two ammonia (H_2N) atoms in *cis* configuration.

Pharmacodynamically, cisplatin activation involves an aquation reaction resulting from the exchange of two chloride-leaving groups with water or hydroxyl ligands (Rosenberg, 1979). In the presence of high chloride concentration (as in isotonic saline or extracellular fluid), cisplatin remains neutral and biologically inactive, hence the aquation reaction does not take place (Rosenberg, 1979; Litterst, 1984). However, intracellular fluid has about one-thirteenth the chloride concentration of extracellular fluid, and such condition favours aquation reaction with resultant DNA damage (Rosenberg, 1979).

Cisplatin primarily inhibits DNA synthesis in cancerous cells through the formation of adducts, with its damage to DNA assuming a similar fashion to that caused by alkylating agents (Barabas *et al.* 2008 and references therein). Upon aquation of the platinum compound, the two chloride groups and replaced with water and will bind to two sites in DNA (figure 1.9), forming DNA adducts (if the binding sites are on same DNA strand) or DNA cross-link (if binding sites are on different DNA strands) (Reed, 2006).

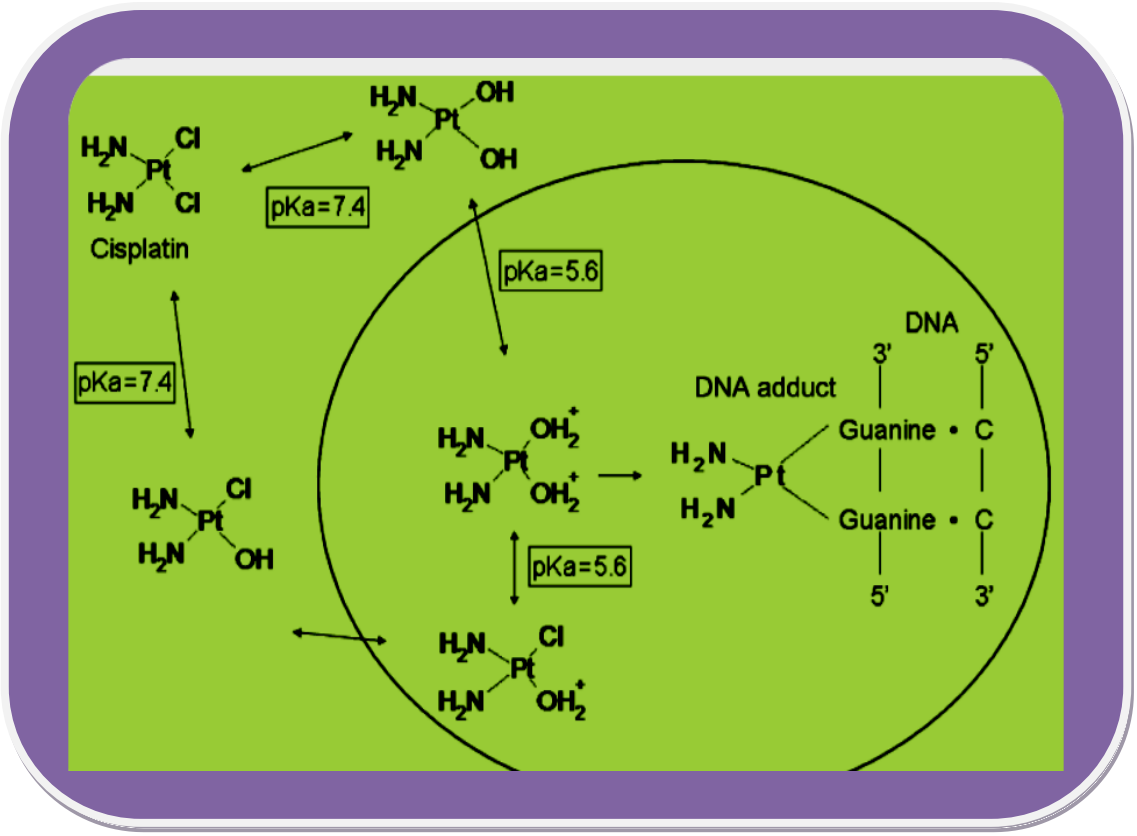


Figure 1.9: Representation of the pharmacodynamics of cisplatin, showing how cancerous cells are killed by DNA damage resulting from DNA adducts formation through aquation reaction (Barabas *et al.* 2008).

1.6.4: Mechanisms of 5-FU action

The anticancer effects of 5-fluourouracil (5-FU) occurs through two mechanisms (figure 1.10): (a) inhibition of DNA synthesis through the inactivation of thymidylate synthase via the formation of a complex between methylenetetrahydrofolate (CH2FH4) and 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), which is synthesized from 5-FU. (b) Interference with RNA metabolism by blocking the uptake of phosphated 5-fluorouridine 5'-triphosphate into RNA (reviewed by Nagai & Sumino, 2008).

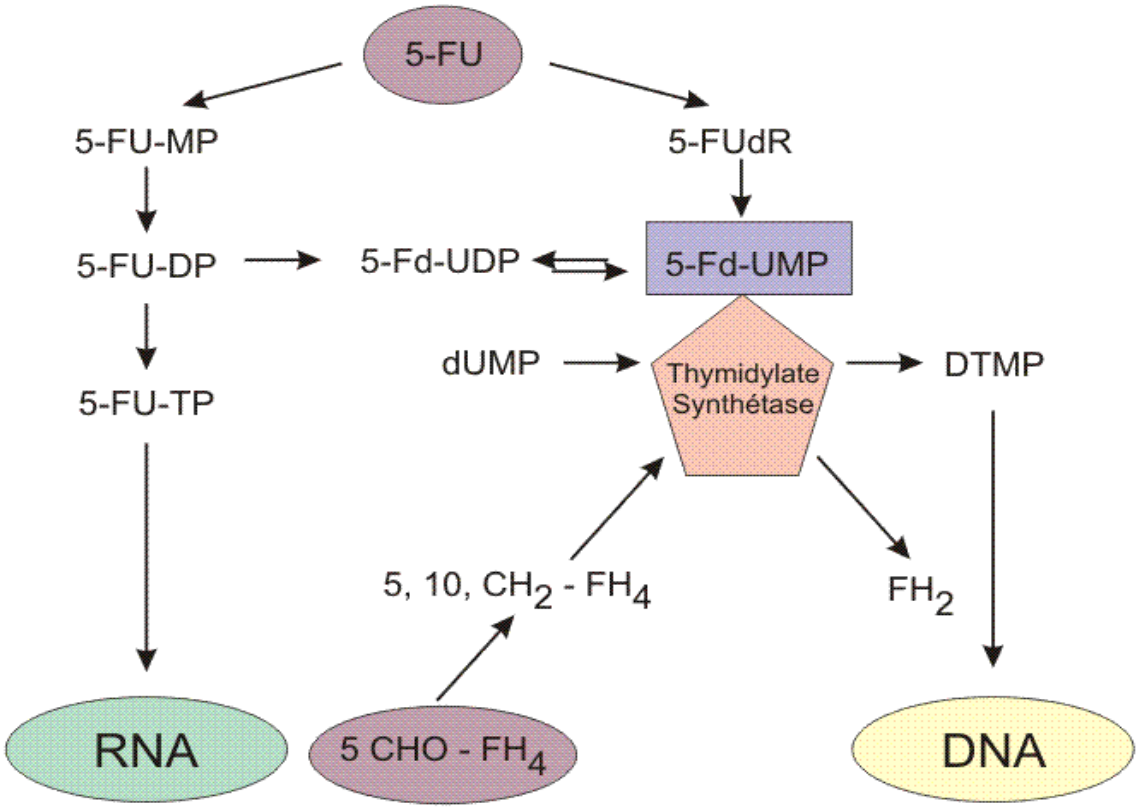


Figure 1.10: Antineoplastic mechanisms of 5-FU, RNA dysfunction and inhibition of DNA synthesis. Metabolism of 5-FU follows a reduction in 5-fluordeoxyuridine 5'monophosphate (FdUMP), which binds to thymidylate synthetase (TS) and blocks the methylation of uracil towards thymine. Phosphorylation of 5-FU to triphosphate (FUTP) and its incorporation in RNA instead of uracil results in blockage of RNA transcription (Nagai & Sumino, 2008).

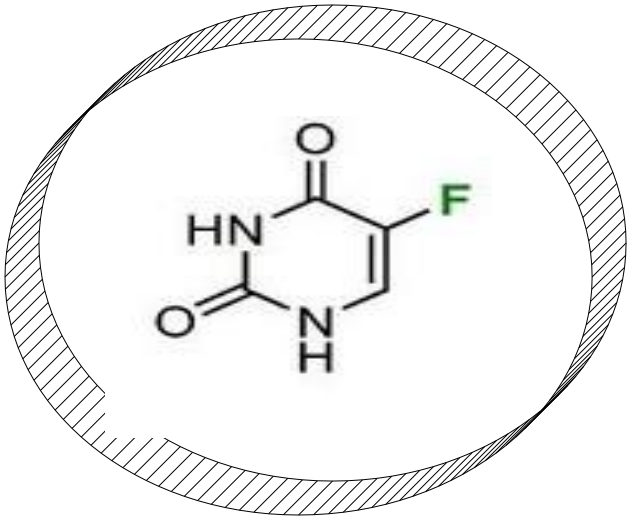


Figure 1.11: The molecular structure of 5-fluourouracil (Nagai & Sumino, 2008).

1.7: FURTHER PERSPECTIVES

Regardless of the wide range of biological functionalities associated with TG2, amidst its unique cellular biochemistry, its exact physiological function remains unclear. This could be substantiated by the fact that homozygous deletion of TG2 in mouse does not result in an embryonic lethal phenotype (De Laurenzi & Melino, 2001; Nanda *et al.* 2001); suggesting a compensation for its absence by other family members. However, Bernassola *et al.* (2002) observed that TG2- deficient mice displayed characteristic glucose intolerance and hyperglycaemia due to reduced insulin secretion, a condition equivalent to a subtype of diabetes called maturity-onset diabetes of the young (MODY). In humans, TG2-deficiency disease is yet to be identified.

1.7.1: Compensation for TG2

Whilst there is existing evidence suggesting the possible compensation for the absence of TG2 by another member of the transglutaminase family, it is rational to think that the enzyme is actually involved in many physiological processes. This could be substantiated by the understanding that TG2 is relatively more abundant than other members of Tgase family. Consequently, its wide tissue distribution and the possession of specialised functional structure that allows for flexibility of interaction with assorted proteins are some of the factors that give TG2 a physiological advantage over other Tgases. Additionally, it is appropriate to argue that the compensation for TG2 function could only be possible for a role that is determined by its cross-linking activity, which is a common feature of the transglutaminase family. For example, TG2-mediated functions that are independent of its cross-linking actions such as its role as a G-protein, and regulation of energy metabolism, are seemingly impossible roles to be undertaken by any other member of the Tgase family. Similarly, TG2-mediated integrin-FN interaction which is one of the major primary routes of

extracellular survival signalling activation and consequent apoptotic evasion; is independent of TG2 cross-linking activity and could not be compensated for by another Tgase (reviewed by Odii & Coussons, 2014).

1.8: RESEARCH AIMS AND OBJECTIVES

Primary liver cancers, especially human hepatocellular carcinoma (HCC), are associated with very low survival rate and high mortality rate, occasioned by high degree of chemo-resistance. TG2 is highly abundant in human liver cells, hence, its common name, "liver transglutaminase". For advanced HCC patients, intra-arterial combination chemotherapy is one of the few successful options, and continuous use of cisplatin /5-fluourouracil (5-FU) has been shown to prolong such patients' survival and yield highest response rate of 47% (Marin *et al.* 2008).

From the foregoing review, the involvement of TG2 in cancer drug resistance and metastasis has become evident. Ironically, however, amidst the low survival rate of HCC due to its specialised ability to resist chemotherapy, the abundance of TG2 in liver cells and reports of its involvement in the development of chemotherapeutic resistance and metastatic potentials by many cancer types; its role in liver cancer remains to be determined. Consequently, the investigation of TG2 abundance in liver cells, its link with drug resistance/metastasis in several cancer types, and high mortality rate of HCC due to chemo-resistance are some of the rationales for this research.

1.8.1: Research aims

1. Investigation of the expression and activity profiles of TG2 in parental and drug-resistant hepatocarcinoma cell lines with the view to gaining insight into its role in liver cancer drug resistance and metastasis.
2. Development of an in vitro model of TG2-based liver cancer therapy that may subsequently be extended to an in vivo mouse model, and possibly clinical applications in the future.

1.8.2: Research objectives

1. To develop a simplified model of drug-resistant HCC that could allow for a probe into its mechanism of resistance and possible metastatic potential.
2. To investigate the behaviour of TG2 in response to different stress conditions in different cell lines.
3. To investigate the effects of down-regulation of TG2 expression by RNA interference and inhibition of its activity in parental and drug-resistant HEPG2 cell lines, with the views to understanding the exact implications of the protein in liver cancer drug resistance and metastasis.

CHAPTER TWO

ESTABLISHMENT OF DRUG-RESISTANT CLONES OF HEPATOCELLULAR CARCINOMA (HEPG2) CELL LINE

2.1: INTRODUCTION

Patients whose liver tumours are at advanced stage are mostly refractory to chemotherapy, resulting in disease progression and death (Tomuleasa *et al.* 2010). The etiological agents of HCC are not well known and its molecular and cellular pathogeneses are yet to be properly understood (Tomuleasa *et al.* 2010), probably due to the complex routes through which the disease can originate. In recent years, there has been an emerging model for the development of drug-resistant tumours that invokes a pool of immortal, self-renewing malignant progenitors called tumour initiating cells or cancer stem cells (CSCs) (Tomuleasa *et al.* 2010; Mondello *et al.* 2011).

Clinically, the critical problem of the emergence of tumour recurrences after therapy has been ascribed to the inherent high resistance ability of CSCs to chemotherapy (Singh *et al.* 2004; Al-Hajj *et al.* 2004). Hence, irrespective of the shrinkage of bulk of the tumour, the remaining recalcitrant CSCs can eventually reproduce the entire malignant phenotype (Clarke & Fuller, 2006; Dalerba *et al.* 2007). Like other cancers, HCC is composed of heterogeneous cell populations; with subset of cells (CSCs) peculiarly characterised by the ability to induce tumours when grafted into host animals (self-renewal and immortality), giving rise to differentiated progeny (Mondello *et al.* 2011).

Many resistant tumour cells in humans are gradually acquired during chemotherapeutic administration and isolation of CSCs based on chemoresistance can provide vital tools for the

validation of drugs targeted at them (Masters, 2000). In addition to their isolation based on chemotherapeutic resistance, tumour initiating cells can also be isolated based on other conditions, including specific surface marker repertoires, ability to exclude fluorescent dyes, or particular culture conditions (Mondello *et al.* 2011). Drug-resistant cell lines, selected by exposure to anticancer agents therefore may serve as valuable tools for the illumination of factors underlying drug resistance (Yan *et al.* 2007). The development of good experimental model of drug resistant cell line is therefore, a prerequisite for any study to understand the cellular mechanisms that determine drug resistance.

Tumour initiating cells were originally isolated in leukemic tumour and then in solid tumours (Bonnet & Dick, 1997; Hemmati *et al.* 2003; Collins & Maitland, 2006; Li *et al.* 2007). Since the advent of drug-based selection of drug-resistant cell lines, various cell types have been successfully selected for resistance pharmacologically. For instance, human leukemic (KG1a) cell line selected against 5-fluorouracil (Zhang *et al.* 2010); breast cancer cell line selected against doxorubicin and nicotine (Calcagno *et al.* 2010; Hirata *et al.* 2010); ovarian cancer cell line selected against cisplatin and paclitaxel (Ma *et al.* 2010; Bapat, 2010); prostate cancer cell line selected against inorganic arsenic (Achanzar *et al.* 2002; Benbrahim-Tallaa & Waalkes, 2008; Tokar *et al.* 2010); and human lung cancer (H460) cell line against cisplatin or doxorubicin (Levina *et al.* 2008). For HCC, the first isolation of tumour initiating cells was reported by Sell *et al.* (2002) and more recently Tomuleasa *et al.* (2009). However, they did not select the cells based on clinical treatment patterns. This approach was recently reported by Odii & Coussons (2012), where selection of drug-resistant HCC was achieved based on chemotherapeutic resistance by mimicking clinical treatment pattern for hepatocarcinoma.

Cisplatin and 5-fluourouracil (5-FU) are frequently used but are mechanistically different antineoplastic agents with wide range of anti-tumour activities. The use of cisplatin and 5-fluourouracil in liver cancer treatment has been discussed earlier (see section 1.6).

2.2: MATERIALS AND METHODS

2.2.1: Pharmacologic agents

Cisplatin and 5-FU (Sigma Aldrich, UK) were dissolved in double distilled water to yield working stocks at concentration of 2mM and 20mM respectively. The working stocks were stored at room temperature, and diluted into cell culture media as required. Working stocks were kept away from light and discarded after one month.

2.2.2: Cell line and culture conditions

The parental human hepatocellular carcinoma (HEPG2) cell line was obtained from the European Collection of Animal Cell Cultures (ECACC). Cells were cryopreserved and then rapidly thawed and grown in RPMI 1640 medium (Invitrogen, UK), fully supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen, UK), 1% glutamine (Invitrogen, UK) and 1% penicillin streptomycin (Invitrogen, UK). The physiological conditions of the cells were maintained at 5% CO_2 and 37ºC in a humidified atmosphere. Logarithmically growing cells were at the second passage when they were cryopreserved with a freezing medium containing 10% dimethylsulphoxide (DMSO) (Sigma Aldrich, UK) and 90% supplemented RPMI 1640 medium (Invitrogen, UK). The freezing vials containing the cells were cryo-preserved in a cryovial containing isopropanol and maintained at -80ºC for 24 hours; subsequently, the vials containing the parental cells were stored and maintained as working stock immersed in liquid nitrogen at -196ºC.

2.2.3: Assay of HEPG2 cells' susceptibility to cisplatin- and 5-FU-induced death

The susceptibility of HEPG2 cells to chemotherapy-induced death and the degrees of cytotoxicity of cisplatin or 5-FU on the cells were measured using cell counting kit (CCK-8) (Sigma Aldrich, UK). The CCK-8 is made up of Dojindo's highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which produces a water-soluble, yellowish formazan dye upon reduction in an electron carrier (Shibata *et al.* 2006). The number of viable cells is directly proportional to the amount of the formazan dye generated in cells.

Assays were conducted following the manufacturer's instructions. Briefly, cells were seeded at density of 10^4 cells per well in 96-well plates, then pre-incubated overnight to induce adherence, at physiological conditions of 5% CO_2 and 37ºC, in a humidified atmosphere. The cells were then treated at concentrations of 0µM to 16µM for cisplatin; or 0µM to 100µM for 5-FU respectively. After twelve to twenty four hours incubation, 10µl of CCK-8 was added to each well and incubated at 37ºC for 2 hours; followed by the measurement of absorbance using an automated micro-plate reader ELx 800 (BioTek, UK) at 450nm. Each experiment was done in triplicate and the results were calculated as the mean of at least three independent measurements in relation to the absorbance of untreated control cells ± standard deviation.

2.2.4: Determination of median effective concentrations (EC_{50})

The median effective concentration, otherwise known as $EC_{50,}$ is the concentration of a drug at which half-maximal effectiveness is achieved (Zhang *et al.* 2010). Determination of this concentration (EC_{50}) is a prerequisite to the selection of drug-resistant cells against any given anticancer drug. Briefly, cells at the logarithmic growth phase (80% confluence) were harvested and seeded at a density of 10^4 cells per well in 96-well plates and incubated

overnight for adherence. Subsequently, the drug-free medium was replaced with pre-warmed, fully supplemented, fresh medium containing 0µM to 100µM of 5-FU or 0µM to 16µM of cisplatin. The cells were incubated for 24 hours, while maintaining the physiological conditions at 5% CO_2 and 37ºC, in a humidified atmosphere. After twenty four hours incubation, cell susceptibility to pharmacological agents was conducted as previously described (section 2.2.3). Cell response to chemotherapy was calculated as a measure of the optical density (OD) of treated cells relative to the optical density of the untreated controls, excluding the OD of blank controls. Concentration-dependent response curves were plotted and the respective EC_{50}(s) of cisplatin and 5-FU were calculated using the GraphPad Prism 6 software and following the software instruction manual for such command. Specifically, determination of EC_{50} was done automatically using the command (enter 50 in the last column of the data table) that enables the GraphPad Prism software to estimate the EC_{50}. A student t-test was carried out to check the difference between the EC_{50} of parental and those of the drug-resistant HEPG2 cells. Statistical significance was defined at p values less than 0.05 ($p < 0.05$) and 95% confidence interval of mean differences.

2.2.5: Flow cytometric analysis of cell death

The EC_{50}(s) were confirmed by flow cytometric analysis of cell death using Annexin V-FITC kit (BD Biosciences, San Diego). Briefly, HEPG2 cells were grown to 80% confluence, and detached using 0.25% (w/v) trypsin in 5mM EDTA (Invitrogen). The cells were seeded at the rate of $2x10^5$ per ml of RPMI 1640 medium (Invitrogen), supplemented with 10% foetal bovine serum (Invitrogen), 1% Penicillin streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen). After twenty four hours incubation in appropriate concentrations of cisplatin (0-16µM) or 5-FU (0-100µM), under physiological conditions of 5% CO_2, 37ºC, in a humidified atmosphere; the cells were detached by trypsinization, washed and re-suspended in binding

buffer. The cells were analysed for apoptosis following mixing with Annexin V-FITC and propidium iodide (PI), and incubation in the dark for five minutes. Analysis of cell death was done using flow cytometer FACSCalibur (BD Biosciences, Europe).

2.2.6: Selection of cells for drug resistance

In selection of the cells for resistance, clinical method of treatment was mimicked by treating the parental HEPG2 cell line in pulse pattern, at the EC_{50} of either cisplatin or 5-FU for four to six hours. Induction was repeated six times, whilst allowing the cells to attain at least 70% confluence between induction intervals. After six complete cycles of induction, selected cells were maintained in drug-free RPMI 1640 medium containing appropriate supplements, and allowed to reach 70-80% confluence. No further experiment was performed on the cells until after four weeks maintenance in drug-free medium.

2.2.7: Assay for drug resistance

The stabilities of the selected clones (HEPG2CR) and (HEPG2FR) were tested after two weeks, one month and three months of drug withdrawal. Briefly, the selected clones were harvested at exponential growth phase using 0.25% trypsin-EDTA. Cell counting was performed using haemocytometer, and the cells were seeded in 96-well plate at the rate of 10^4 cells per well, in triplicate. Plates were maintained at 37ºC in a humidified atmosphere of 5% CO_2. After overnight incubation for attachment, cells were incubated for twenty four hours in RPMI 1640 medium containing appropriate concentration of cisplatin (0-16µM) or 5-FU (0-100 µM). Following the twenty four hours incubation, the cells were further incubated for two hours in the presence of 10 µl of CCK-8 per well. The optical densities were measured and the new EC_{50} was obtained from a concentration-dependent cytotoxicity curve for each of the drugs as previously described. The difference between the EC_{50} of the resistant clones and

that of the parental cell line defines the degree of resistance to the drug against which the cells are selected; and the significance of the difference was determined statistically by student t-test.

2.3: RESULTS

2.3.1: Pharmacological induction of cell death, cellular susceptibility to chemotherapy and determination of median effective concentrations (EC_{50}).

To understand the effect of cisplatin or 5-FU on HEPG2 cell viability and establish the degree of cytotoxicity due to these drugs, HEPG2 cells were incubated for twenty four hours with 0µM to 16µM of cisplatin or 0µM to 100µM of 5-FU. Cellular susceptibility to drug-induced death was measured by CCK-8 assay and cells were found to be more susceptible to cisplatin than 5-FU. Furthermore, with 5-FU, cytotoxicity was found to be directly proportional to drug concentration and time. However, for cisplatin, an approximately similar degree of cytotoxicity was obtained at both twelve hours treatment in drug-containing medium, followed by forty eight hours incubation in drug-free medium and twenty four hours continuous treatment in drug-containing medium at similar concentration (Figure 2.1). The respective EC_{50} were calculated from concentration-dependent response curves, following analysis of cell viability by CCK-8 assay. For cisplatin, cell survival was minimal beyond 4µM (Figure 2.1), however, in the case of 5-FU, cell survival was minimal beyond 50µM (Figure 2.2). Hence, the EC_{50} of cisplatin was found to be 4µM while that of 5-FU was 50µM. The EC_{50}(s) were confirmed by the flow cytometric analyses of cell death due to cisplatin or 5-FU (section 3.3.3).

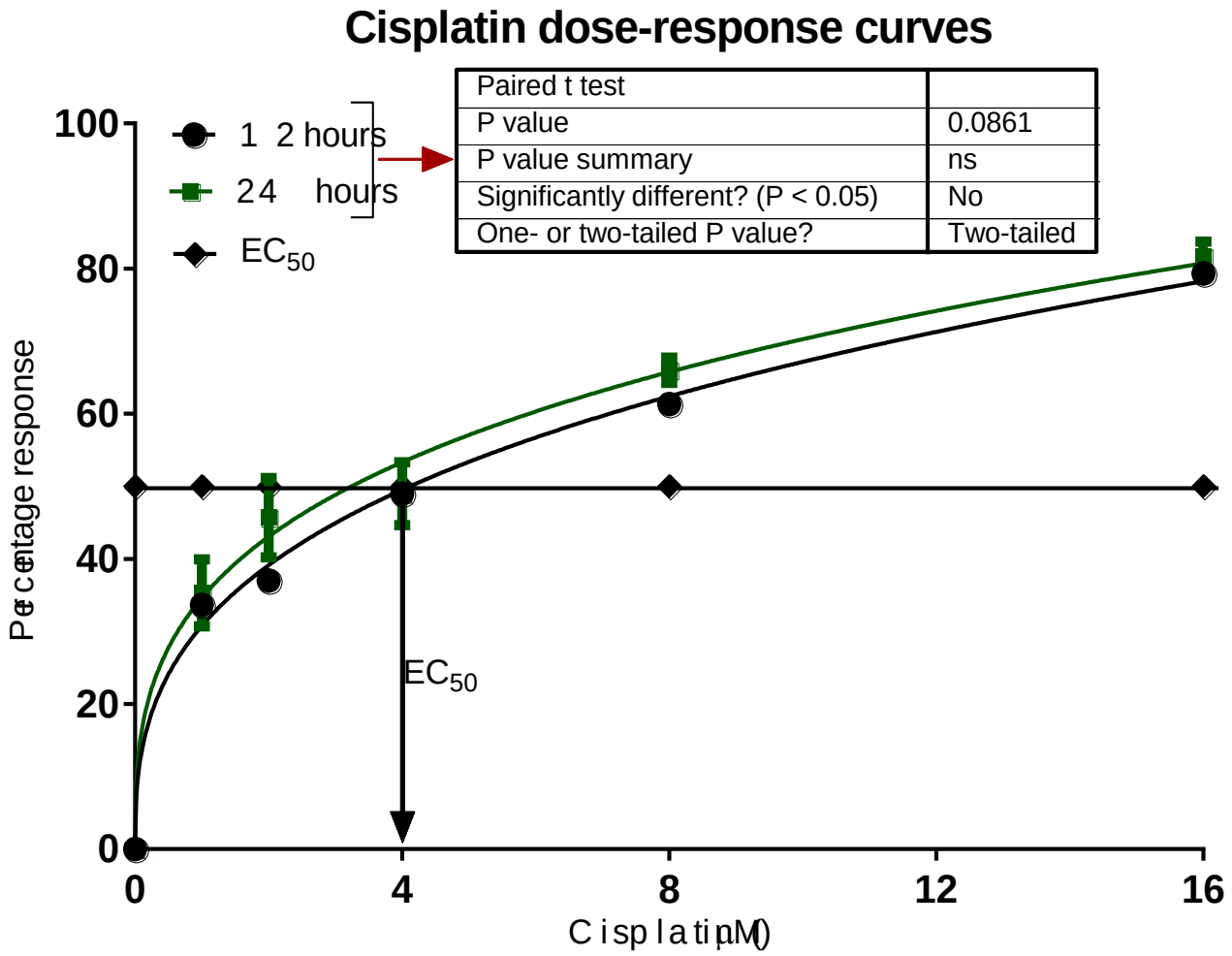


Figure 2.1: Response of HEPG2 Cells to cisplatin treatment for 12 and 24 hours and determination of the median effective concentration at which half-maximal response is obtained (EC50). A student t-test shows that there is no significant difference between the effectiveness of cisplatin at 12 and 24 hours, as indicated by a p value of 0.0861 which is > 0.05; n = 6 as shown in appendix 4, tables A4.0 and A4.1.

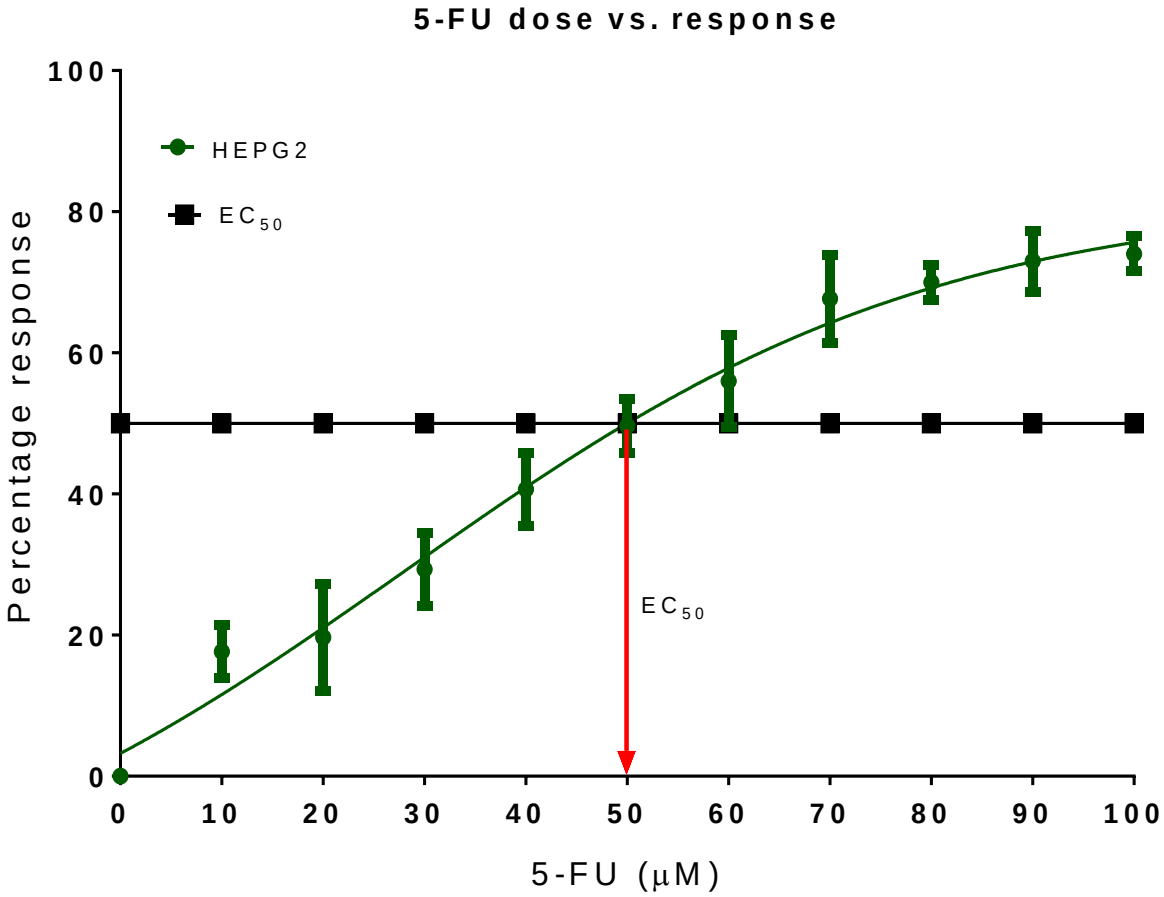


Figure 2.2: CCK-8 assay of HEPG2 cells' susceptibility to 5-FU after 24 hours continuous treatment and determination of the EC_{50} as shown by the red arrow. The EC_{50} was automatically computed using the GraphPad Prism6 software command as described in section 2.2.4 (appendix 4, table A4.2)

2.3.2: Development of drug resistant sub-lines and test for resistance

Following the establishment of the concentration of cisplatin or 5-FU at which HEPG2 cell viability is reduced by 50%, the resistant sublinesHEPG2CR and HEPG2FR were established by incubating the parental HEPG2 cell line at the EC_{50} of cisplatin (4µM) or 5-FU (50µM), whilst imitating the clinical method of treatment. The cells were found to have developed resistance to either CDDP or 5-FU following six intervals of induction. A test for resistance

was conducted following maintenance of the cells in drug-free medium, and a comparison of the EC_{50} of the parental cells and those of the resistant cells revealed an increase in resistance. For cisplatin, the EC_{50} for HEPG2CR is significantly different from that of the parental cell line, rising from 4µM to approximately 8µM, with p value of < 0.0001 at statistical significance defined by $p < 0.05$; where n = 12 (Figure 2.3) (see details in appendix 6, tables A6.3 and A6.4). However, for 5-FU, the EC_{50} for HEPG2FR is significantly different from that of the parental cell line, increasing from 50µM to 65µM (Figure 2.4), with p value of 0.0317, where statistical significance is defined by $p < 0.05$; n = 12, and the strength of the statistical significance is indicated by * (see appendix 4, tables A4.5 and A4.6).

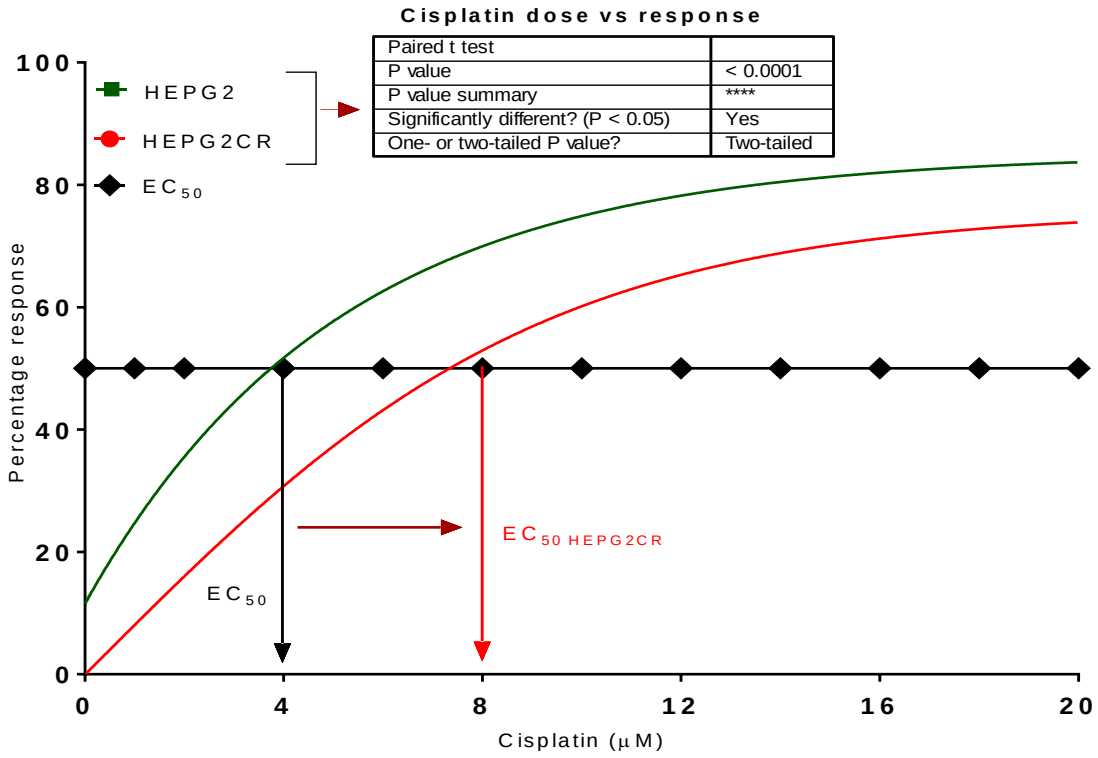


Figure 2.3: Comparison of the EC_{50} of cisplatin for parental HEPG2 cell line and that of the resistant sub-line (HEPG2CR). After a student t-test, a p value of < 0.0001 shows that HEPG2 and HEPG2CR are significantly different from each other, as per susceptibility to cisplatin treatment and the strength of the statistical significance is indicated by ****. The black arrow crossing shows the EC_{50} of cisplatin for HEPG2 as 4µM and the red arrow indicates the rise from 4µM to 8µM as the parental cells develop resistance.

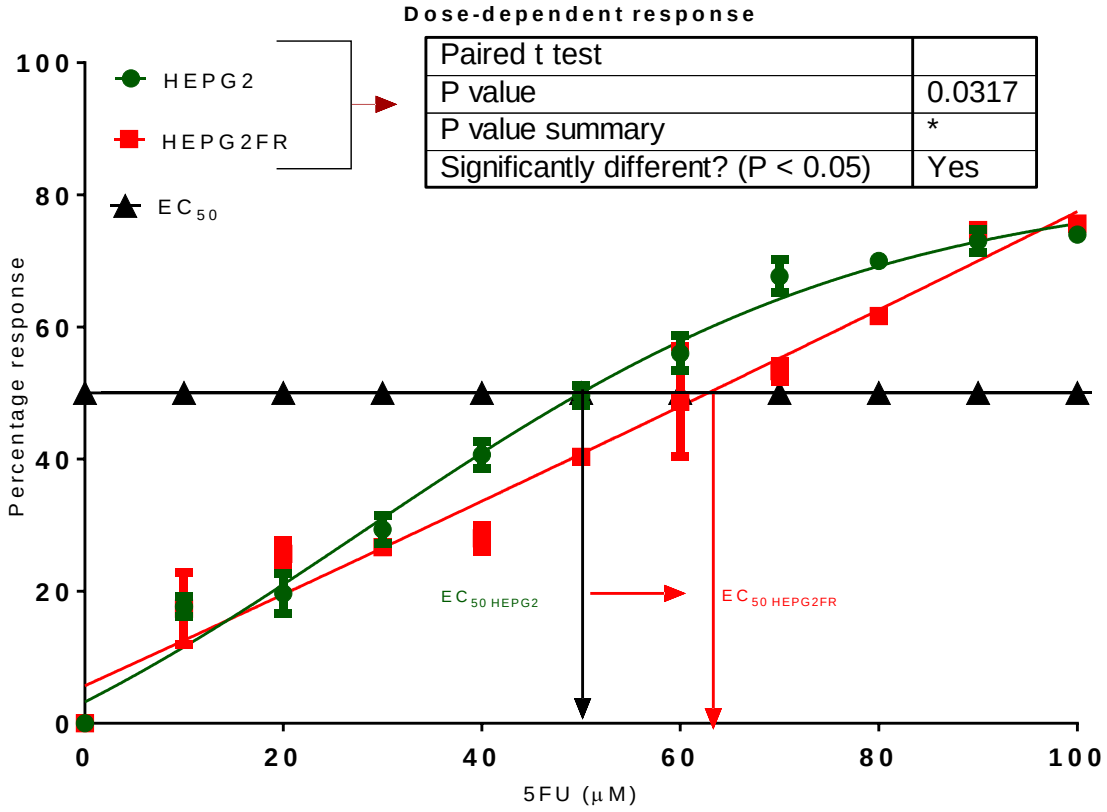


Figure 2.4: Comparison of the EC_{50} of 5-FU for parental HEPG2 cells and that of the resistant sub-line (HEPG2FR). A student t-test shows that the resistant clone is significantly different from the parental cells with p value of 0.0317, where statistical significance is defined by $p < 0.05$; at 95% confidence interval, n = 12, and the strength of the statistical significance is indicated by * (see appendix 4, tables A4.5 and A4.6).

2.3.3: Evaluation of the stability of drug-resistant cells in drug-free conditions

The ability of the selected drug-resistant cells to retain resistance in drug-free conditions was tested after two weeks, one month, and three months. The results show that both HEPG2CR and HEPG2FR are stable and there is no significant change in EC_{50} following a one-way ANOVA analysis, with multiple comparisons of each test period against another. For HEPG2CR, the EC_{50} remained unchanged at 8µM as shown in figure 2.5 (data shown in appendix 4, table A4.51); the p value is 0.9982, which is far greater than 0.05 which defines

statistical significance at 95% confidence interval (see appendix 4, table A4.52 and table A4.53). Similarly, the EC_{50} of 5-FU for HEPG2FR remained unchanged at 65µM as shown in figure 2.6 (see appendix 4, table A4.54 for data); the p value is 0.9992, thus > 0.05 at 95% confidence interval as shown in appendix 4, table A4.55). Multiple comparisons of each test interval against another show that there is no significant change in EC_{50} after three months of growth in drug-free medium (appendix 4, table A4.56).

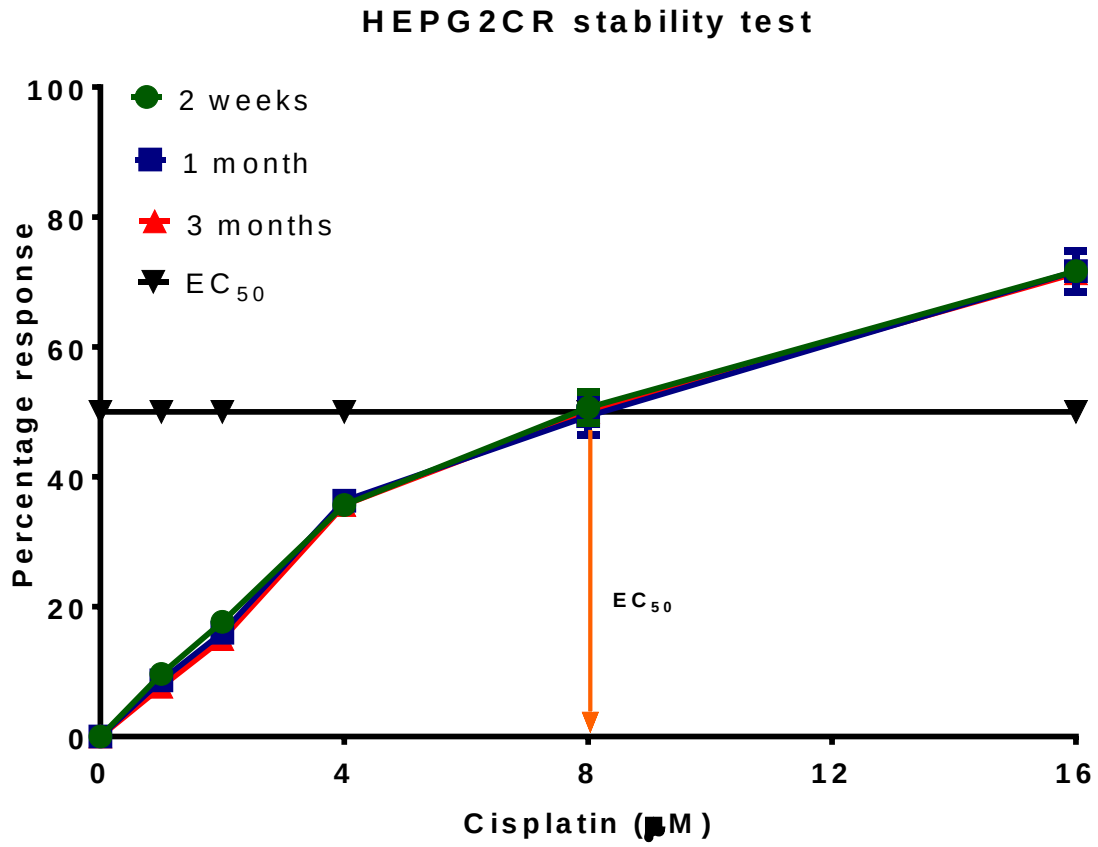


Figure 2.5: Assessment of the stability of HEPG2CR cells in drug-free conditions after two weeks, one month and three months maintenance in drug-free medium, showing consistent EC_{50} of 8µM. One-way ANOVA analysis of the results show an insignificant difference in EC_{50} after two weeks, one month and three months with p value of 0.9982, which is far greater than 0.05 at which statistical significance is defined at 95% confidence interval (appendix 4, table A4.52). Also, multiple comparisons of each test interval against another by one-way ANOVA show no difference in EC_{50} in all cases (appendix 4, table A4.53).

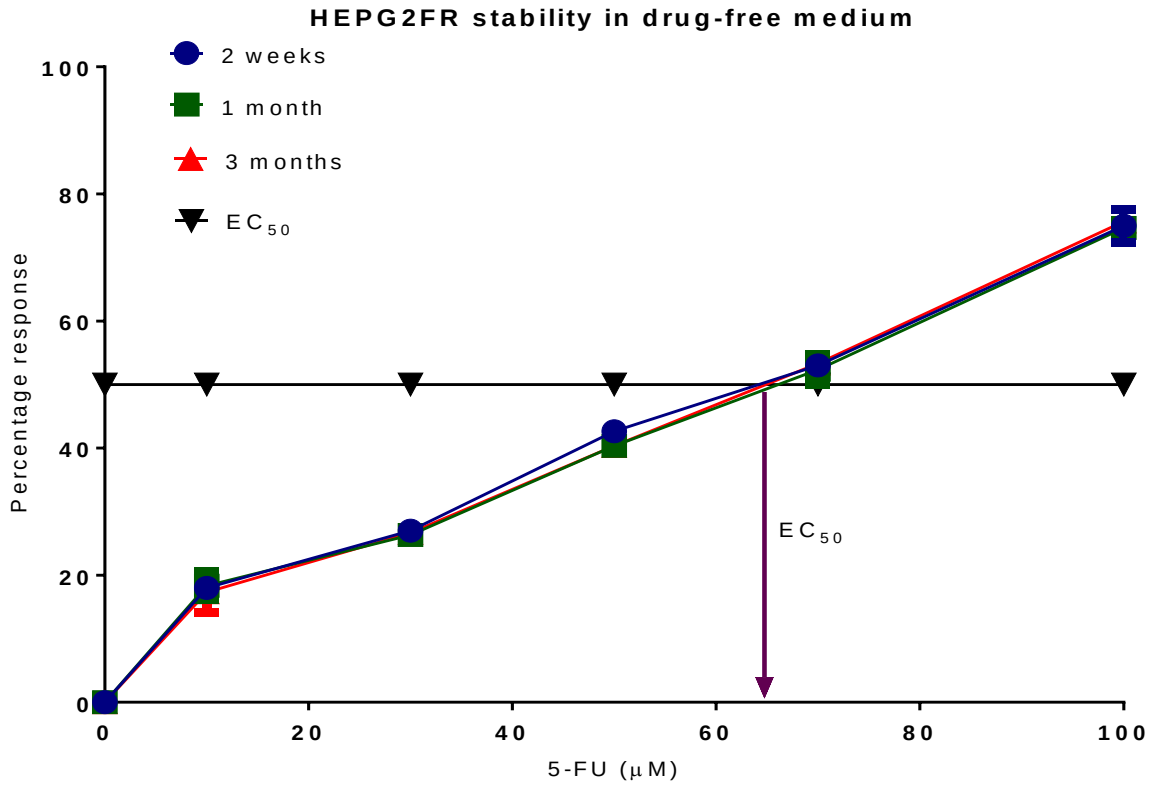


Figure 2.6: Assessment of the stability of HEPG2FR cells in drug-free conditions after two weeks, one month and three months maintenance in drug-free medium showing consistent EC_{50} of approximately 65µM. One-way ANOVA analysis of the results show an insignificant difference in EC_{50} after two weeks, one month and three months with p value of 0.9992, which is statistically insignificance at p value < 0.05 at 95% confidence interval (appendix 4, table A4.55). Also, multiple comparisons of each test interval against another by one-way ANOVA show no difference in EC_{50} in all cases (appendix 4, table A4.56).

2.4: DISCUSSION

Drug resistant tumour cells in humans gradually accumulate during chemotherapy. Development of good drug-resistant cell line models serve as useful paradigms for the clinical scenario of anticancer drug resistance. Cell line models with acquired resistance to a variety of anticancer agents have been variously developed in the quest to unravel the

mechanisms underlying clinical drug resistance (Chen *et al.* 1994; Takeshita *et al.* 1996; Yan *et al.* 2007; Zhang *et al.* 2010).

The first step during the development of drug-resistant cell line is the choice of parental cell line. This is subject to a number of factors, such as tumour type and its relevance to the selecting agents under consideration (Coley, 2004). The human hepatocellular carcinoma (HEPG2) cell line was chosen because it is the most prevalent (accounting for up to 90% of liver cancer cases) and deadliest (Nowak *et al.* 2004; Parkin *et al.* 2005; Kumar *et al*. 2011) form of liver cancer. In relation to the selection agents, cisplatin and 5-FU combination therapy is the most sensitive and preferred treatment option for patients with advanced HCC (Nagai & Smino, 2008).

The results of the assays revealed that HEPG2 cell line is more sensitive to cisplatin than 5-FU as evidenced in the respective concentrations at which EC_{50} was achieved for both drugs (figure 2.1 and 2.2). The EC_{50} of cisplatin or 5-FU was calculated as 4µM or 50µM respectively, from concentration-dependent cytotoxicity curves. Also, the results showed distinct initial decline in cell viability upon addition of cisplatin or 5-FU. This could be due to the heterogeneity of the cell population, where the cells are at different stages of cell cycle. Consequently, the initial decline may represent population of cells that have committed to division prior to drug introduction, hence were more susceptible to drug-induced death. Though, the initial drop in cell viability was also observed in drug-resistant cell lines, the decline was more gradual. This could be due to the fact the population of the drug-resistant cell lines was less heterogeneous.

On establishing the appropriate concentrations of cisplatin and 5-FU at which the resistant sub-lines of HEPG2 cell line could be produced, the resistant cell lines were then maintained at these concentrations. The selection process mimicked the clinical method of treatment, as the parental cell line was treated in pulse, whilst allowing the cells to recover between treatment intervals. The treatment intervals are synonymous to the clinical cycles of treatment while the numbers of inductions are equational to the clinical treatment courses. After the sixth induction, an increase in the EC_{50} of both drugs was an indication of resistance development. Statistical analysis using student t-test shows that HEPG2 and HEPG2CR are significantly different from each other, as per susceptibility to cisplatin treatment, with a p value of < 0.0001. The EC_{50} of cisplatin for the sub-clone of HEPG2 selected for cisplatin resistance doubled that of the parental cell line. Similarly, a student t-test shows that 5-FU resistant clone is significantly different from the parental cells with p value of 0.0317; however, the EC_{50} of 5-FU for the sub-clones of HEPG2 selected for 5-FU resistance increased by a shorter margin compared to cisplatin (from 50μM to 65μM). This shows that HEPG2 cell line has stronger cytotoxicity memory for 5-FU, hence, smaller change (increase) in concentration was needed for the cells to develop resistance to 5-FU relative to cisplatin. This can also be attributed to the stronger toxicity strength of cisplatin relative to 5-FU.

The chemo-resistant cell lines were found to be very stable in drug-free medium, as indicated by insignificant statistical difference in the EC_{50} of cisplatin and 5-FU after months of maintenance in drug-free medium (figure 2.5 and figure 2.6, repectively). This contradicts some reports that drug-resistant cell lines need to continually grow in drug-containing medium in order to retain resistance (Twentyman *et al.* 1986). Therefore, this study provides the basis for the establishment of stable model of drug-resistant cell line, with minimal cost of production and maintenance.

CHAPTER THREE

REDUCTION OF CISPLATIN CYTOTOXICITY IN HEPATOCARCINOMA CELLS BY OPTIMIZATION OF TREATMENT CONDITIONS

3.1.0: INTRODUCTION

Most organs of the body accumulate cisplatin, especially the kidneys, liver, prostate, spleen, bladder, testicles, pancreas, bowel, adrenal glands, heart, lungs, cerebrum, and cerebellum (as reviewed in Sancho-Martínez *et al*., 2012). However, the pattern of tissue accumulation does not always correlate with the pattern of tissue toxicity (Staffhorst *et al.* 2008; Sancho-Martínez *et al.,* 2012). For instance, Junior *et al.* (2007) reported higher accumulation of cisplatin in liver and spleen than the kidneys, whilst the main toxicity was witnessed in the kidney. This observation supported previous findings reported by Stewart *et al.* (1982), where tissue platinum concentration was higher in liver and prostate while nephrotoxicity was evident in the kidneys.

Tumours also accumulate cisplatin but the tumour-to-plasma coefficient is lower in many organs (Staffhorst *et al.* 2008). For instance, Sancho-Martinez *et al.* (2011) reported that cell cycle arrest requires lower concentrations of cisplatin relative to cell death induction. This could explain why cisplatin is able to exert antineoplastic activity at low dosages (Sancho-Martínez *et al.,* 2012), as low as 7 mg/m^2 (table 3.0).

3.1.1: Mechanism of cisplatin-induced death

Cisplatin-induced cell death occurs through both apoptotic and non-apoptotic, necrotic-like processes (Price *et al.* 2004; Cepeda *et al.* 2007; Ramirez-Camacho *et al.* 2008). The mode of cell death is concentration-dependent; and for both tumour and non-tumour cells, low

cisplatin concentrations induce apoptotic cell death, whereas necrosis occurs at higher concentrations (Sancho-Martinez *et al.* 2011). Cisplatin is highly cytotoxic for proliferating cells, because of its characteristic interaction with DNA, during which it forms covalent adducts with certain DNA bases and impedes DNA replication and mitosis (Yang *et al*., 2006). Unfortunately, the therapeutic use and efficacy of cisplatin are limited by its strong side effects, especially nephrotoxicity, ototoxicity, neurotoxicity, cardiotoxicity, gastrointestinal toxicity and bone marrow suppression (El-Sayed *et al*., 2011; López-Hernández *et al*., 2012).

3.1.2: Side-effects and clinical use of cisplatin

Cisplatin, like most anticancer drugs, is not action-specific (Bodiga *et al.* 2012). Hence, it acts on both proliferating and non-proliferating cell types, with resultant side effects, especially at high dosage and prolonged exposure (Barabas *et al*., 2008; Rybak *et al*., 2009; Sanchez-Gonzalez *et al*., 2011a; Jaggi and Singh, 2012). Evidently, cisplatin cytotoxicity is at cross-roads of its therapeutic and side effects and for many years, various strategies have been adopted in clinical settings to curtail these side effects. One of these approaches is to synthesize and screen for novel cisplatin analogues that have lower toxicity in normal tissues. To this end, several cisplatin analogues, like carboplatin, have been identified and shown to have less severe side effects (Pasetto *et al*., 2006). Additionally, hydration of patients with 1 to 2 litres of fluid infused 8 to 12 hours prior to cisplatin treatment is another strategy that has been used with some success (Cornelison and Reed, 1993). Haemodialysis (Brivet *et al.* 1986; Lagrange *et al.* 1994), plasmapheresis (Guenter *et al.* 2006), molecular mediators (Pabla and Dong 2012) and even natural and synthetic antioxidants (Yinghua *et al.* 2011), have be reported as some of the strategies with proven effectiveness in ameliorating cisplatin

side effects. Irrespective of these efforts, the side effects of cisplatin remain a major factor militating against its use and efficacy in cancer therapy.

The clinical use of cisplatin involves varying dosages depending on the type of cancer and other clinical factors, such as additional therapy, patient's body weight, size and general health state (Solimando, 2010). The approximate doses of cisplatin, methods of administration and types of cancer are presented in table 3.

Table 3: Clinical dosages of cisplatin and methods of administration for various types of cancer (Solimando, 2010)

Cancer type	**Cisplatin adult dosage**	**Method of administration**
Testicular	20 mg/m^2	Intravenously once a day for 5 days per cycle
Ovarian	75 to 100 mg/m^2	Intravenously once every 4 weeks
Bladder	50 to 70 mg/m^2	Intravenously once every 3 to 4 weeks
Neuroblastoma	60 to 100 mg/m^2	Infusion
Non-small cell lung	60 to 100 mg/m^2	Intravenously on day one of every 21 days
Cervical	40 to 70 mg/m^2	Intravenously weekly depending on additional therapy
Liver	7 to 100 mg/m^2	Hepatic arterial infusion once a day for 3 to 5 days per cycle

3.1.3: Optimizing cisplatin therapy

In both clinical and non-clinical settings, it is important to establish the optimal treatment conditions for cisplatin by exploiting potential differences in its handling or response. Knowledge of such optimal conditions of treatment might help improve the pharmaco-toxicological profile of cisplatin, and reduce its cytotoxic effects on non-target cells. Consequently, this study seeks to develop a model to understand more about the kinetics of cisplatin cytotoxic effects, in this case, on hepatocarcinoma cells.

3.2: MATERIALS AND METHODS

3.2.1: Materials

All the materials were obtained from Sigma Aldrich UK and Invitrogen UK unless otherwise stated.

3.2.2: Cell line and cell culture establishment

Cell line and cell culture establishment involved similar methods previously described in section 2.2.2.

3.2.3: Cisplatin-induced cytotoxicity assay

Assays for cisplatin-induced cytotoxicity were conducted following the methods described by Odii & Coussons (2012) (see section 2.2.3).

3.2.4: DNA fragmentation assay of apoptosis

To investigate the ability of cisplatin to induce apoptotic death by DNA fragmentation, preparation of the cell samples was conducted as described by Okamura *et al*. (2008). Briefly, HEPG2 cells were inoculated in 25cm^2 flasks at10^5 cells/ml and pre-incubated for overnight for adherence. The cells were then treated with 0µM to 16µM cisplatin for twelve hours, followed by forty eight hours incubation in drug-free medium, or continuously for twenty four or forty eight hours. Following appropriate incubation, cells were collected by trypsinization using 1ml trypsin-EDTA (0.1% trypsin and 0.02% EDTA in phosphate-buffered saline) (Sigma Aldrich, UK) for every 25cm^2 flask. Genomic DNA was extracted from the collected cells using QIAamp DNA Mini Kit (Qiagen, UK), following the manufacturer's instructions. The isolated DNA was assayed for fragmentation and laddering

feature using 2% agarose gels (Sigma Aldrich, UK). For each concentration of cisplatin, duplicate experiments were carried out and the experiment was repeated three times for the treatment durations investigated.

3.2.5: Time-course induction and analysis of apoptosis by flow cytometry

Flow cytometric analysis of cellular cytotoxicity of cisplatin towards HEPG2 cells at different time intervals was conducted as previously described in section chapter two, section 2.2.5. In addition, the cells were treated with 0µM to 16µM cisplatin for twelve hours, washed once with HBSS (Invitrogen, UK), and incubated for forty eight hours in drug-free medium. Alternatively, the cells were incubated continuously with similar concentration of cisplatin for twenty four hours or forty eight hours before analysis. Preparation of samples for analysis involved cell detachment by trypsinization, followed by washing of cells in phosphate buffered saline (PBS) (Invitrogen, UK) and re-suspension in binding buffer. Finally, the cells were analysed for apoptosis after mixing with Annexin V-FITC and propidium iodide (PI), and incubation in the dark for five minutes. Each experiment was repeated two times and duplicate of experiment was prepared for every concentration of cisplatin tested.

3.2.6: Cell cycle analysis at optimal treatment time by flow cytometry

Cell cycle distribution was assessed by flow cytometric analysis as described by Li *et al.* (2011). Briefly, HEPG2 cells at about 80% growth confluence were prepared as described in section chapter two, section 2.2.5, and then were treated continuously for twelve hours with cisplatin-containing medium at concentrations of 0µM to 16µM cisplatin, followed by forty eight hours incubation in drug-free medium. After harvest, cells were re-suspended in 0.5ml 1X PBS (Invitrogen) and fixed in ice-cold 70% ethanol overnight. Ethanol was decanted and

cells were washed with PBS and stained with propidium iodide (PI) for 4hrs in a dark cupboard before cell cycle distribution was analysed.

3.3: RESULTS

3.3.1: Cisplatin-induced cytotoxicity

The EC_{50} of cisplatin for HEPG2 cells was calculated from the concentration-dependent response charts, and after twelve hours or twenty four hours incubation, it was consistently found to be 4μM (figure 2.1). A comparison of cellular response to cisplatin at both twelve and twenty four hours incubation showed a uniform 30% drop of cell viability on initial introduction of cisplatin to HEPG2 cells followed by a steady decline after the EC_{50} as previously reported in chapter two, section 2.3.1, figure 2.1.

3.3.2: Induction and detection of cisplatin-induced apoptosis by DNA fragmentation assay

Most anticancer drugs including cisplatin, exhibit their antineoplastic actions by apoptosis induction. One of the major markers of apoptosis is the cleavage of double-stranded DNA in the linker regions between nucleosomes by endogenous endonucleases, which generate mono- and oligo-nucleosomes multiples of approximately 180 to 200 bp (Sambrook *et al.*, 2001; El-Sayed *et al.* 2011). To understand the ability of cisplatin to induce apoptosis in HEPG2 cells, time-course analysis of internucleosomal DNA fragmentation was carried out by gel electrophoresis as described in section 3.2.4. The results show that cisplatin can induce apoptosis as early as six hours of treatment (figure 3.0a). After twelve hours of treatment, uniform DNA fragments were produced across concentrations (figure 3.0b), with increased DNA smears, typical of necrosis, as the concentration increases. After twenty four and forty

eight hours of induction, DNA smears were more pronounced across concentrations of cisplatin (figure 3.0 c and d).

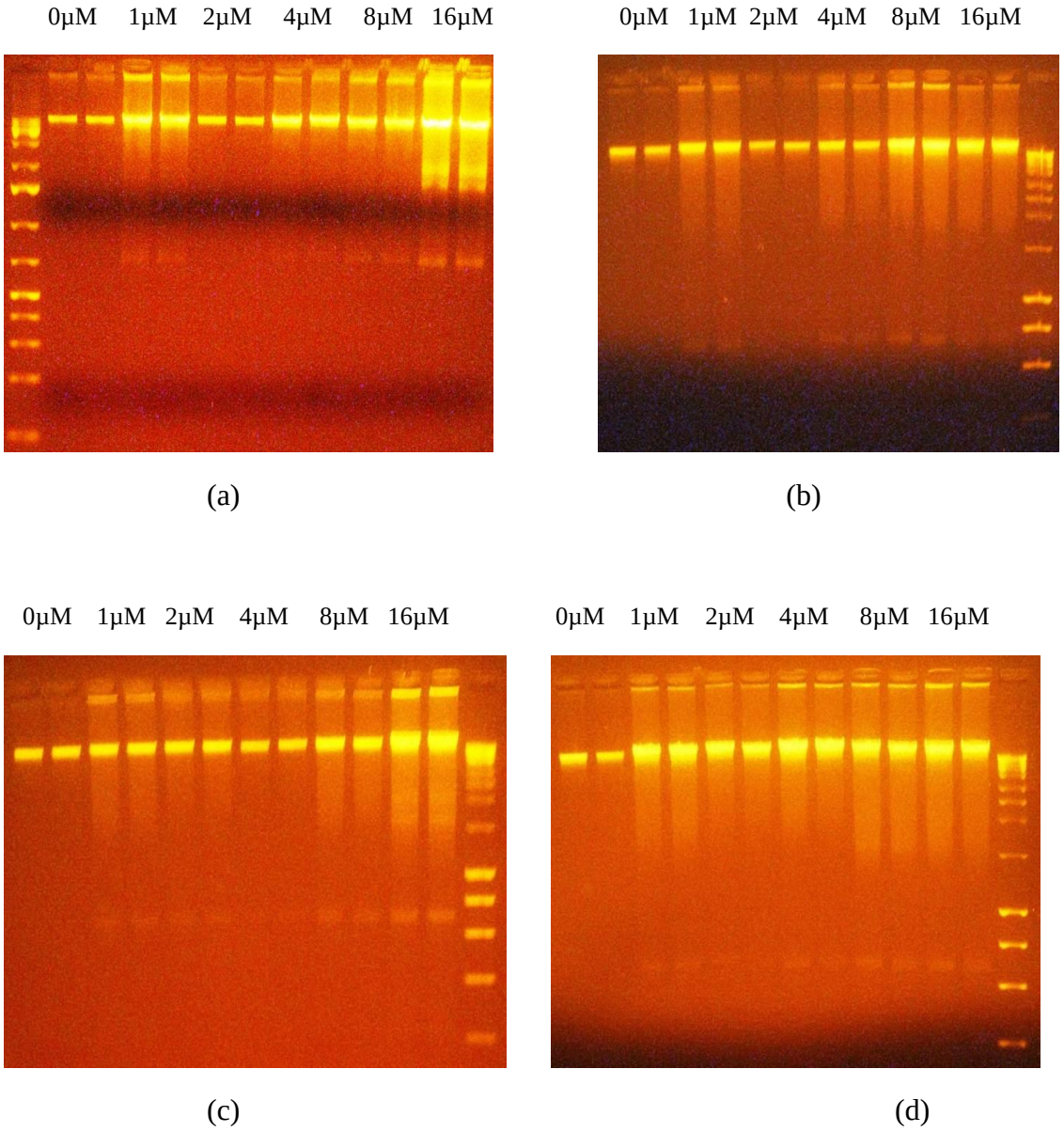


Figure 3.0: Detection of cisplatin-induced apoptosis by DNA fragmentation assay after (a) six hours treatment, showing more intact DNA bands and less smears (b) twelve hours continuous treatment followed by forty eight hours incubation in drug-free medium, with DNA fragments uniformly produced across concentrations (c) twenty four hours treatment, and (d) forty eight hours treatment, where DNA fragmentation was accompanied by prominent smearing, typical of necrotic cell death.

3.3.3: Time-course analysis of cisplatin-induced cell death by flow cytometry

To confirm the EC_{50} of cisplatin and properly establish twelve hours as the optimal treatment time for the drug, cell death analysis by flow cytometry was carried out at intervals of twelve hours in cisplatin-containing medium and forty eight hours incubation in drug-free medium, or twenty four to forty eight hours continuous treatment in drug-containing medium. The EC_{50} remained approximately the same, regardless of duration of treatment and the percentage of apoptotic cells obtained at twelve hours was approximately similar to that obtained at twenty four to forty eight hours continuous treatment (figure 3.1). Interestingly, the EC_{50} remained constant across the various intervals of HEPG2 treatment; however, the stages of cell death were different. At optimal treatment time (twelve hours) 51% of the cells were non-apoptotic, 32% of the cells were at early stage of apoptosis, 13% of the cells were at late phase of apoptosis, while only 1% of the cells were necrotic (figure 3.1). Following a twenty four hours continuous exposure to cisplatin, the proportion of the cells at late state of apoptosis and necrosis increased to 18% and 19% respectively. With continued exposure up to forty eight hours, 27% of the cells were at secondary stage of apoptosis, 26% were at stage of necrosis, and only 4% of the cells were at early stage of apoptosis (figure 3.1).

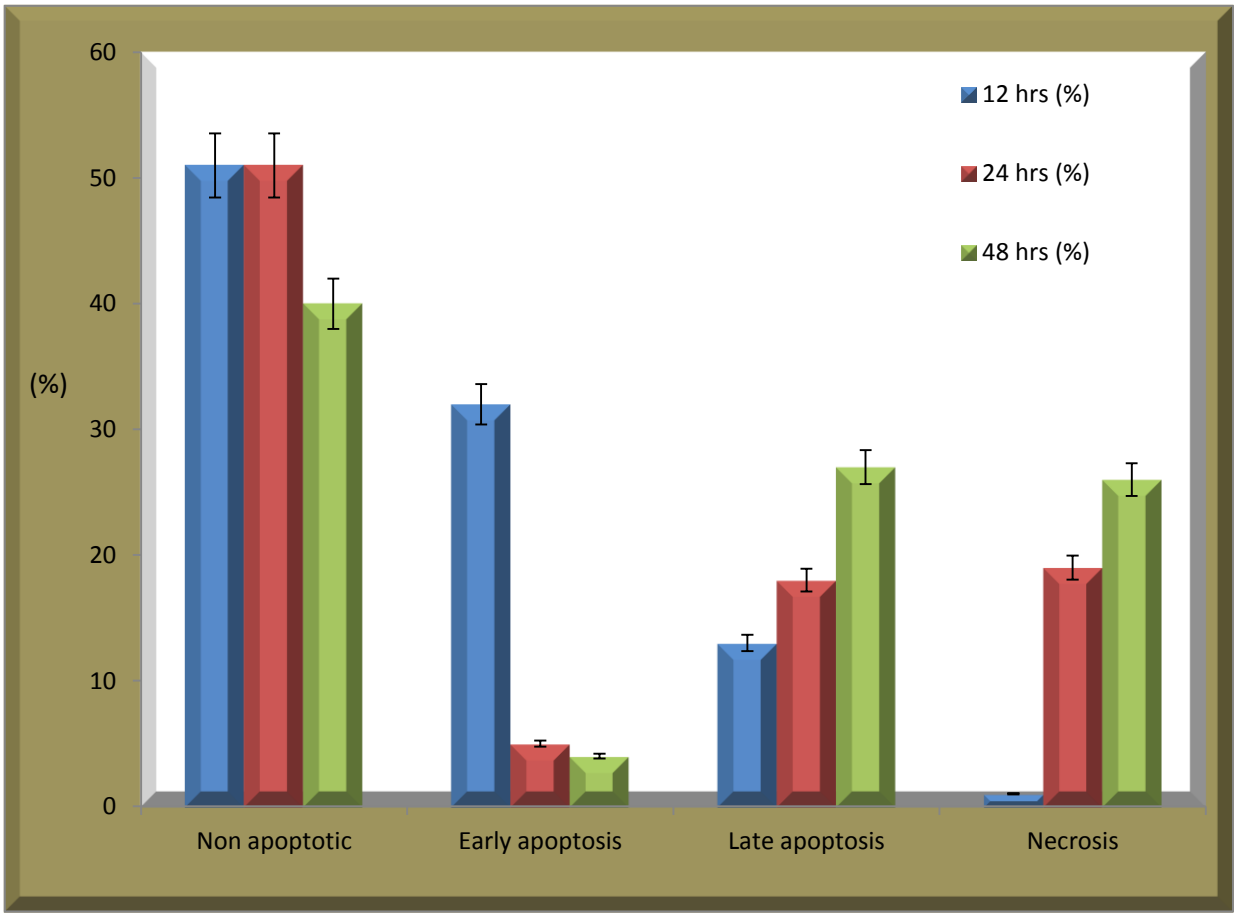


Figure 3.1: Comparison of the patterns of cell death distribution after treatment of HEPG2 cell line with similar cisplatin concentration, EC_{50} (4µM) for different durations, twelve, twenty four and forty eight hours. Necrotic death and late apoptosis increased with increased treatment duration from twelve hours to forty eight hours (see appendix 2, tables A2.2.1, A2.2.2, and A2.2.3).

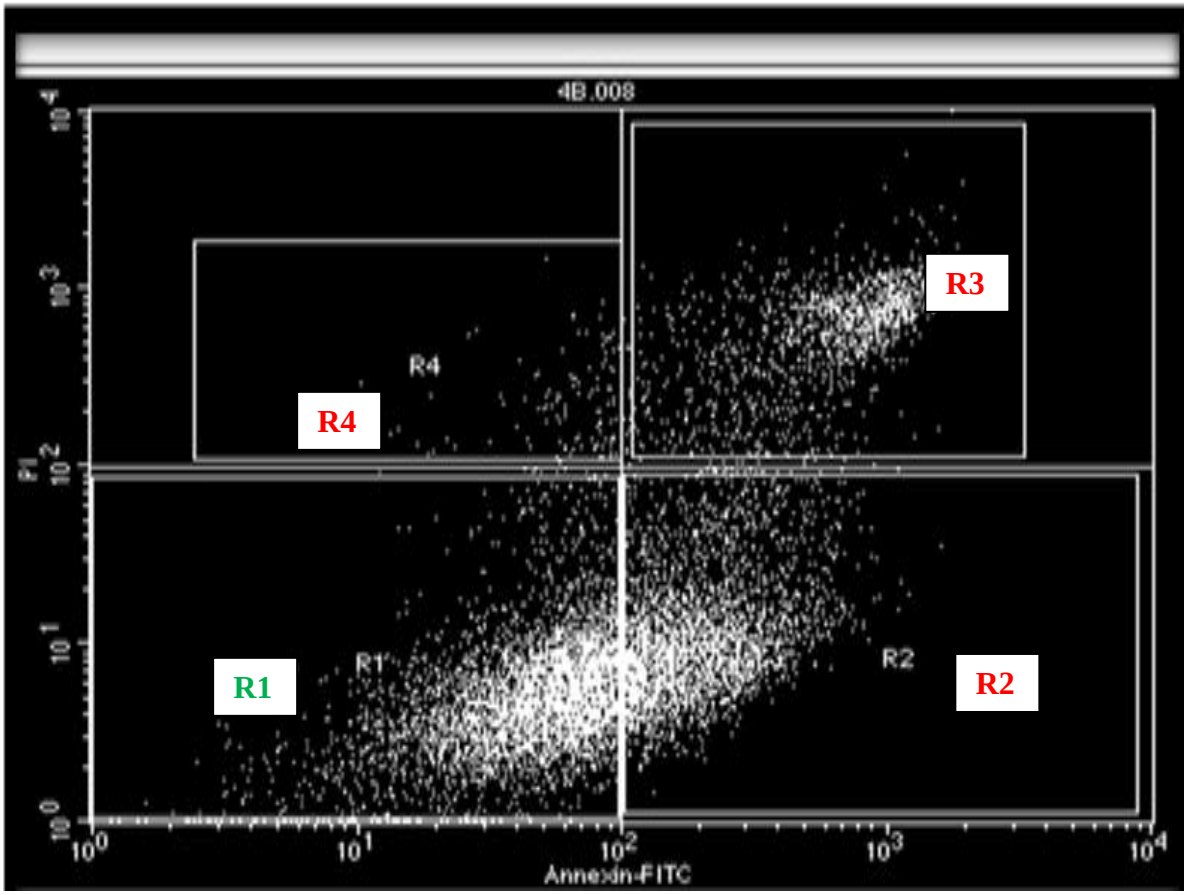
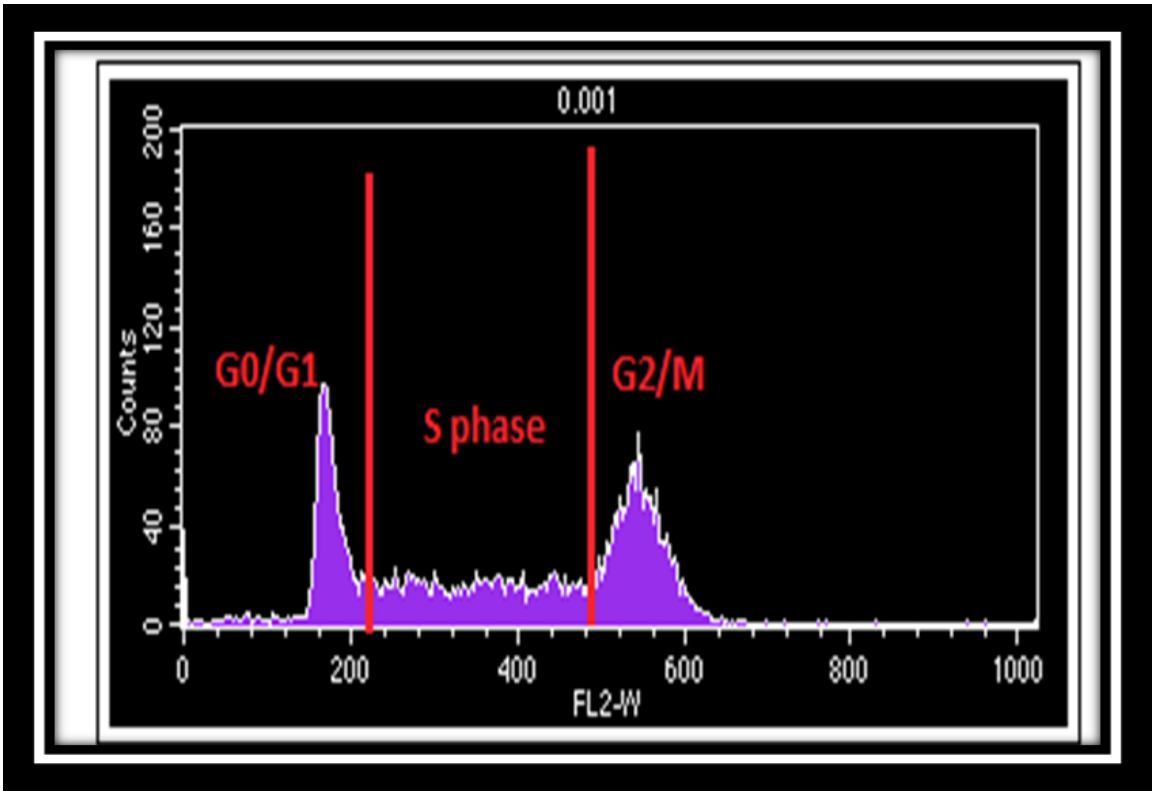


Figure 3.2: An example of FACS result showing cell death distribution at EC_{50} (4µM) after twelve hours cisplatin treatment followed by forty eight hours incubation in drug-free medium, with cell death mainly due to primary and secondary apoptosis (R2 and R3) and minimal necrosis (R4) as earlier summarised in figure 3.1.

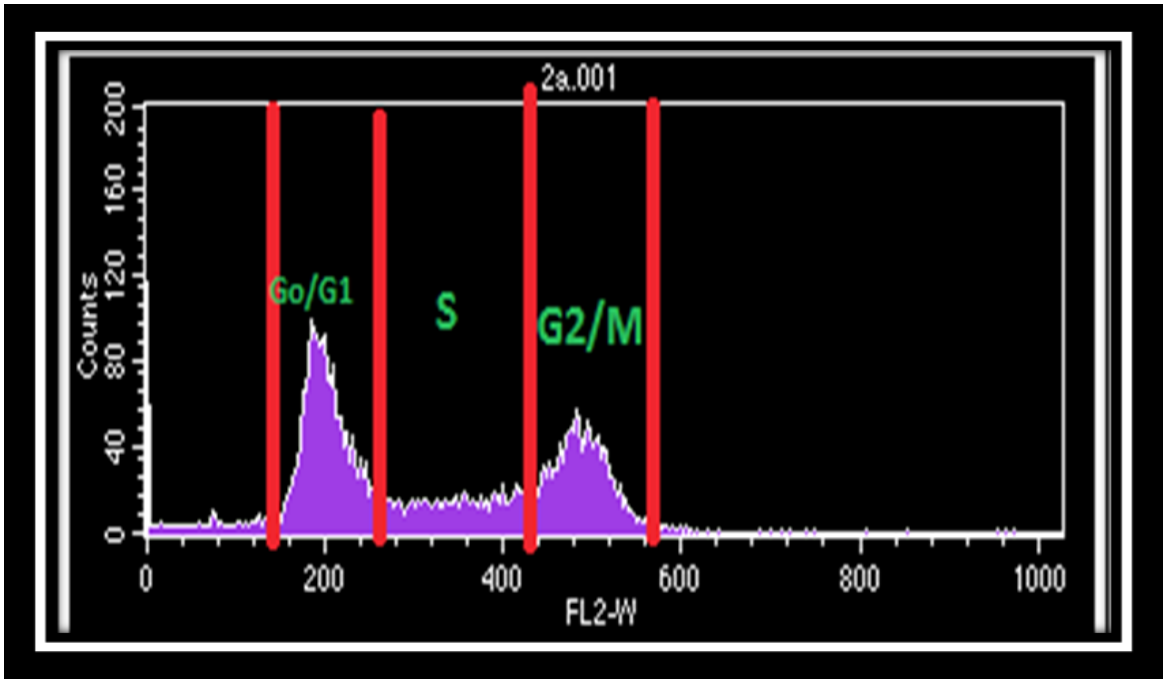
3.3.4: Analysis of cell cycle at optimal treatment time

Cisplatin initiates cell death by intercalating with DNA bases forming adducts and inhibiting DNA synthesis and mitosis (Yang *et al*., 2006). To find out the effect of cisplatin on HEPG2 cell cycle progression at optimal treatment time, cell cycle events were analysed following 12 hours cisplatin treatment at 0µM to 16µM and 48 hours incubation in drug-free medium. The results revealed that cisplatin was only able to almost completely stop cell cycle at G0/G1

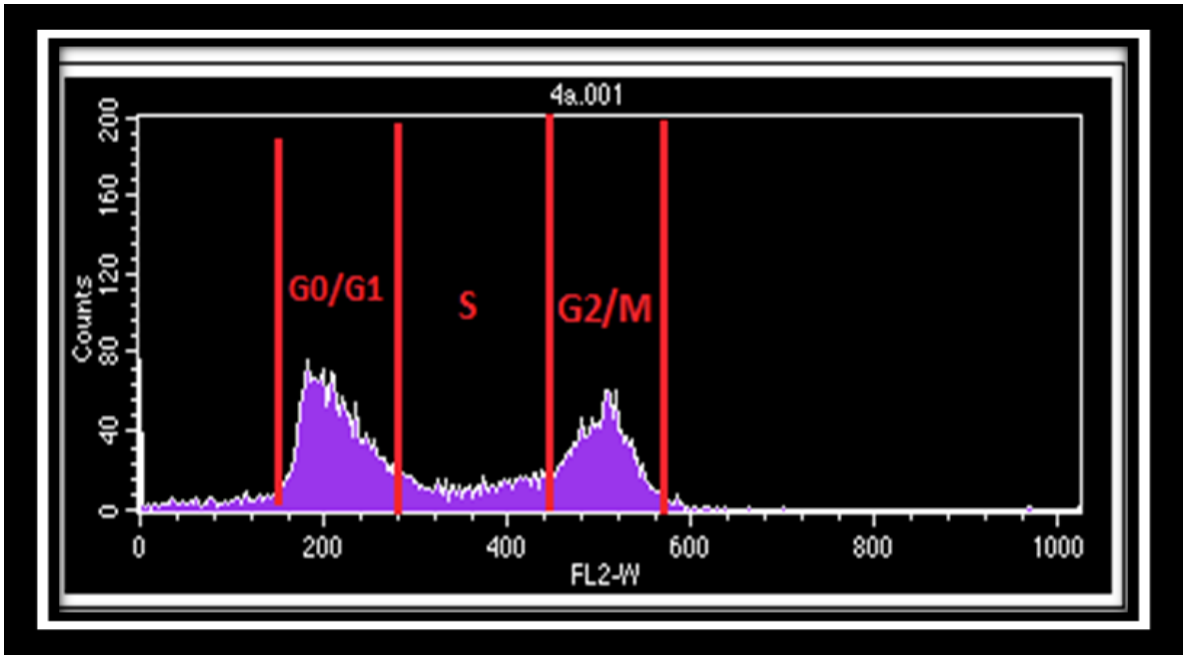
phase at the highest concentration (16µM) (figure 3.3), while at lower concentrations, cell cycle progression was gradual, with significant proportions of the cells at G0/G1, S and G2/M phases. However, S and G2/M phases continued to decrease, while G0/G1 increased as the cisplatin concentration increases.



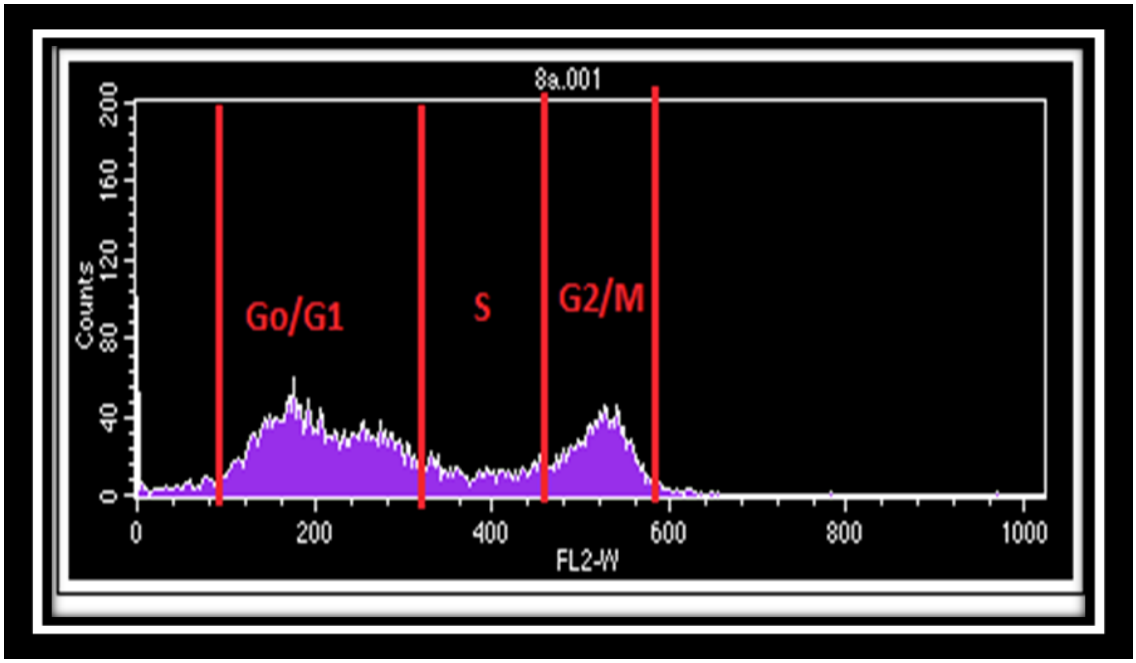
(a) At 0µM, cells were properly spread across the various cell cycle phases, with a large number of the cells at the synthesis phase.



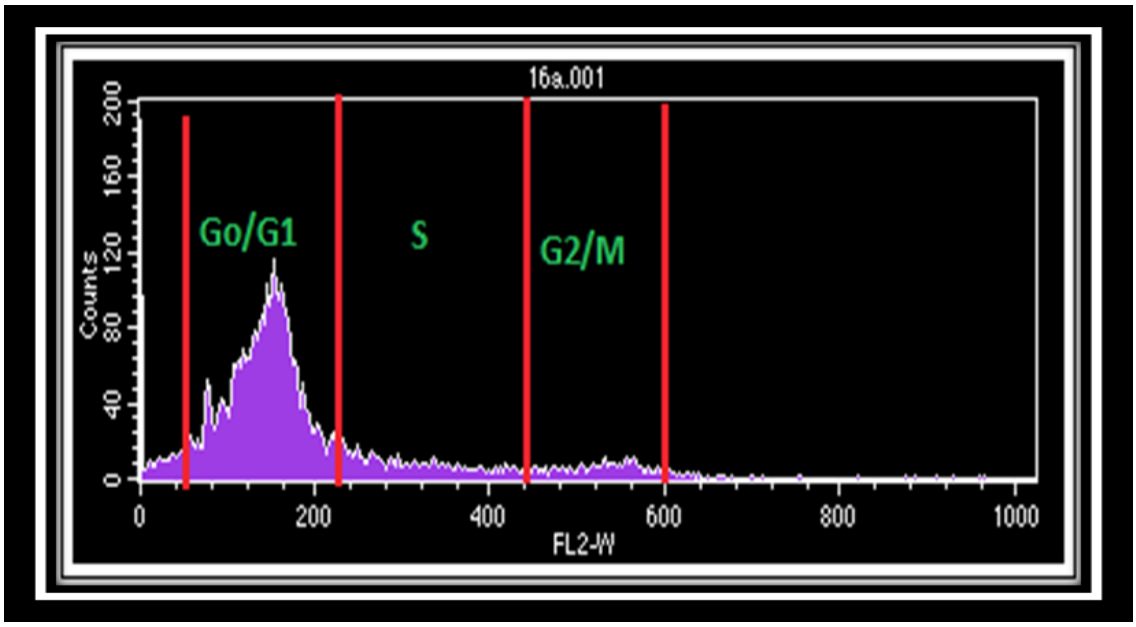
(b) 2µM: Upon introduction of 2µM of cisplatin, the number of cells progressing from G0/G1 through S to G2/M phase decreased with an observed increase in number of cells at G0/G1.



(c) 4µM: The G0/G1 phase continued to increase while S and G2/M phases continued to shrink with further increase in cisplatin concentration to 4µM.



(d) 8µM: At 8µM, G0/G1 became very prominent with further shrinkage of S and G2/M phases.



(e) 16 µM: At highest concentration of cisplatin, the cell cycle process was almost completely halted at G0/G1.

Figure 3.3: HEPG2 cell cycle progression after twelve hours cisplatin treatment followed by forty eight hours incubation in drug-free medium.

3.4: DISCUSSION

Very few studies have attempted to exploit patient or cellular response to cisplatin to establish its optimal treatment conditions. For instance, Drewinko *et al.* (1973) reported that for human lymphoma, prolonging the treatment time of cisplatin from 1 to 8 hours at 5µg/ml produced a magnitude of cytotoxicity similar to what was observed after one hour treatment at 50µg/ml. Similarly, Okamura *et al.* (2008) reported that treatment of various clones of human oral squamous carcinoma cell lines with cisplatin for twelve hours and subsequent thirty six-hour incubation in drug-free medium produced a comparable magnitude of cytotoxicity with that obtained after 48-hour continuous treatment. In agreement with these reports, the results presented in this thesis showed that a consistent EC_{50} of 4µM was obtained following different intervals of HEPG2 treatment with cisplatin (figure 3.1).

The pattern of cell death distribution analysed by flow cytometry, further revealed the consequence of prolonged exposure of HEPG2 cells to cisplatin. This was evident in the phases of cell death observed in the cells at similar concentration (EC_{50}) but different treatment durations (figure 3.1). For instance, after twelve hours treatment in drug-containing medium, followed by forty eight hours incubation in drug-free medium, cell death was mainly due to primary and secondary apoptosis, with minimal necrosis (figure 3.2). However, following an increase in incubation duration from twelve hours to twenty four or forty eight hours continuously in drug-containing medium, cell death was dominantly due to secondary apoptosis and necrosis (figure 3.1).

Additionally, a time-course analysis of internucleosomal DNA fragmentation in HEPG2 cells due to cisplatin revealed a consistent pattern of cell death to the flow cytometry results. Within the first twelve hours of death induction, cell death was mainly due to primary and

secondary apoptosis, with minimal necrosis, hence, the DNA bands were more intact and apoptotic fragments were produced across cisplatin concentrations (figure 3.0 a and b). Increase in treatment duration resulted in more necrotic cell death as indicated by prominent DNA smearing (figure 3.0 c and d). Furthermore, the results show that the drug is capable of inducing detectable DNA fragmentation within six hours of treatment (figure 3.0 a) and the extensive fragmentation observed at 1μM across treatment duration could be attributed to the similar case of heterogeneous cell population, where dividing cells are more prone to death as previously observed in chapter two, figure 2.1 and 2.2.

Flow cytometric analysis of HEPG2 cell cycle distribution after treatment with cisplatin for twelve hours, followed by forty eight hours incubation in drug-free medium indicated that the effect of cisplatin on cell cycle progression was subtle at lower concentration, and cells were able to progress to G2/M phase until 8μM. However, cell cycle was almost completely halted at G0/G1 phase when cisplatin concentration was raised to 16μM (figure 3.3). This is an indication that at optimal treatment time, cellular cisplatin toxicity may be gradual and manageable. A comparison of cell cycle arrest and cell death due to cisplatin reveals that the extent of cell cycle arrest obtainable at lower concentrations was marginally higher than the degree of cell death. This is in agreement with the findings reported by Sancho-Martinez *et al.* (2011); that cell cycle arrest requires lower concentrations of cisplatin relative to cell death induction. The results presented in this chapter therefore, suggest that prolonged exposure of HEPG2 cells to a given dose of cisplatin might be responsible for tissue damage and associated toxicity characteristic of the drug.

CHAPTER FOUR

TRANSGLUTAMINASE2 EXPRESSION AND ACTIVITY PROFILES IN PARENTAL AND DRUG-RESISTANT HEPATOCARCINOMA CELL LINES

4.1: INTRODUCTION

Many cancer deaths occur due to the ability of tumour cells to develop resistance to conventional drug therapy and metastasize successfully to distant sites (Verma & Mehta, 2007). Determination of the changes in the pattern of expression of genes in tumours that contribute to the development of drug-resistance can reveal novel therapeutic targets for the treatment of cancer (Mehta *et al.* 2010). For over three decades, the molecular basis of drug resistance has been under investigation and several genes that may be involved in the mechanisms of drug resistance and metastasis have been identified (Jiang *et al.* 2003a, b; Mehta *et al.* 2010). Among these genes, TGM2, a stress-responsive gene which encodes transglutaminase 2 (TG2) enzyme, has been identified as a putative gene involved in tumour evasion of apoptosis, drug resistance, and metastasis (Iacobuzio-Donahue *et al* 2003; Agnihotri *et al.* 2013).

A significant feature among drug-resistant and metastatic cancer cells is an increased level of TGM2 gene expression (Budillon *et al.* 2011). Elevated expression levels of TGM2 and its gene product TG2, have been demonstrated as a predominant feature of many advanced types of cancer cells, including pancreatic carcinoma (Verma *et al*. 2006), ovarian carcinoma (Satpathy *et al*. 2007; Hwang et al. 2008), malignant melanoma (Fok et al. 2006), lung carcinoma (Park *et al*. 2010), glioblastoma (Yuan *et al*. 2007), and breast carcinoma (Mehta *et al*. 2004).

TG2 has been shown to play a major role in apoptotic evasion, development of drug resistance, and metastasis in many cancer types (see Mehta *et al.* 2010 for a review). When aberrantly regulated, TG2 may aid tumour cells to evade apoptosis and have direct consequences on cancer drug resistance (Mehta, 1994; Chen *et al.* 2002 and metastatic progression (Mehta *et al.* 2004). For instance, Park *et al* (2009) reported that TG2-specific cross-linking activity resulted in the polymerization and inhibition of nucleophosmin, and concomitant increase in drug resistance potential of cancer cells.

Recent evidence shows that aberrant expression of TG2 in mammary epithelial cells bestows stem cell characteristics on the cells (Kumar *et al.* 2011). Similarly, Kumar *et al* (2011) reported that high basal expression of TG2 in breast cancer cells promotes the development of stem cell features. Additionally, Caffarel *et al* (2013) observed that the activation of TG2:integrin-α5ß1 interactions through the stimulation of oncostatin M receptor in cervical squamous cell carcinoma, induced pro-malignant changes. Clinically, TG2 has been reported to serve as a predictive indicator of anticancer therapeutic efficacy. For instance, Jeong *et al* (2013) suggested that TG2 expression is a promising indicator of the effectiveness of epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) therapy in patients suffering from non-small cell lung cancer. Similarly, Assi *et al.* (2013) reported that the accumulation of TG2 in tumour stroma can serve as an independent risk factor for the identification of invasive ductal carcinomas (IDCs) of breast, and establish breast cancer patients at high risk of recurrence. They also observed that overexpression of TG2 can serve as an indicator of poor prognosis for IDC of the breast.

Agnihotri *et al.* (2013) proposed that inflammation induced progression of breast cancer and promoted acquisition of survival and invasive capabilities by breast cancer cells. In acute

myeloid leukaemia, Pierce *et al* (2013) demonstrated that increased expression of TG2 characterized a more advanced state of the disease in relapse patients. They further established that increased TG2 expression is correlated with the expression of proteins involved in apoptosis, motility and extracellular matrix association, processes that have been linked with leukaemia development and progression. This is a testament to the specialized ability of TG2 to interact with several proteins as substrates in various biological events, probably due to the unique biochemical structure of TG2 that is uncharacteristic of any other transglutaminase enzyme.

From the available literature, it has become evident that TG2 is involved in cancer drug resistance and metastasis. Though, TG2 expression profile has been widely studied and reported in different cancer cell types as reviewed above, the involvement of TG2 in primary liver cancers such as human hepatocellular carcinoma (HCC) has not been reported; even though, the protein is abundant in liver cells. To date, no report has shown the expression pattern of TGM2 gene and its product, TG2, in parental hepatocellular carcinoma cell line in relation to the drug-resistant and possibly metastatic sub-clones. This loophole exists, notwithstanding the low survival rate of HCC due to its specialized ability to resist chemotherapy. TG2 abundance in liver cells, its link with drug resistance and metastasis in several cancer types, and the high mortality rate of HCC due to chemo-resistance, are some of the factors that informed the entire thesis. Here, the patterns of expression and activity of TG2 in parental and drug-resistant HEPG2 cell lines were investigated, with the view to gaining insights into the roles of TG2 in cancer drug-resistance and metastasis.

4.2: MATERIALS AND METHODS

4.2.1: Materials

Materials were obtained from Sigma Aldrich UK or Invitrogen UK, except RNA easy plus mini kit (Qiagen, UK), QuantiTect reverse transcription kit (Qiagen, UK), Superscript II reverse transcriptase with SYBR Green (Qiagen, UK), TG2-specific test kit (TG2-covtest) (Covalab, UK), recombinant human TG2 (rhTG2) produced in *E. coli* (Covalab, UK).

4.2.2: RT-PCR analyses of TGM2 gene expression

The pattern of expression of TGM2 gene in parental and drug-resistant hepatocellular carcinoma cell lines was determined following anticancer drug administration. The process involved total RNA extraction from cells, complementary DNA (cDNA) synthesis, and real-time polymerase chain reaction (RT-PCR) as described by Kim *et al.* (2009) and Wang *et al.* (2012).

Briefly, total RNA was extracted from parental and drug-resistant HEPG2 cell lines after 24 hours incubation in medium containing cisplatin at 0µM to 16µM or 5-FU at 0µM to 100µM, using the RNA easy plus mini kit. Following cleanup of the RNA isolate through DNase treatment, cDNA was synthesized from 1µg of total RNA by reverse transcription process, using QuantiTect reverse transcription kit. Polymerase chain reaction was carried out using TGM2-specifc primers, forward primer 5'TAA GAG ATG CTG TGG AGG AG-3' and reverse primer 5'CGA GCC CTG GTA GAT AAA-3'. Absolute mRNA molecules were normalized against actin forward primer AGCAGTTGTAGCTACCCGCCCA and reverse primer GGCGGGCACGTTGAAGGTCT. The amplification conditions used were 40 cycles of denaturation for 30 seconds at 94^{o}C, annealing for 30 seconds at 55^{o}C and 10 minutes

extension at 72^{o}C. Potential DNA contamination was tested by the inclusion of one control reaction without RT enzyme.

Values of TGM2 gene expression in the experimental samples were obtained by the interpolation of cycle threshold (Ct) values on standard amplification curves, derived from known amounts of cognate, amplicon-specific synthetic RNA; in this case, actin. Each set of experiment was done in duplicate and the actual expression level of TGM2 gene was the mean values of the duplicates plus or minus the standard deviation, represented as fold increase or decrease and represented using bar charts. The quality of the assay and the amplification of target gene were shown by the resulting single peaks obtained in the melting curves for all the reactions, which demonstrated that the experiments were devoid of contamination as shown in appendix three.

4.2.3: Western blot analysis of TG2 protein expression

Parental HEPG2 cell line and drug-resistant clones were treated as previously described in section 2.2.3. Afterwards, Western blot analyses were performed with the cell lysates following the methods described by Kumar *et al.* (2011), Wang *et al.* (2012), and Yakubov *et al.* (2013). Briefly, drug-treated cells were lysed directly on flasks with ice-cold RIPA buffer containing 50mM Tris-HCl (pH 8.0), 150mM sodium chloride, 1% igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS); and protease inhibitor cocktail. Protein concentrations were determined using Bradford method as described by Lin *et al.* (2011), and lysates were snap-frozen in liquid nitrogen before storage at -80^{o}C. Cell lysates were thawed on ice and mixed at the ratio of 1:1 with Laemmli buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris HCl, pH approx. 6.8. The mixture containing 50µg of total protein was heated for ten minutes

at 70^{o}C before separating the proteins on 10% to 12% NuPAGE Bis-Tris Mini Gels at a constant voltage of 100V and 70mA for one hour.

Upon separation, the proteins were transferred onto nitrocellulose membranes at constant voltage of 100V and 400mA, using ice-cold blotting buffer containing 1% transfer buffer salt 10X, 2% methanol, and 70% distilled water at 4^{o}C. The membrane blots were subsequently blocked for two hours in blocking buffer containing 5% milk in TBS-Tween 20 (TBS-T), containing 20mM Tris-HCl, (pH 7.6), 0.8% (w/v) glycine, and 0.25% Tween-20. This was followed by incubation with primary antibody (anti-TGM1 (ab1), antibody produced in rabbit) (Sigma Aldrich, catalogue number: AV47471-50UG) in blocking buffer at the ratio of 1:25,000, for forty minutes at room temperature. The blots were then washed three times with TBS-T for twenty minutes intervals, before further incubation with secondary antibody (ant-rabbit IgG (whole molecule)-peroxidase antibody produced in goat) (Sigma Aldrich, catalogue number: A9169) in blocking buffer, at the ratio of 1:25, 0000 for 30 minutes at room temperature. Afterwards, the blots were washed further times with TBS-T for twenty minutes interval, before incubation in horse radish peroxidase (HRP) substrate for twenty minutes to reveal the protein bands.

4.2.4: General transglutaminase (Tgase) activity assay

Transglutaminase activity was assayed by colorimetric hydroxamate method, following the recipe described by Montero *et al.* (2005) as originally designed by Folk and Cole, (1965). Firstly, to ascertain extent of activity of total Tgase in HEPG2 cell line and establish the optimal cell number with which Tgase activity is detectable, the activity of total Tgase was measured relative to cell number. Briefly, exponentially growing HEPG2 cells were harvested by trypsinization, reseeded at the range of 10^6 cells to 40 x 10^6 cells. Subsequently,

the cells were harvested at 4^{o}C by direct lysis with ice-cold RIPA buffer containing 20% of protease inhibitor cocktail. This was followed by the preparation of a reaction mixture containing 200µl of 100mM CBZ-Glyn-Gly, 50µl of 100mM DTT, 50µl of 100mM calcium chloride, 100µl of 100mM hydroxylamine, 0.5ml of 50mM Tris-base (pH 8.0), and 50µg of total protein. The final reaction mixture was incubated for 10 minutes at 37^{o}C before the addition of 0.5ml of 20mg/ml iron III chloride, following which product formation was measured spectrophotometrically on the basis of absorbance at 525nm. After measuring the enzyme activity in parental HEPG2 cell line relative to cell number, lysates from the drug-resistant clones were tested for similar activity following treatment with cisplatin (0µM to 16µM) for cisplatin-resistant cells or 5-FU (0µM to 100µM) for 5-FU resistant cells respectively. Each experiment was conducted in duplicate and the enzyme activity was represented as mean value ± standard deviations.

4.2.5: Transglutaminase 2-specific activity assay using TG2-covtest kit

Specific tissue transglutaminase (tTG/TG2) colorimetric microassay kit (TG2-CovTest) is based on the method described by Parez Alea *et al.* (2009). Essentially, it uses biotinylated T26 peptide (biotin-pepT26) as the first substrate (amine acceptor/acyl donor) and spermine as second substrate (amine donor/acyl acceptor). Samples thought to contain TG2 are incubated in the wells of microtiter plates with covalently bound spermine, in the presence of 11.6mM calcium chloride, 50mM DTT, and biotin-pepT26. If TG2 is present in the samples, spermine is incorporated into the γ-carboxamide of the glutaminyl residue of biotin-pepT26, forming biotin-pepT26-γ-glutamyl spermine. The system is coupled to streptavidin labelled peroxidase (SAv-HRP), and subsequently revealed using hydrogen peroxide as HRP substrate and tetramethyl benzidine as electron acceptor.

TG2-specific activity was assayed in the three different cell lines, based on the assay principle described above. Accordingly, the cells from the exponentially growing cell lines were harvested by trypsinization, washed with 1X PBS, reseeded in T-75cm^2 flasks, then incubated overnight for attachment, before appropriate treatment with cisplatin (0µM to 16µM) or 5-FU (0µM to 100µM), and further twenty four hours incubation for drug action. Cell lysates were prepared as earlier described (section 4.2.3). TG2 activity was measured in duplicate samples for each test concentration, as described in the manufacturer's protocol.

4.3: RESULTS

4.3.1: Analysis of TGM2 gene expression by RT-PCR

The expression level of TG2 gene in untreated cells was used as the standard for the establishment of fold change. For each of the anticancer drugs under investigation, the pattern of expression of TGM2 gene in response to drug treatment was investigated in both parental and drug-resistant cell lines. Analyses of TGM2 gene expression in HEPG2 cell line following cisplatin treatment revealed that TGM2 gene expression level increased by 27%, 44%, 52%, 27% and 20% at concentrations of 1uM, 2uM, 4uM, 8uM, and 16uM respectively, relative to untreated cells. Interestingly, however, a dramatic increase in TGM2 gene expression was recorded in resistant clone (HEPG2CR) after similar treatment, where the expression level at 4µM nearly tripled relative to controls (figure 4.1). A p value of 0.0313 shows that the expression of TGM2 gene in parental HEPG2 cells differs significantly from TGM2 gene expression in HEPG2CR cells, where significant difference is defined by a p value less than 0.05 ($p < 0.05$).

Additionally, target gene amplification was further confirmed by the subjecting the PCR products to electrophoretic separation. Agarose gel electrophoresis of the products resulted in uniform single banding pattern, matching the target size of about 180bp as shown in figure 4.0.

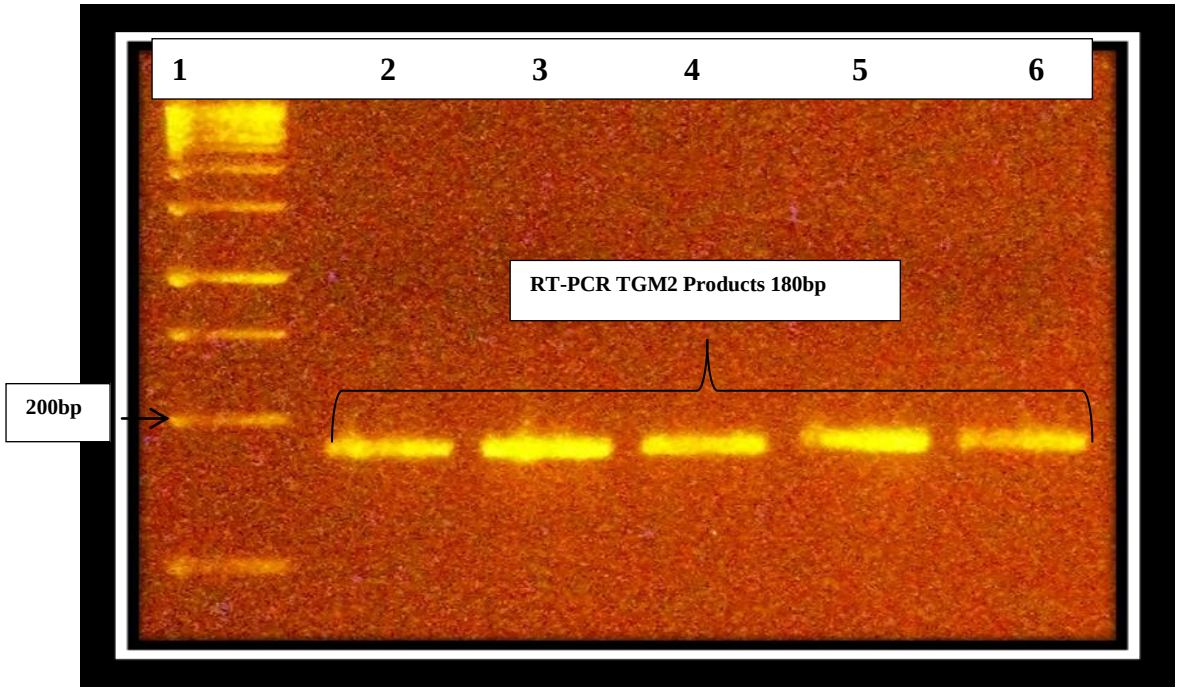


Figure 4.0: Confirmation of RT-PCR amplification of target TGM2 gene by gel electrophoresis; lane 1: marker, lanes 2, 3, 4, 5 and 6 are PCR products equivalent to 180bp target band size, obtained from different replicates of the experiment. The PCR product was sequenced by a colleague (Mark D'Arcy) and confirmed to be TGM2 gene.

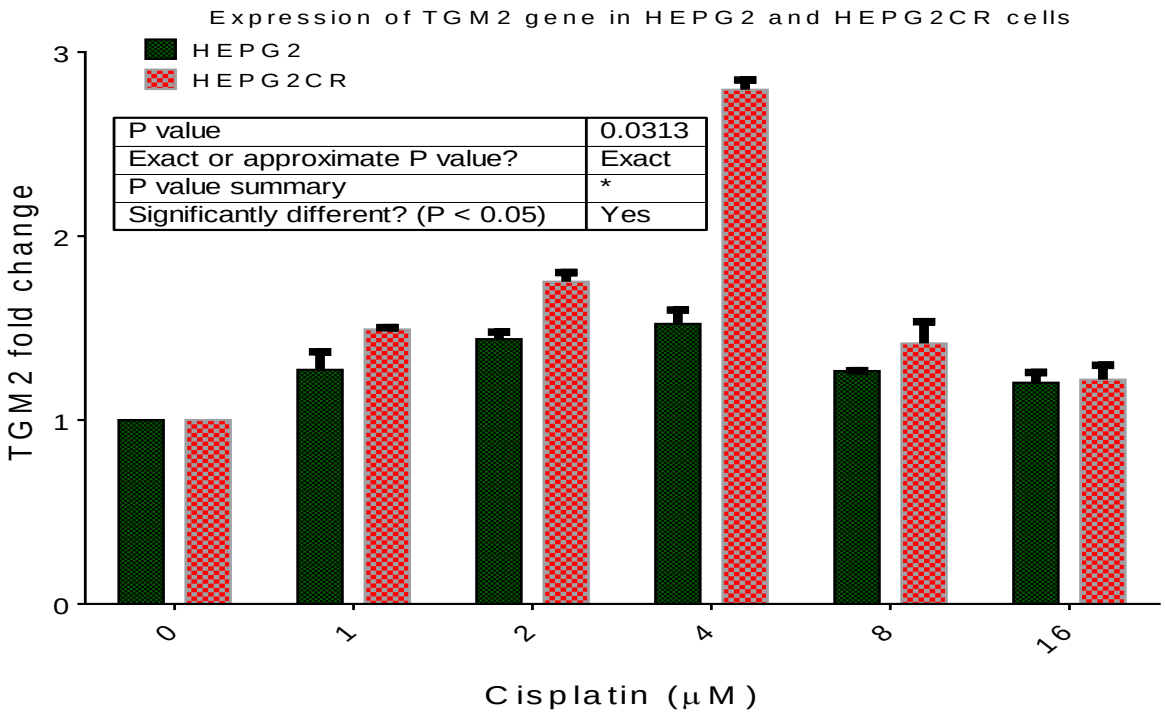


Figure 4.1: Comparison of TGM2 gene expression in parental (HEPG2) and cisplatin-resistant (HEPG2CR) cell lines after twenty four hours treatment with cisplatin, showing increased TGM2 gene expression that peaks at 4μM before decline, in both HEPG2 and HEPG2CR cells. The resistant clone (HEPG2CR) showed significantly higher expression levels of TGM2 gene relative to the parental cell line as indicated by a p value of 0.0313, where statistical significance is defined by a p value less than 0.05 (see appendix 4, table A4.8 and appendix 2, tables A2.3.1 and A2.3.2).

A closer inspection of the pattern of expression of TGM2 gene in both HEPG2 and HEPG2CR revealed two common denominators between the two cell lines, namely: (a) A general pattern of initial increase in expression followed by a decline in expression with increase in cisplatin concentration. (b) A maximum expression level of TGM2 gene at 4μM of cisplatin in both cell lines. However, in addition to the similarity in the pattern of TGM2 gene expression in both cell lines, the results also revealed a significant increase in the gene expression in the drug-resistant cell line relative to the parental cell line (p value = 0.0313).

For 5-FU, a comparison of TGM2 expression in HEPG2 and HEPG2FR cell lines after treatment with varying concentrations of 5-FU as described in section 4.2.2, revealed that the pattern of the gene expression was considerably different from what was obtained in cisplatin-treated lines. Though, a steady initial increase and final uniform decrease was recorded in both cell lines, the expression of TGM2 gene in HEPGFR cell line was lower than the expression level of the gene in the parental cell line (figure 4.2). This is a direct opposite of the results obtained in cisplatin-resistant cell line and the parental cell line.

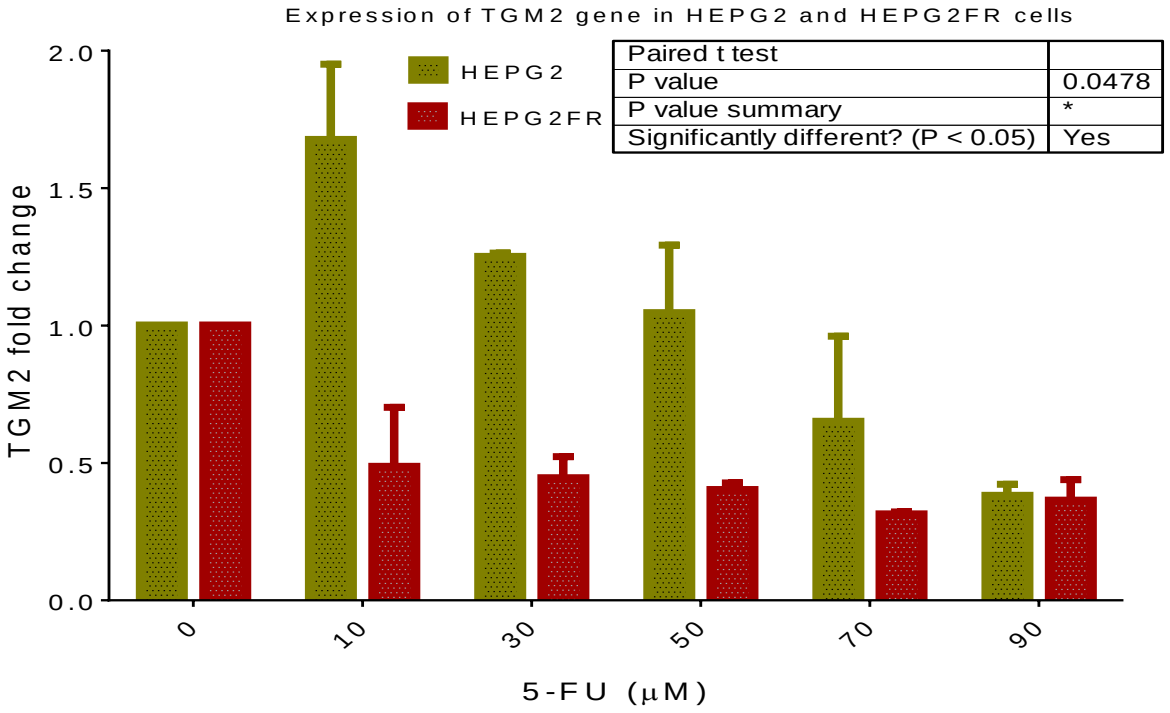


Figure 4.2: Expression of TGM2 gene in HEPG2 and HEPG2FR cell lines after twenty four hours treatment with 5-FU, showing opposing patterns of TGM2 gene expression relative to figure 4.1. In this case, a dramatic decrease in TGM2 gene expression was observed in the resistant clone relative to the parental cell line. A student paired t-test shows that TGM2 gene expression in HEPG2 cells is significantly different from that of HEPG2FR with p value of 0.0478 where statistical significance is defined by a p value less than 0.05 at 95% confidence interval (see appendix 4, table A4.10 and appendix 2, tables A2.3.3 and A2.3.4).

4.3.2: Western Blot Analyses of TG2 Expression

To understand the pattern of expression of TGM2 gene product, transglutaminase 2 (TG2), in both parental and drug-resistant hepatocellular carcinoma cell lines, Western blot analyses were carried out on the lysates from the cell lines. Essentially, the cell lines were treated as described in section 4.2.3 and 50µg of total protein in the lysate from each of the cell line was subjected to SDS-PAGE gel electrophoresis for separation as shown in figure 4.3. From the image shown below, the strength of the protein bands as an indication of the amount of soluble protein present in cell extracts prepared from six different replicates.

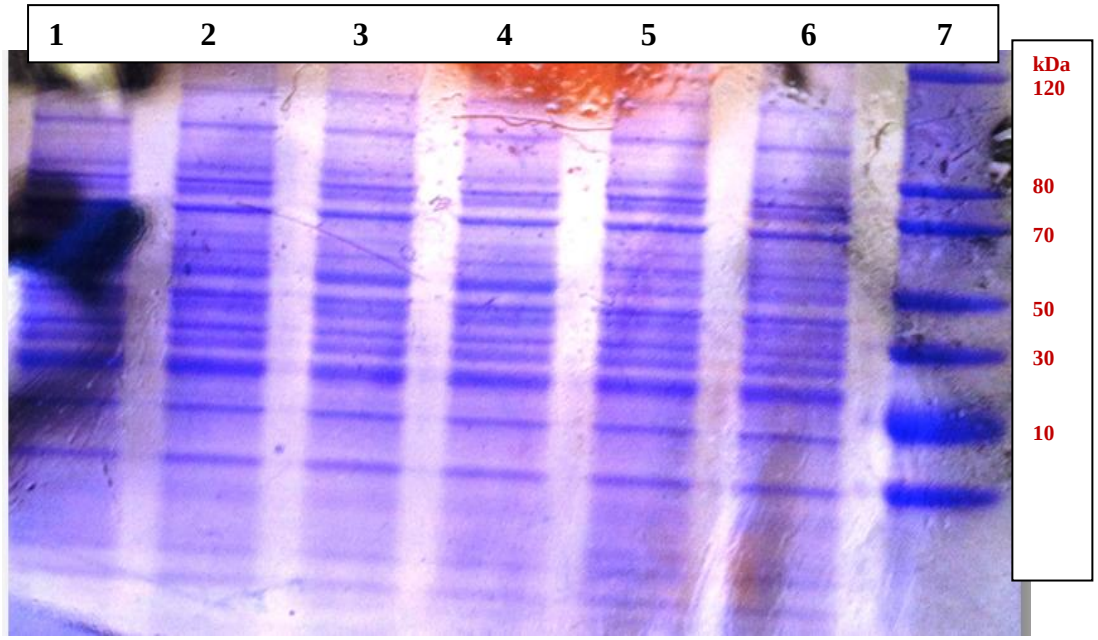


Figure 4.3: SDS-PAGE gel image showing protein bands before Western blotting; lanes 1 to 6: protein bands from six different cell extracts, containing 50µg of protein each; lane 7: marker of different sizes, up to 120 kDa as shown.

4.3.2.1: Effect of cisplatin on TG2 protein

A comparison of the expression pattern of TG2 with that of its gene, TGM2, in HEPG2 and HEPG2CR cell lines after cisplatin treatment for twenty four hours, the results showed a sustained increase in expression of TGM2 and TG2 in the drug-resistant cell line relative to

the parental cell line. Taken together, cisplatin-resistant hepatocellular carcinoma cell lined expressed higher level of TG2 messenger RNA and TG2 protein than the parental cell line in response to drug treatment. Additionally, results from the Western blot analysis showed that the expression TG2 increases with increased concentration of cisplatin in both HEPG2 and HEPG2CR as shown in figure 4.4 and figure 4.5. Interestingly, similar to TGM2 gene expression which was at its peak at 4µM in both parental and cisplatin-resistant cell lines, highest expression of TG2 protein was obtained at 4µM in HEPG2CR, before a gradual decline, also characteristic of the gene expression.

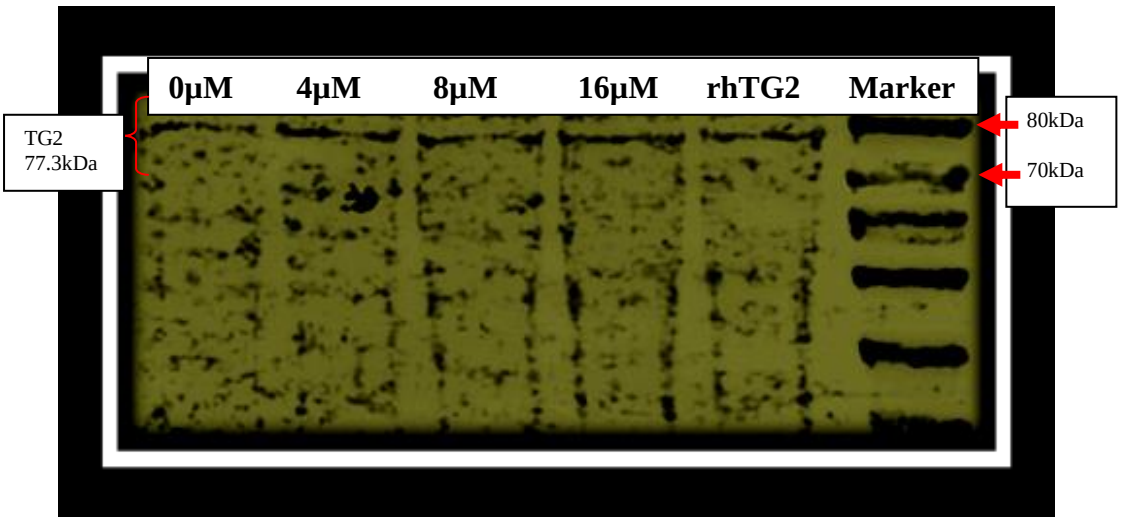


Figure 4.4: Transglutaminase 2 protein (77.3kDa) expression in HEPG2 cell line following twenty four hours cisplatin treatment at different concentrations; lane 1: 0µM, lane 2: 4µM, lane 3: 8µM, lane 4: 16µM, land 5: 2.2µg of recombinant human TG2 (rhTG2), and lane 6: marker.

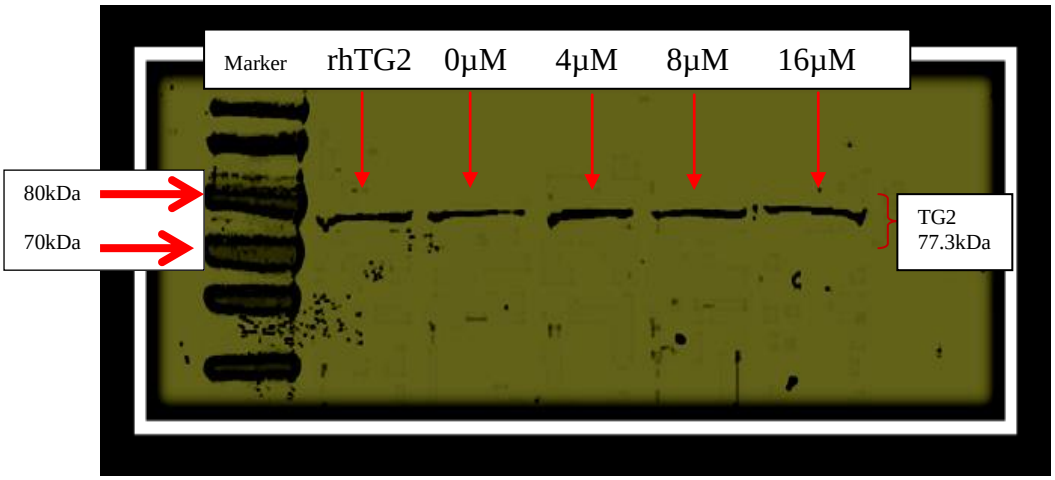


Figure 4.5: Expression of transglutaminase 2 protein (77.3kDa) in HEPG2CR cell line following twenty four hours cisplatin treatment different concentrations; lane 1: marker, lane 2: 2.2µg of rhTG2, lane 3: 0µM, lane 4: 4µM, land 5: 8µM, and lane 6: 16µM.

4.3.2.2: Effect of 5-FU on TG2 expression

The expression of TG2 in parental and 5-FU-resistant hepatocellular carcinoma cell lines after 5-FU treatment assumed different pattern relative to the expression pattern of TGM2 gene. Essentially, the results obtained from the Western blot analyses of cell extracts from both cell lines following drug induction, showed a steady increase in TG2 expression in both HEPG2 and HEPG2FR cell lines, relative to what was obtained in the gene expression results. A direct comparison of TG2 expression in HEPG2 and HEPG2FR cell lines showed a higher expression level in HEPG2FR than in HEPG2, however, increased TG2 expression with increasing 5-FU concentration was a common denominator between both cell lines (figure 4.6 and figure 4.7).

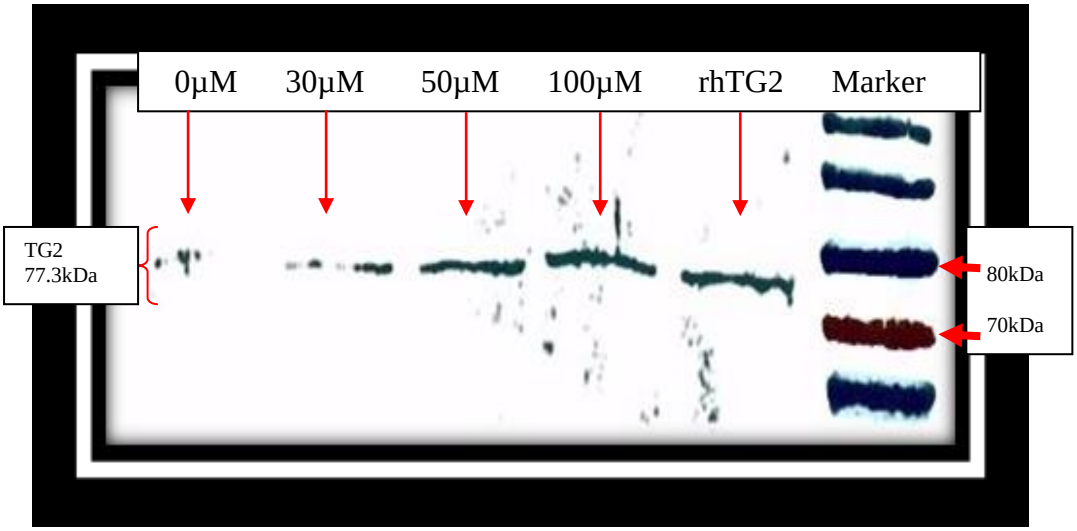


Figure 4.6: The expression pattern of TG2 protein (77.3kDa) in HEPG2 cell line in response to twenty four hours treatment with different concentrations of 5-FU; lane 1: 0µM, lane 2: 30µM, lane 3: 50µM, lane 4: 100µM, land 5: 2.2µg of rhTG2, and lane 6: marker.

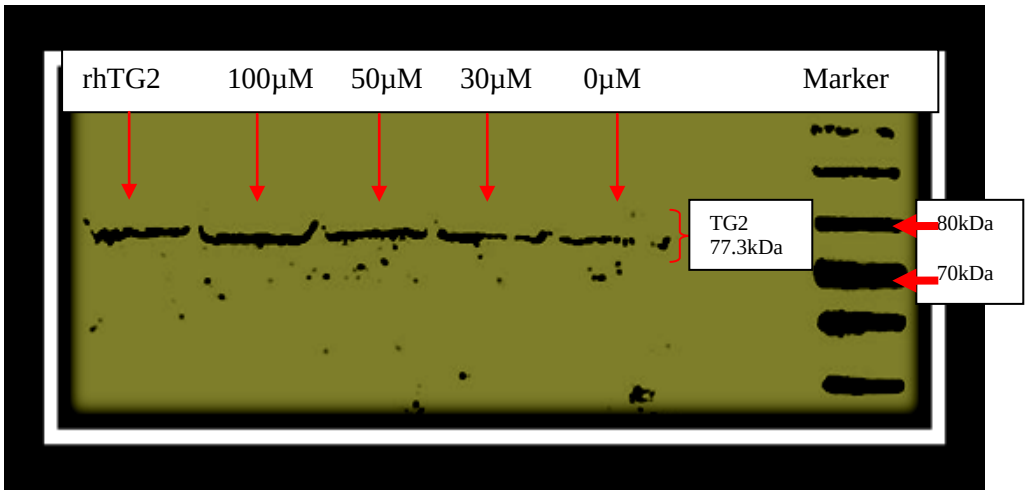


Figure 4.7: Transglutaminase 2 protein (77.3kDa) expression in HEPG2FR cell line in response to twenty four hours treatment with different concentrations of 5-FU; lane 1: 2.2µg of rhTG2, lane 2: 100µM, lane 3: 50µM, lane 4: 30µM, land 5: 0µM, and lane 6: empty, lane 7: marker.

4.3.3: Transglutaminase (Tgase) Activity Profiles in Parental and Drug-Resistant Hepatocellular Carcinoma Cell Lines

In order to get general idea of the activity profiles of the entire transglutaminase (Tgase) family existent in hepatocarcinoma cells, and to establish the number of cells at which activity level of Tgase is optimum; an initial investigation of Tgase activity relative to cell number was carried out (section 4.2.4). The results obtained from this assay showed that Tgase activity is directly proportional to cell number (figure 4.8). Additionally, the optimal number of cells that could be cultivated in $75cm^2$ flask to obtain reasonable activity was established to as 10 x 10^6 cells at 10^6 cells per ml.

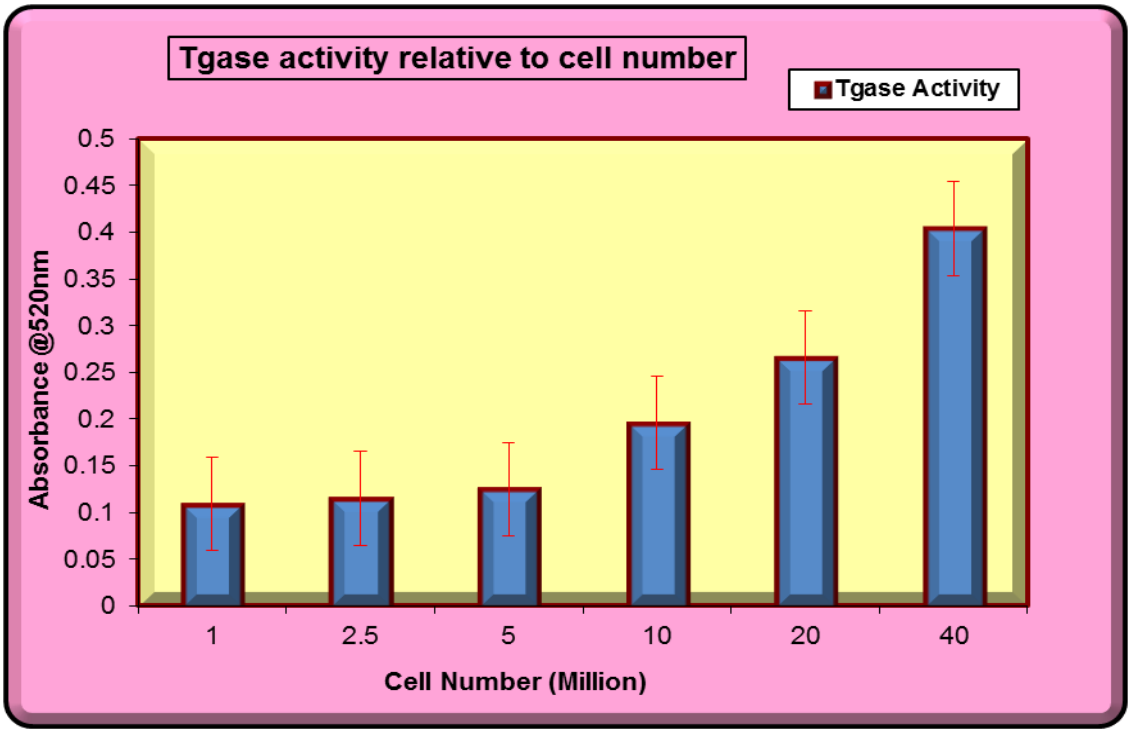


Figure 4.8: CBZ-Hydroxamate-based assay of Tgase activity relative to HEPG2 cell number, using similar amount of total protein (50µg) from lysates produced from flasks containing different number of cells. Increased total Tgase activity as the cell number increases was recorded, though the activity level was approximately the same below five million cells.

4.3.3.1: Effect of cisplatin on Tgase activity

A comparative analysis of total Tgase activity in parental (HEPG2) and cisplatin-resistant (HEPG2CR) cell lines after cisplatin treatment revealed a pattern of expression that followed the same pattern with the patterns of TGM2 gene and TG2 protein expression in the same cell lines after similar treatment. Again, the enzyme activity initially increased, peaked at 4µM in both cell lines, before declining at higher concentrations. The activity of Tgase was shown to be a significantly higher in resistant (HEPG2CR) compared to HEPG2 as indicated by a p value of 0.0041, where statistical difference is defined by p value less than 0.05 ($p < 0.05$) at 95% confidence interval (figure 4.9).

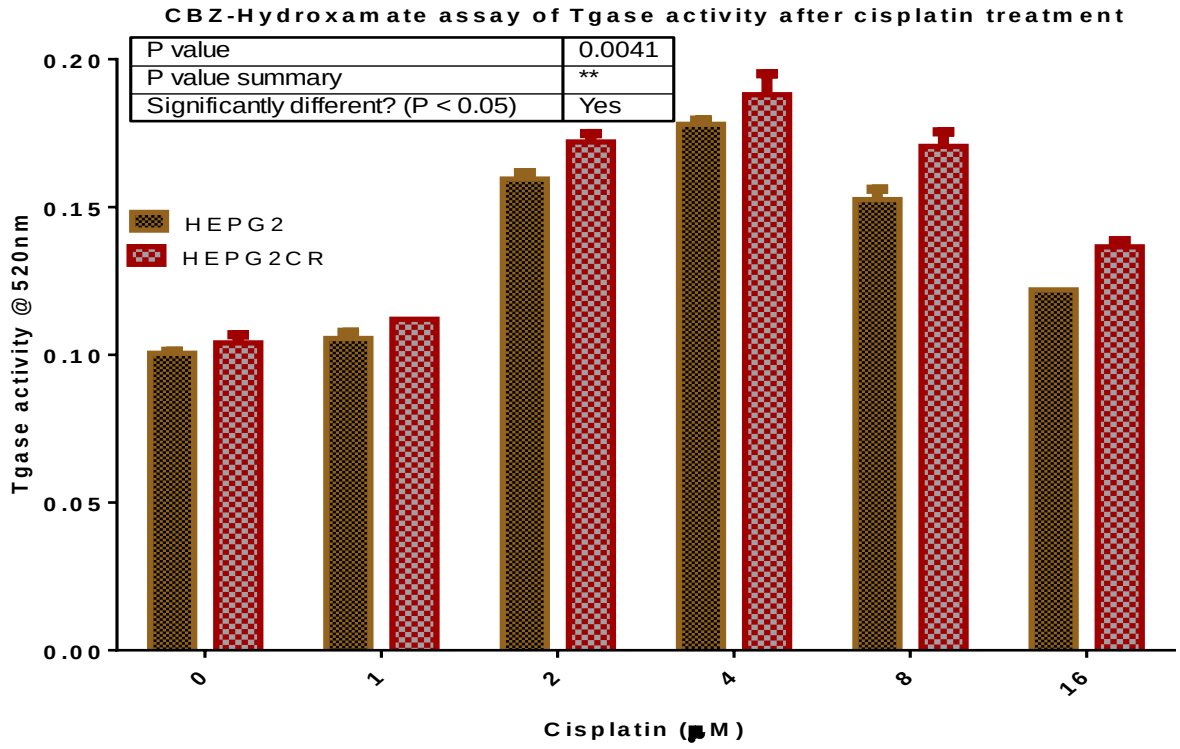


Figure 4.9: CBZ-Hydroxamate-based assay of Tgase activity in HEPG2 and HEPG2CR cells after twenty four hours cisplatin treatment, showing increased Tgase activity in HEPG2CR cells compared to the parental HEPG2 cells. The activity of Tgase in HEPG2CR is significantly higher than its activity in HEPG2 cells as indicated by a p value of 0.0041, where statistical difference is defined by p value less than 0.05 ($p < 0.05$) at 95% confidence interval (appendix 4, table A4.12 and appendix 2, tables A2.3.5 and A2.3.6).

4.3.3.2: Effect of 5-FU on Tgase activity

For 5-FU, analysis of Tgase activity in both parental (HEPG2) and 5-FU-resistant (HEPG2FR) cell lines showed a steady increase in enzyme activity with increasing drug concentration (figure 4.10). The pattern of Tgase activity conformed to the expression pattern of TG2 protein in HEPG2 and HEPG2FR cell lines under similar treatment with 5-FU. Though, increased activity of Tgase was recorded in both cell lines, the enzyme activity was slightly higher in HEPG2FR than in HEPG2, across concentrations.

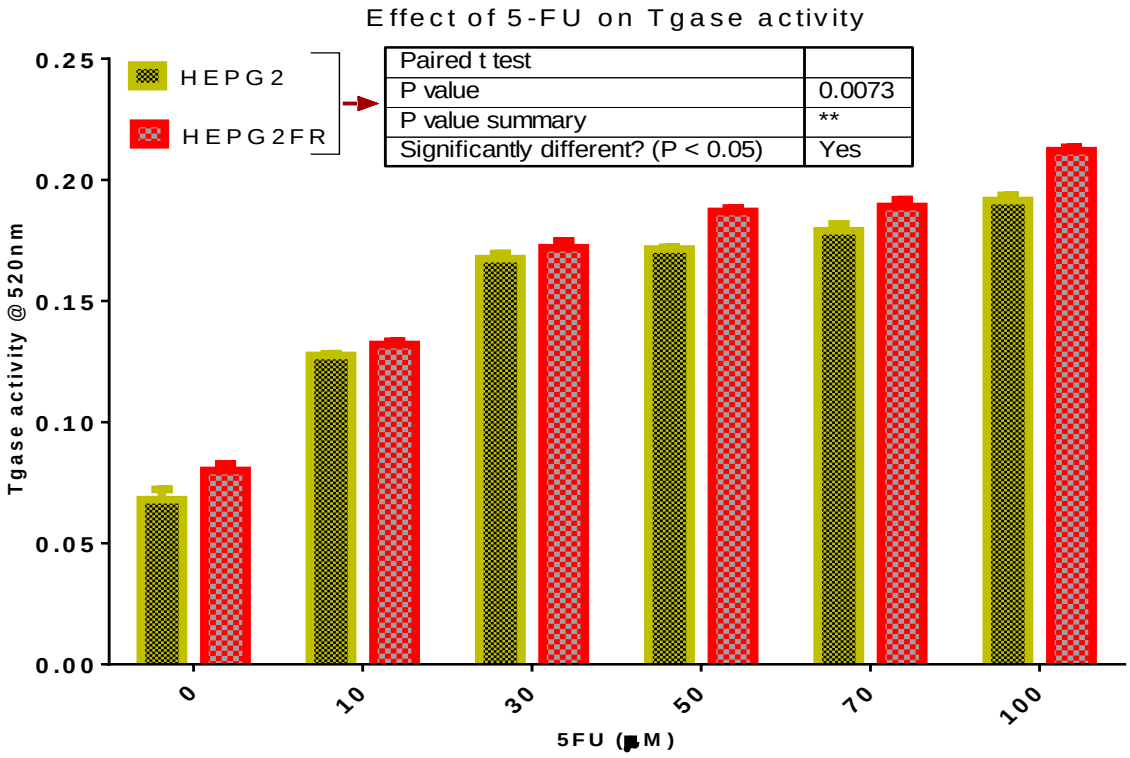


Figure 4.10: CBZ-Hydroxamate-based assay of Tgase activity in HEPG2 and HEPG2FR following twenty four hours 5-FU treatment, showing a sustained increase in Tgase activity as drug concentration increases in both parental cell line and drug-resistant clone, with greater activity in HEPG2CR. A student paired t-test shows that the activity of Tgase in HEPG2FR is significantly different from its activity in HEPG2 as indicated by a p value of 0.0073 which is less than 0.05 at 95% confidence interval (see appendix 4, table A4.14 and appendix 2, tables A2.3.7 and A2.3.8).

4.3.4: Transglutaminase 2-Specific Activity Profiles in Parental and Drug-Resistant Hepatocellular Carcinoma Cell Lines

Following the analyses of total Tgase activities in parental and drug-resistant hepatocellular carcinoma cell lines, it became pertinent to establish the proportion of the total transglutaminase constituted by TG2 as opposed to total Tgase activity. Additionally, it was imperative to establish TG2-specific activity in the cell lines following drug treatment. This was done with the view to comparing the activity of TG2 with its expression at transcriptional and post-transcriptional levels in the cell lines. Consequently, total cell lysates from the three cell lines (HEPG2, HEPG2CR and HEPG2FR) treated accordingly with appropriate drug concentrations were subjected to TG2-specific activity assay (section 4.2.5).

4.3.4.1: Effect of cisplatin on TG2 activity

The activity of TG2 in HEPG2 and HEPG2CR maintained similar pattern as TGM2 and TG2 expressions, again with an initial increase in activity and peak activity at 4μM (optimal concentration) in both cell lines followed by a uniform, steady decrease in activity. Unlike TGM2 and TG2 expression, there is no significant difference in activity of TG2 in parental and cisplatin-resistant HEPG2 cells (figure 4.11).

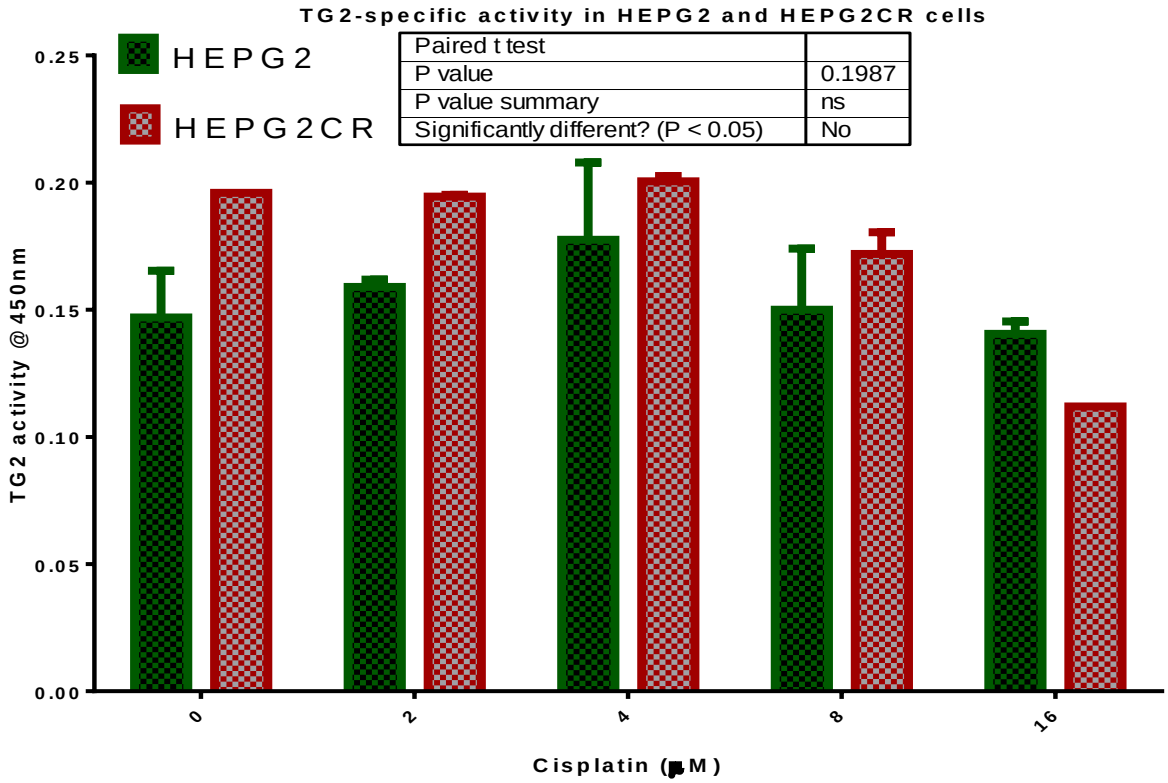


Figure 4.11: TG2-specific activity in HEPG2 and HEG2CR cell lines after twenty four hours treatment with cisplatin. From a student paired t-test, p value of 0.1987 which is greater than 0.05 at 95% confidence interval indicates that there is no significant difference between TG2 activities in parental and cisplatin-resistant cells (see appendix 4, table A4.16 and appendix 2, table A2.3.9).

4.3.4.2: Effect of 5-FU on TG2 activity

The enzyme activity was higher in HEPG2FR cell line relative to the parental cell line (Figure 4.12). Analysis of TG2 activity in 5-FU-treated cells revealed that TG2 activity initially increased, followed by a decreased activity before another rise in activity level as 5-FU concentration increases in both parental (HEPG2) and 5-FU-resistant (HEPG2FR) cell lines. The pattern of TG2 activity in both cell lines after drug induction followed similar pattern as that of TGM2 gene expression, with maximum activity at maximum concentration.

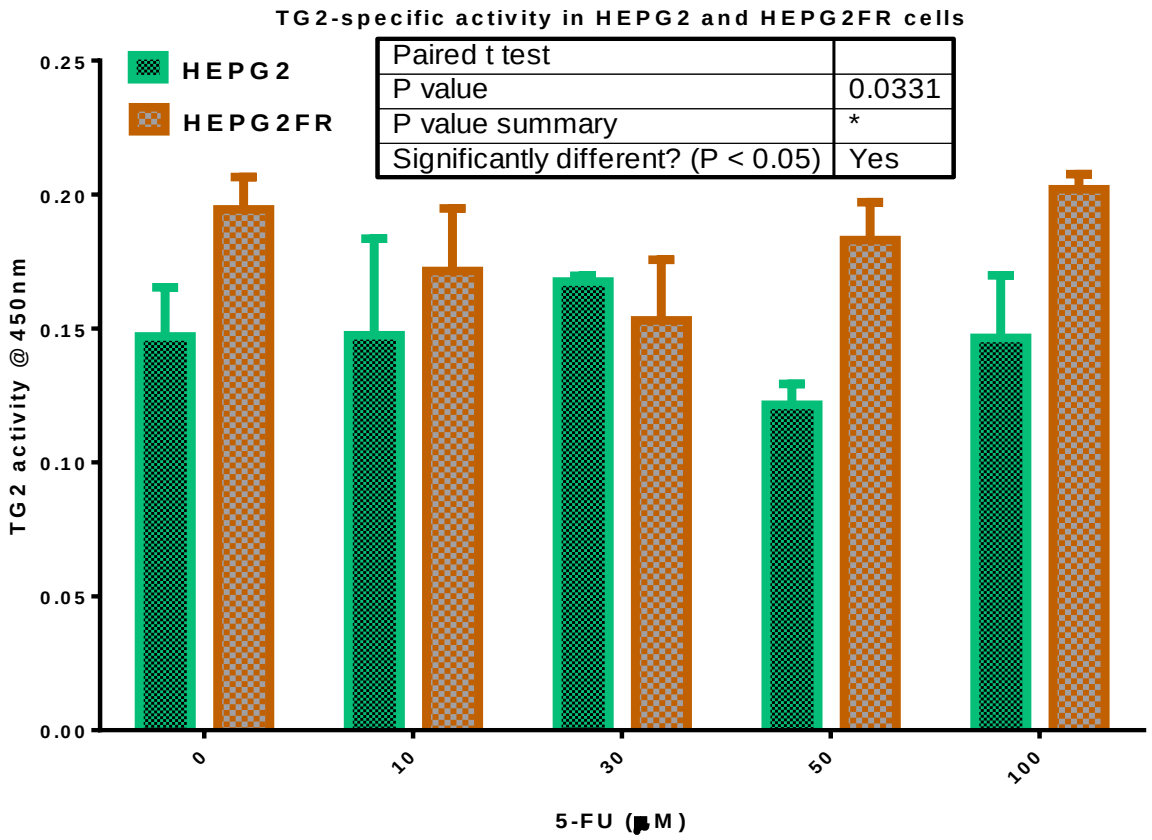


Figure 4.12: TG2-specific activity in HEPG2 and HEG2FR cell lines following twenty four hours incubation with 5-FU-containing medium, showing a fluctuation in TG2 activity across drug concentrations. A statistical analysis of this result by student paired t-test shows that TG2 activity in HEPG2FR is significantly different from that of the parental HEPG2 cells, regardless of the fluctuation in pattern of activity. Hence, a p value of 0.0331 which is less than 0.05 indicates statistical difference, where significant difference is defined by $p < 0.05$ at 95% confidence interval (see appendix 4, table A4.18 and appendix 2, table A2.3.10).

4.4: DISCUSSION

Analyses of the patterns of expression of TGM2 gene in parental (HEPG2) and cisplatin-resistant (HEPG2CR) cell lines following repeated cisplatin treatments indicated an elevated expression level of TGM2 gene in HEPG2CR than in HEPG2 cells (figure 4.1). This pattern of expression is in agreement with earlier reports of elevated expression levels of TGM2 gene

in drug-resistant and metastatic cell lines derived from many cancer types, including pancreatic carcinoma (Verma *et al*. 2006), ovarian carcinoma (Satpathy *et al*. 2007; Hwang et al. 2008), malignant melanoma (Fok et al. 2006), lung carcinoma (Park *et al*. 2010), glioblastoma (Yuan *et al*. 2007), and breast carcinoma (Mehta *et al*. 2004). Comparing these patterns of expression of TGM2 gene with cell death patterns following similar treatment with cisplatin (as shown in previous chapters), it is rational to argue that increased expression of TGM2 gene may be elicited as an apoptosis evasion tool, in agreement with the anti-apoptotic function of TG2 reviewed in Mehta *et al.* (2006), especially judging from the fact that the gene expression level is higher in drug-resistant cell. However, owing to the dual roles of TGM2 and its gene product in apoptosis, it is tempting to suggest that increased expression of TGM2 gene may be necessary to excite the cells to death due to cisplatin, in agreement with Mehta *et al.* (2006) and Verma & Mehta (2007).

Additionally, a critical observation of the pattern of TGM2 gene expression in HEPG2 and HEPG2CR following induction with cisplatin revealed two significant commonalities between both cell lines: (a) A general pattern of initial increase in expression followed by a decline in expression with increase in cisplatin concentration. (b) A maximum expression level of TGM2 at 4µM of cisplatin in both cell lines. Comparison of these patterns of expression to cell death patterns shown in cell viability assay results in previous chapters (sections 2.3.1 and 3.3.1), it shows that the initial increase in TGM2 gene expression corresponds to the initial drop in cell viability in both cell lines following cisplatin introduction. In one hand, the initial elevated TGM2 gene expression level may be interpreted as an early defence response by the cells, and on the other hand, it may be a death signal corresponding to the initial sub-set of dividing cells of the cell population that are more susceptible to death due to chemotherapeutic stress, in this case, cisplatin. This is in

agreement with the proposition that TGM2 gene expression is elevated in the events of cellular stress, such as chemotherapeutic induction, where it is necessary to ensure the integrity of dying cells and prevent inflammation (Fesus & Szondy, 2005; Mehta *et al.* 2006)

The expression of TGM2 gene steadily increased in the cell lines, peaking at 4µM cisplatin, and then declining afterwards with increasing drug concentration. The peak expression of TGM2 gene at 4µM, which was also the EC_{50} of cisplatin for parental cells, is particularly interesting and this may be due to the domination of the remaining cellular population by cells with more resistance potential. Beyond the optimal concentration of cisplatin, decrease in TGM2 gene expression seems to provide an avenue for drug action, hence, the corresponding increase in cell death.

In 5-FU-treated cells, the expression pattern of TGM2 in parental (HEPG2) and 5-FU-resistant (HEPG2FR) cell lines was directly opposite to the pattern of the same gene expression in cisplatin-treated hepatocarcinoma cell lines. This pattern of expression is suggestive of the fact that TGM2 gene expression is not only stress-dependent (Agnihotri *et al.* 2013), but stress-type-dependent. Essentially, TGM2 gene expression level was grossly decreased in the resistant clone (HEPG2CR) compared to the parental cell line (figure 4.2). This is in disagreement with previous reports of increased expression of TGM2 gene in cell lines derived from other cancer types (as reviewed in Mehta *et al.* 2010). However, merging the expression patterns of TGM2 gene with cell death patterns following similar treatment with 5-FU, it is tempting to assert that the role of TGM2 gene and TG2 in apoptosis is dependent on the type of apoptosis-inducing stimulus, as originally suggested by Milakovic *et al* (2004). Furthermore, it is tempting to suggest that the decreased expression of TGM2

gene in HEPG2FR may be due to poor extractability of RNA as a consequence of massive TG2 cross-linking effect upon drug induction.

Western blot results of TG2 protein expression in HEPG2 and HEPG2CR cell lines after twenty four hours cisplatin treatment showed that the patterns of TG2 protein expression assumed similar patterns as TGM2 gene expression in the two cell lines. Elevated TG2 protein expression was obtained in HEPG2CR relative to the parental HEPG2 cell line (figure 4.4 and 4.5). This expression pattern of TG2 at both gene and protein levels is in agreement with previous reports that drug-resistant cells express high levels of TG2 relative to the parental cell line in many cancer types (Mehta *et al* 2010). Interestingly, the expression of TG2 peaked at 4µM as reported in the TGM2 gene expression results, hence, further strengthening the view that the presence of highest number of more resistant cells at EC_{50} of cisplatin might be responsible for the peak expression levels at both gene and protein levels.

Analysis of TG2 protein expression in HEPG2 and HEPG2FR cell lines after 5-FU treatment for twenty four hours showed an increase in TG2 expression in both cell lines upon drug introduction. However, the expression of TG2 protein in the resistant clone was higher relative to the parental cell line (figure 4.6 and 4.7), and opposite to the expression pattern obtained at gene level. The elevated expression level of TG2 protein in resistant clone relative to the parental cell line again, is in agreement with previous reports in other cancer types (as earlier presented). However, the decreased level of TGM2 gene expression in HEPG2FR relative to the elevated expression level of TG2 protein in same cell line after similar treatment, suggests that the expression pattern of TG2 may not always necessarily be the same at transcriptional and post-transcriptional levels. On the other hand, this may further

substantiate the earlier assertion that lower expression of TGM2 gene in drug-resistant clone relative to the parental cell line may be due to the impediment of extractability by TG2 crosslinking, hence, the use of stronger lysis buffer (RIPA buffer) during protein extraction relative to the weak buffer used during RNA extraction, resulted in increased protein yield.

Analyses of general transglutaminase (Tgase) activity in parental and drug-resistant (HEPG2, HEPG2CR and HEPG2FR) cell lines following appropriate and corresponding drug treatment, revealed that Tgase activity increased with increased cell number and increased drug concentration across the cell lines (figure 4.8, 4.9, and 4.10). The activity of Tgase in HEPG2CR is significantly higher than its activity in HEPG2 cells as indicated by a p value of 0.0041, where statistical difference is defined by p value less than 0.05 ($p < 0.05$) at 95% confidence interval (figure 4.9); in agreement with TGM2 gene and TG2 protein expression profiles discussed previously. In same vein, a student t-test shows that the activity of Tgase in HEPG2FR is significantly different from its activity in HEPG2 as indicated by a p value of 0.0073 which is less than 0.05 at 95% confidence interval (figure 4.10).

Interestingly, however, TG2-specific activity assay in all the cell lines showed a similar pattern of activity for HEPG2FR versus HEPG2, with a p value of 0.0331 which is less than 0.05 indicates statistical difference, where significant difference is defined by $p < 0.05$ at 95% confidence interval (figure 4.12). However, there is no significant difference in activity of TG2 in HEPG2CR versus HEPG2 as indicated by a p value of 0.1987, where statistical difference is defined by p value less than 0.05 ($p < 0.05$) at 95% confidence interval (figure 4.11). This implies that 5-FU and cisplatin have different effects on TG2 activity. Furthermore, a comparative observation of Tgase and TG2 activity levels in all the cell lines

revealed that TG2 is the predominant member of transglutaminase enzymes present in liver cells, in agreement with its name liver transglutaminase.

Comparing TG2 activity profiles with apoptosis profiles for parental HEPG2 cell line and cisplatin-resistant (HEPG2CR), there appears to be a relationship between TG2 activity and apoptotic progression. This is because decreased TG2 activity just after 4μM (figure 4.11) corresponds to increased cell death recorded after the same concentration of cisplatin (section 2.3.2, figure 2.3). Furthermore, the initial increase in TG2 activity may be a pro-survival signalling mechanism employed by the cells to evade cisplatin action. From these results, it is therefore, reasonable to suggest that increase in TG2 activity may account for the ability of HEPG2 cells to evade cisplatin-induced apoptosis, and any attempt to decrease TG2 activity may sensitise cells to cisplatin-induced death. In the case of 5-FU, TG2 activity continued to increase with increased 5-FU concentration, in a similar fashion as the TG2 protein expression reported in section 4.3.2.2. This may account for the lower susceptibility of HEPG2 cells to 5-FU compared to cisplatin.

CHAPTER FIVE

EFFECTS OF TG2 SILENCING ON DRUG RESISTANCE AND METASTASIS POTENTIAL OF HEPATOCARCINOMA

5.1: INTRODUCTION

Cell migration and invasion are important steps in a variety of physiological processes, including implantation, morphogenesis, embryogenesis, neurogenesis, angiogenesis, wound healing and inflammation (von der Mark *et al.* 1999; Cho & Klemke, 2000). However, cell migration and invasion are also involved in the pathophysiology of many diseases such as cancer (Bozzuto *et al.* 2010). The ability of cancer cells to spread from its primary site of origin and its subsequent growth in another distant organ within the body is called metastasis (Sahai, 2005; Valestyan and Weinberg, 2011). Metastatic spread of cancer cells from the site of their origin rather than primary tumours, are responsible for the high mortality rates associated with cancer; accounting for over 90% of cancer-related deaths (Hanahan & Weinberg, 2000; Mangala *et al.* 2007). The process of tumour metastasis involves distinct steps, including detachment of tumour cells from primary tumour, invasion of tumour cells into surrounding tissues, entry into blood or lymphatic vessels, dissemination in the blood stream or lymphatic system and, finally, invasion of other host tissues and proliferation at secondary sites (Parker & Sukumar, 2003; Bozzuto *et al.* 2010).

Each of these metastatic processes requires a distinct molecular programme, where the regulation of the adhesive, migratory and cytoskeletal properties of the spreading cancer cells play important roles (Bozzuto *et al.* 2010). Over the past decade, the molecular mechanisms underlying the various steps of tumour metastasis has been under intense investigation. Major efforts have been undertaken to elucidate novel proteins and pathways that are involved in the

transformation of primary tumour cells into metastatic clones, and develop therapeutic protocols that can control the metastasis of cancer cells (Mazzocca and Carloni, 2009; Jung *et al.* 2012). For instance, in an independent study, Jiang *et al.* (2003a, b) reported that TG2 was one of the eleven metastasis-associated proteins that were selectively elevated in metastatic human lung and breast carcinomas. Similarly, Mehta *et al.* (2004) observed that metastatic cancer cells isolated from parental breast cancer cell line expressed elevated levels of TG2; and metastatic lymph node tumours from patients with breast cancer showed consistent higher level of TG2 relative to primary tumours from same patients (Mangala *et al.* 2007).

Furthermore, several reports have suggested that elevated TG2 expression enhances invasive, metastatic and drug resistance potentials of cancer cells (see Yakubov *et al.* 2013 for reviews). Mangala *et al.* (2007) suggested that TG2 expression in metastatic breast cancer cells promotes integrin-mediated cell attachment, survival signalling pathways, as well as cell migration and invasive capacity; whilst conferring apoptosis-resistance capability on the cells. Similarly, TG2 has been suggested to regulate ß-integrin-dependent cell adhesion to the ECM through the extracellular signalling activation of focal adhesion kinase; thus, contributing to increased cell survival and invasiveness (Verma *et al.* 2008; Satpathy *et al.* 2009). Recently, Yakubov *et al.* (2013) reported that extracellular TG2 induces epithelial-to-mesenchymal transition (EMT) through a distinct pathway that results in the activation of the transcription factor, nuclear factor kappa β (NF-ƙβ) and consequent increase in cellular invasiveness and peritoneal metastasis. TG2 has also been reported to enhance tumour aggressiveness, facilitating distant metastasis in both xenografts animal models and in patients with advanced breast cancer (Oh *et al.* 2011).

As previously reviewed in chapter one (section 1.5.3.2) elevated level of TG2 expression is a common denominator in drug-resistant and metastatic cancer cells derived from different cancer types. Indeed, TG2 down-regulation by small interfering RNA (siRNA) has been variously reported to attenuate cell adherence, survival and migration, whilst promoting the susceptibility of cancer cells to chemotherapy-induced death (section 1.5.3.2). The role of TG2 in cancer drug resistance and metastasis is suggested to be determined by its non-enzymatic activation of extracellular survival signalling pathways, as typified by its interaction with integrins and fibronectin, as well as phospholipase C (reviewed by Odii and Coussons, 2014). However, it is yet to be established if the actual causal relationship between TG2 and cancer drug resistance and metastasis is dependent on its activity or expression. It is therefore pertinent to investigate, and to compare the effects of TG2 down-regulation and activity inhibition on cancer cells' sensitivity to anticancer agents, as well as metastatic potential. It is for this reason that the implication of TG2 down-regulation in liver cancer drug resistance and metastasis was investigated, and the results are described below.

5.2: MATERIALS AND METHODS

5.2.1: Materials

All the materials were obtained from Sigma Aldrich UK and Invitrogen UK, except Annexin V-FITC kit (BD Biosciences, Europe), flow cytometer FACS Calibur (BD Biosciences, Europe), matrigel coated plates (BD Biosciences, Europe), transmembrane inserts (BD Biosciences, Europe), and TG2-specific test kit (TG2-covtest) (Covalab, UK).

5.2.2: Cell culture

Cell line and cell culture establishment involved similar method previously described in chapter two, subsection 2.2.2.

5.2.3: Matrigel cell invasion and migration assay

The invasive and migration potentials of parental and drug-resistant cells were investigated using biocoat matrigel invasion chamber (BD Biosciences, Europe). This experiment was adapted from the method reported by Choi *et al.* (2011), and following manufacturer's instructions. Briefly, matrigel-coated 6-well companion plates (BD Biosciences, Europe) were thawed in ice. Cells from either parental or drug-resistant clones of HEPG2 were prepared by trypsinization and re-suspension at a final concentration of 10^5 per ml, in serum-free RPMI 1640 medium (Invitrogen, UK). Subsequently, trans-membrane inserts were carefully placed in coated and uncoated wells (negative controls) using sterile forceps. Then, 2ml of cell suspension was transferred to each insert, followed by careful addition of 1.5ml of chemo-attractant (FBS) to the wells of the test companion plates (except the negative controls) via the access port; avoiding air bubbles. This was followed by incubation of the invasion chambers at 37^oC, 5% CO_2, and humidity for twenty four hours.

After twenty four hours incubation, non-invading cells were removed using moistened cotton swab. Afterwards, cells were fixed with 100% methanol and stained with 1% Toluidine blue containing 1% borax. Staining procedure involves transfer of inserts containing fixed cells into Toluidine stain for two minutes, after which excess stain was rinsed in distilled water. Inserts were allowed to air dry and migrated cells were pictured and counted under the microscope. Data was expressed as percentage of invasion through the matrigel matrix membrane relative to the migration of cells through the uncoated membranes, as shown:

$$\% \text{ Invasion} = \frac{\text{Mean number of cells invading through matrigel matrix insert}}{\text{Mean number of cells migrating through the uncoated insert}} \times 100\%$$

5.2.4: Assessment of siRNA uptake using BLOCK-iT

The BLOCK-iT fluorescent oligo (Invitrogen, UK) is a fluorescein-labelled double-stranded RNA (dsRNA) oligomer designed for use in RNA interference (RNAi) analysis to facilitate optimization and assessment of lipid-mediated delivery of dsRNA oligonucleotides into mammalian cells. It is a double-stranded RNA duplex with similar length, charge, and configuration as standard small interfering RNA (siRNA) (Ciccarone *et al.* 1999).

Prior to TG2 down-regulation by siRNA, BLOCK-iT was used to check transfection efficiency, following manufacturer's instructions. Briefly, 20μM stock of BLOCK-iT was thawed on ice and diluted to 2μM using Opti-MEM® I Reduced Serum Medium and incubated at room temperature for five minutes. Also, lipofectamine 2000 reagent was diluted with Opti-MEM® I Reduced Serum Medium at the ratio of 1:25, and incubated at room temperature for five minutes. Afterwards, dilute Block-iT and lipofectamine 2000 reagent were mixed and incubated at room temperature for twenty minutes to form a complex. After twenty minutes incubation, BLOCK-iT-lipofectamine complex was added to HEPG2 cells (except for the control) seeded onto a special slide at the density of $2x10^4$ per ml of serum-free Opti-MEM® I Reduced Serum Medium (Invitrogen, UK), and incubated for twenty four hours at 37^oC, 5% CO_2, and humidified atmosphere. After twenty four hours post-transfection incubation, oligo uptake was qualitatively assessed using fluorescence microscope.

5.2.5: TG2 down-regulation by small interfering RNA (siRNA)

Down-regulation of TG2 by siRNA was carried out as after the establishment of transfection protocol (described in section 5.2.4), with the method originally reported by Kim *et al.* (2006). Briefly, parental and drug-resistant cells growing at 80% confluence were trypsinized, re-suspended and seeded in $25cm^2$ flasks at seeding density of 10^6 cells per millilitre of serum-free Opti-MEM® I Reduced Serum Medium (Invitrogen, UK). After overnight incubation for cell attachment, siRNA duplex (5'-GCUCAUGUUCUCAGCACUU-3') targeting human TG2, was introduced into the cells (except for the control cells) using lipofectamine 2000 reagent as described in section 5.2.4. After twenty four to forty eight hours incubation, cells were harvested directly on flasks, using ice-cold RIPA buffer and the cell lysates were examined for TG2 by Western blot analysis, as previously described in chapter four, section 4.2.3.

5.2.6: Post-silencing cell invasion and migration assay

After siRNA silencing of TG2 in parental and drug resistant cell lines was carried out as described in sections 5.2.4 and 5.2.5, the cells were subjected to cell invasion and migration assessment using matrigel-coated companion plates and transmembrane inserts as previously described in section 5.2.3.

5.2.7: Post-silencing chemosensitivity assay

Following the successful down-regulation of TG2 in parental and drug-resistant HEPG2 cells as previously described in sections 5.2.4 and 5.2.5, the susceptibility of the cells to the chemotherapeutic drugs with which they were selected was tested using the method previously described in chapter two, section 2.2.7.

5.2.8: Post-silencing apoptosis assay by flow cytometry

After TG2 down-regulation by siRNA, the three cell lines under investigation were treated with appropriate concentrations of cisplatin or 5-FU and cell death distribution was analysed using flow cytometry, as previously described in chapter three (section 3.2.5).

5.2.9: Cystamine inhibition of TG2 activity

The therapeutic benefit of cystamine (mecaptoethanolamine (MEA) disulfide) is partly attributed to its ability to inhibit transglutaminase activity (Karpuj *et al.* 2002; Dedeoglu *et al.* 2002). Within cells, cystamine has been shown to be reduced to MEA with the amine group of MEA acting as substrate to TG2, resulting in the formation of N^{β}-(γ-L-glutamyl)-MEA bonds, consequently competing with TG2-catalysed reactions like polyamination, deamination, and cross-linking reactions (Jeitner *et al.* 2005).

To ascertain the best concentration of cystamine at which TG2 activity is inhibited, parental and drug resistant cells growing at logarithmic phase were harvested by trypsinization, washed with 1% PBS and re-suspended in fully supplemented RPM 1640 medium. The cells were seeded in six-well plates at density of 10^6 cells per millilitre of medium, incubated overnight at 37^oC, 5% CO_2, and humidified atmosphere. Subsequently, the cells were treated with different concentrations of cystamine, ranging from 0mM to 4mM, and maintained at similar incubation conditions for twenty four hours. Afterwards, the cells were harvested and the resulting cell lysates were examined for TG2 activity using TG2-specific colorimetric microassay kit (TG2-CovTest) as described in chapter four, section 4.2.5.

5.2.10: Chemosensitivity assay after cystamine inhibition of TG2 activity

After the inhibition of TG2 activity using cystamine, sensitivity of parental and drug resistant cells to cisplatin or 5-FU was examined using similar method previously reported in chapter two (section 2.2.7).

5.2.11: Analysis of cell invasion and migration after the inhibition of TG2 activity

Following the inhibition of TG2 activity by cystamine, parental HEPG2 cell line and the drug-resistant clones were assessed for invasion and migration capabilities using matrigel-coated companion plates and transmembrane inserts as earlier described in section 5.2.3.

5.2.12: Analysis of cell death distribution after cystamine inhibition of TG2 activity

Following the inhibition of TG2 activity using cystamine, parental and drug resistant cells were treated with appropriate concentrations of cisplatin or 5-FU, and cell death distribution was analysed by flow cytometry as previously described in chapter three (section 3.2.5).

5.3: RESULTS

5.3.1: *In vitro* assay of invasive and migration potentials of parental and drug-resistant HEPG2 cells

The ability of parental and drug-resistant HEPG2 cells to invade and migrate to distant sites was investigated *in vitro* using matrigel coated plates and transmembrane inserts as schematically represented in figure 5.0. This test also provided room to compare the invasive and migration capacities of the parental HEPG2 cell line and the drug-resistant clones. The results indicate that the three cell lines are capable of migrating to distant sites and they all invaded the membrane inserts as shown in figure 5.1b.

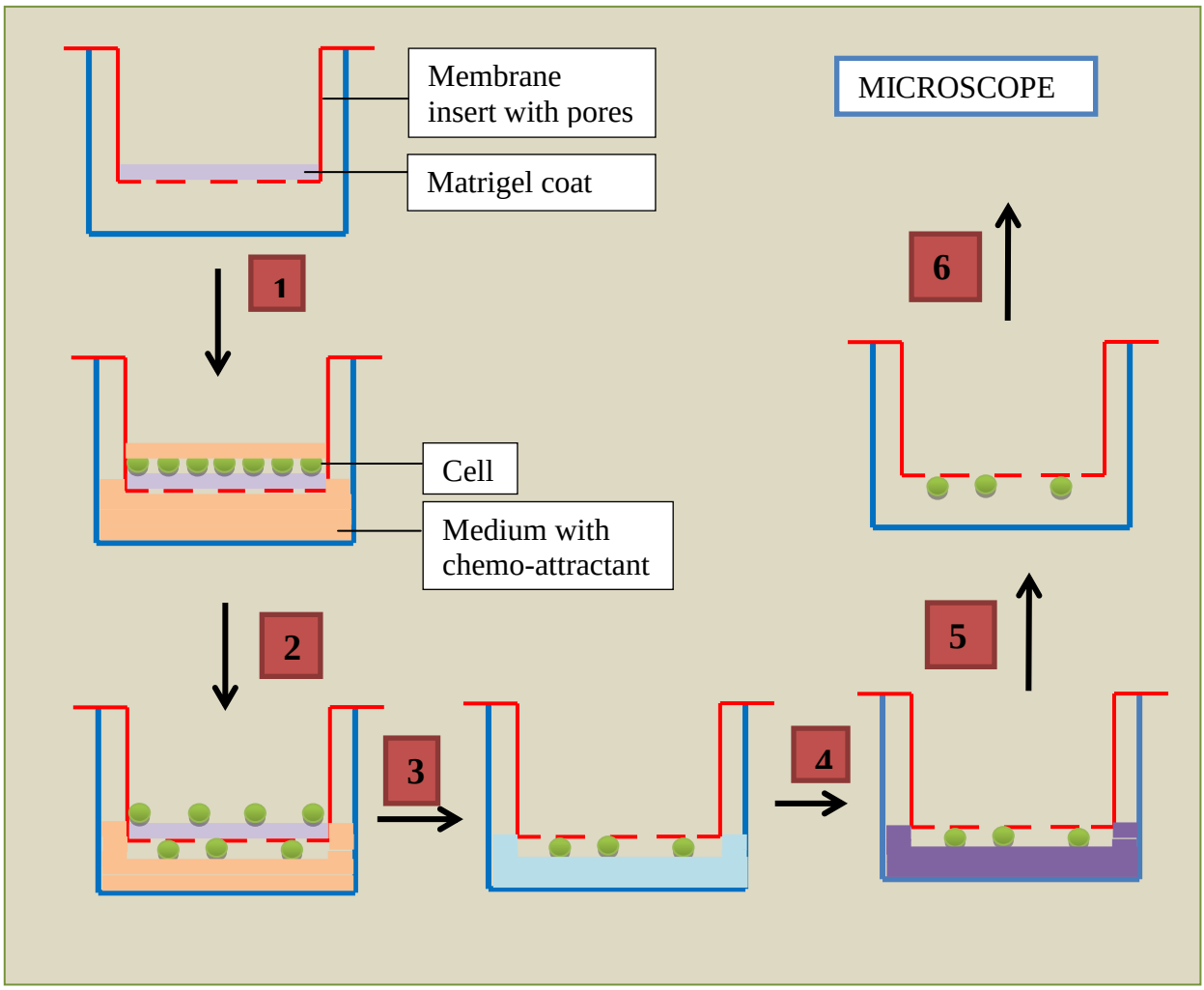
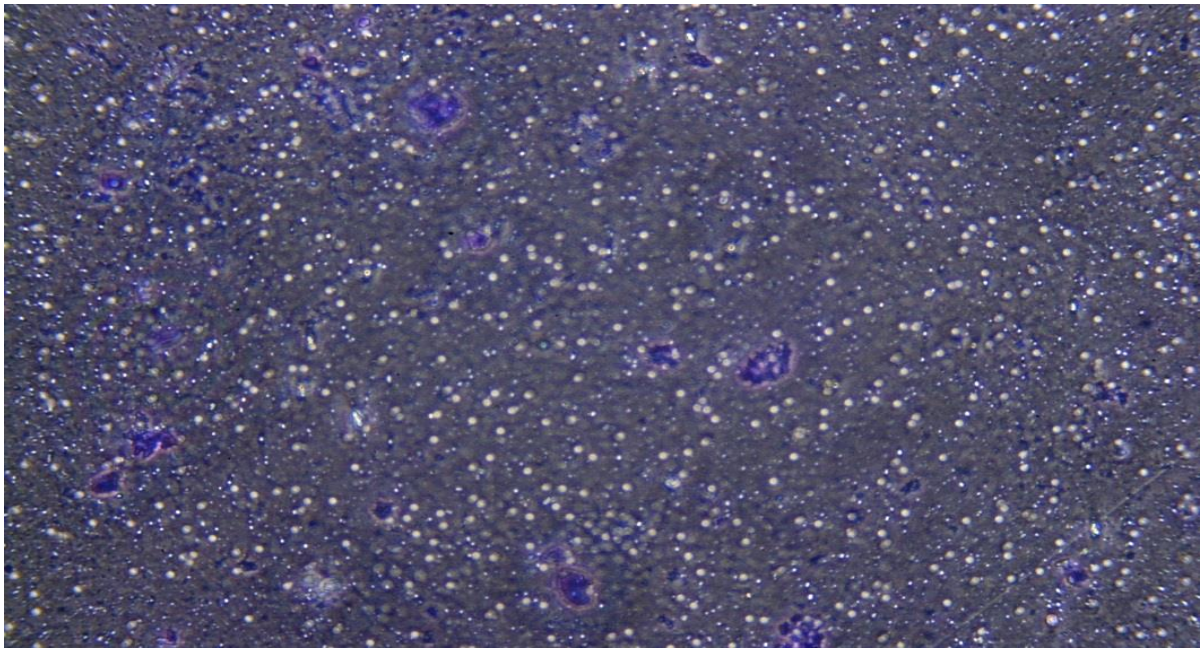
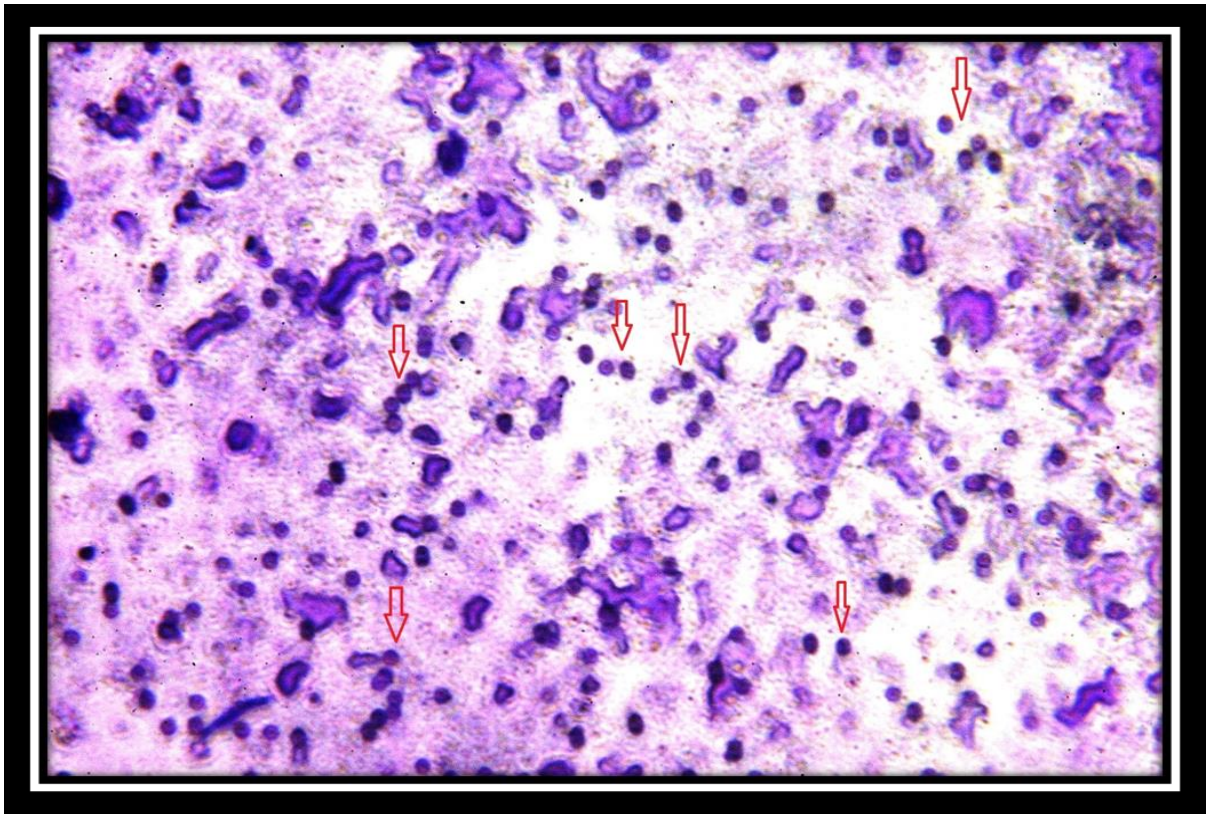


Figure 5.0: Schematic representation of matrigel invasion assay processes: (1) thaw matrigel at 37^{o}C and seed cells in culture medium (2) incubate cells for twenty four hours at 37^{o}C in culture medium containing chemo-attractant (foetal bovine serum in this case) (3) scrape un-migrated cells using cotton bud and fix the migrated cells in methanol (4) stain cells with Toluidine blue containing 1% borax (5) wash excess dye and allow to dry (6) count migrated cells under microscope.



(a)



(b)

Figure 5.1: (a) Surface of matrigel showing un-migrated cells before scrapping to reveal (x400) (b) migrating cells invading the membrane insert as shown by the arrowheads (x400).

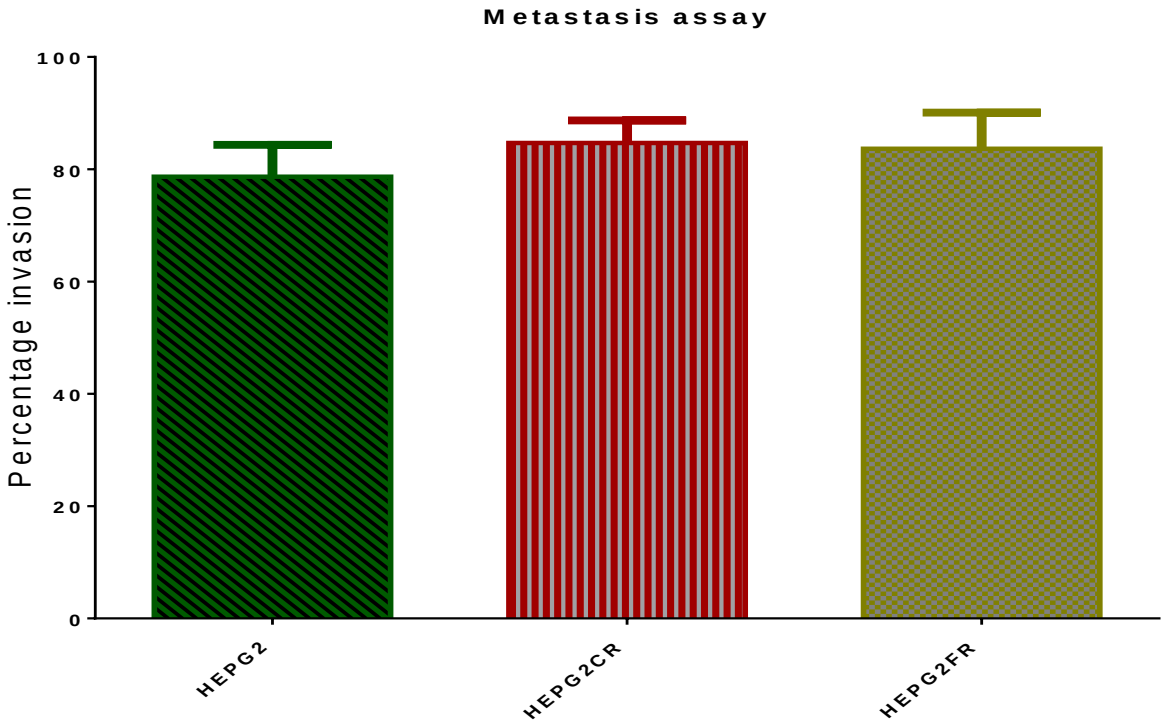
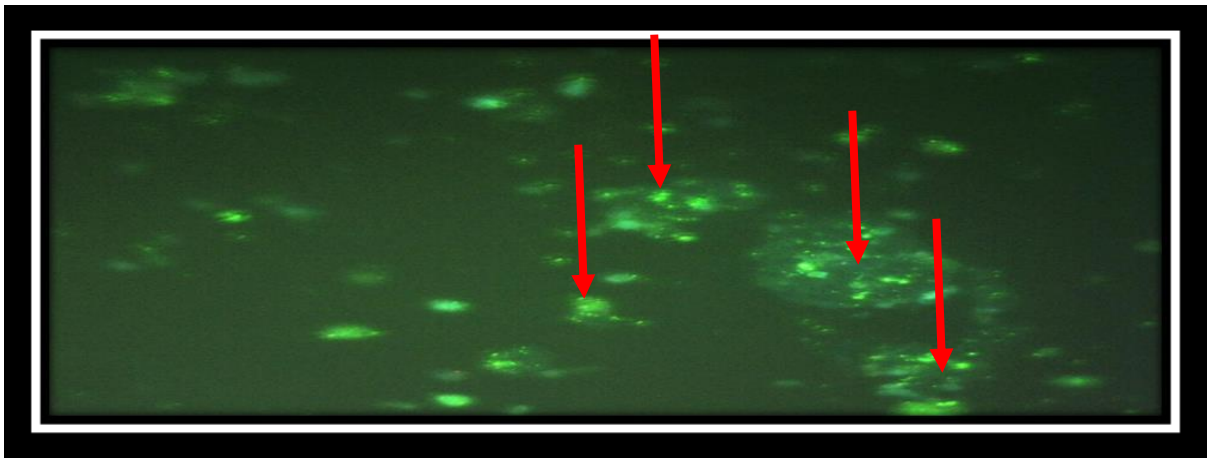


Figure 5.2: Percentage invasion and migration of parental and drug-resistant hepatocarcinoma cell lines on matrigel-coated surface. The three clones of HEPG2 displayed invasive and migration potential and a statistical analysis by ANOVA shows that there is no significant difference in the invasiveness of parental HEPG2 cells and the drug-resistant clones; p value = 0.4115 which is greater than 0.05 which defines significance (appendix 4, table A4.19). Additionally, student t-test comparing the invasive abilities of resistant clones with that of the parental cells shows that they have similar invasive propensity. For HEPG2 vs HEPG2CR, p value = 0.2539; HEPG2 vs HEPG2FR, p value = 0.0820; HEPG2CR vs HEPG2FR, p value = 0.8581; all of which are greater than 0.05 at 95% confidence interval (appendix 4, tables A4.20, A4.21 and A4.22 respectively, and appendix 2, table A2.4.1).

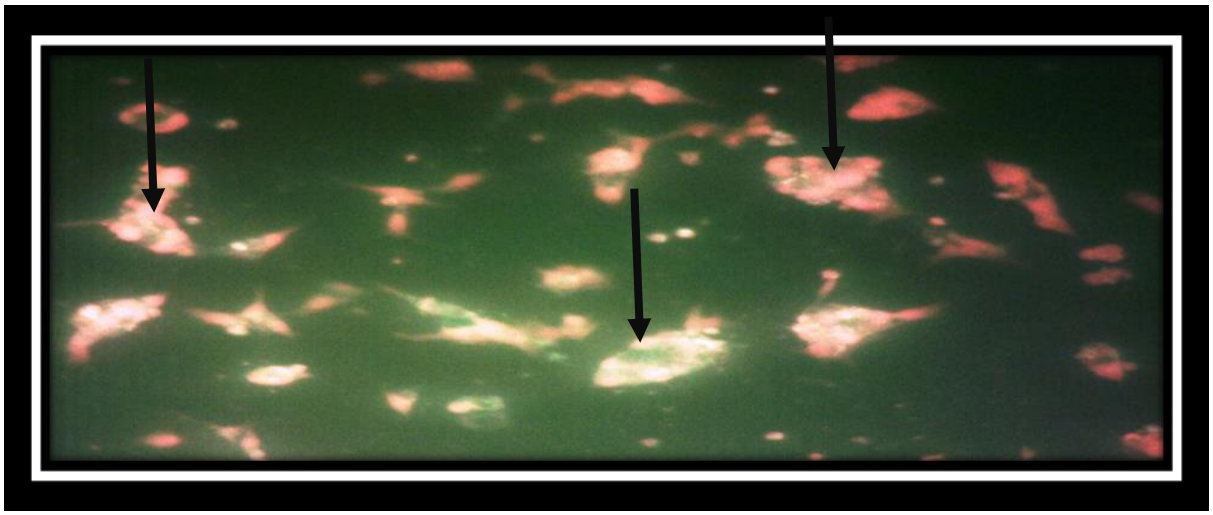
5.3.2: Qualitative assessment of siRNA uptake

Prior to TG2 down-regulation by siRNA, it is important to ascertain the possibility of lipid-mediated siRNA incorporation into the cells under investigation. To achieve this, a fluorescein-labelled double-stranded RNA duplex with similar length, charge, and

configuration as standard siRNA was introduced into the cells using similar lipid-based protocol for siRNA transfection. The intake of siRNA was qualitatively assessed under fluorescence microscope. The result revealed that the lipid-mediated transfection protocol is very efficient and suitable for the planned assay. Over 90% of the transfected cells showed fluorescence, while cells in the control (without BLOCK-iT) showed no fluorescence (figure 5.3 a and b).



(a)

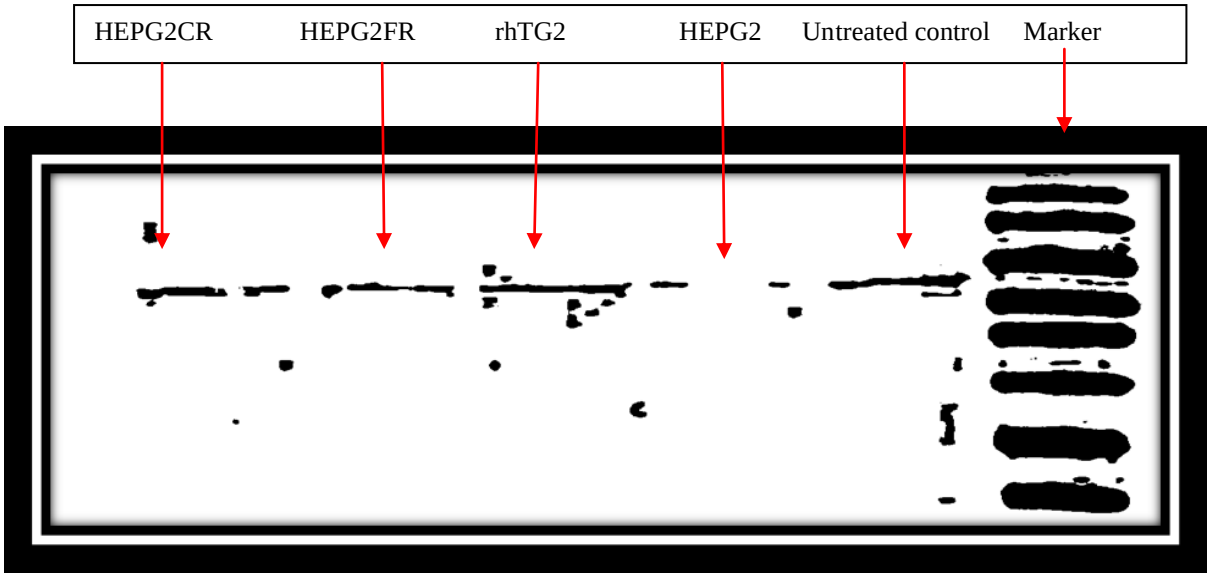


(b)

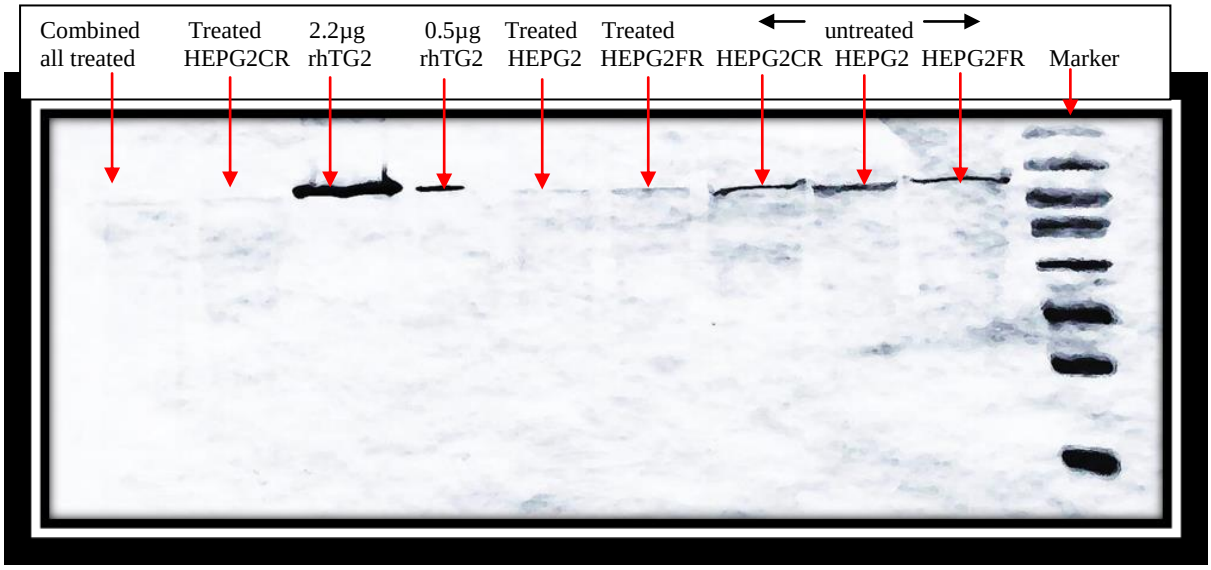
Figure 5.3: Fluorescent microscopy analysis of siRNA incorporation into cells (a) uptake of siRNA by the cells as shown by the red arrowheads (x100) (b) Untreated control showing no fluorescence as indicated by the black arrowheads (x100).

5.3.3: TG2 down-regulation in parental and drug-resistant HEPG2 cells

To determine the role of TG2 in cancer drug resistance and metastasis, siRNA was used to down-regulate its expression. After twenty four hours incubation of either parental HEPG2 cell line or the drug-resistant clones with siRNA, results obtained from the Western blot examination of the cell lysates revealed only a slight reduction in TG2 expression in the treated cells relative to the untreated control. This could be seen in figure 5.4 a, lanes 1 (HEPG2CR), 2 (HEPG2FR), and 4 (HEPG2), relative to lane 5 (untreated control). However, Western blot analysis of cell lysates obtained from HEPG2 clones forty eight hours after siRNA was introduced into the cells showed a significantly reduced expression of TG2 in the lysates of treated cells relative to the untreated cells. The expression levels of TG2 in the three cell lines after forty eight hours incubation with or without siRNA are shown in figure 5.4b, where lane 1 contains a combined lysates from all siRNA-treated cells; lane 2 (treated HEPG2CR); lanes 3 and 4 (2.2μg rhTG2 as positive control); lane 5 (treated HEPG2); lane 6 (treated HEPG2FR); lanes 7 to 9 (untreated controls for HEPG2CR, HEPG2 and HEPGFR respectively); and lane 10 (molecular marker).



(a)



(b)

Figure 5.4: Western blot analysis of TG2 protein expression following the introduction of siRNA into the cells and incubation for (a) twenty four hours: lane 1 (HEPG2CR), lane 2 (HEPG2FR), lane 3 (rhTG2 as positive control), lane 4 (HEPG2), lane 5 (untreated control), and lane 6 (molecular marker); (b) forty eight hours: lane 1 contains a combined lysates from all siRNA-treated cells; lane 2 (treated HEPG2CR); lanes 3 and 4 (2.2µg and 0.5µg rhTG2 respectivels, as positive controls); lane 5 (treated HEPG2); lane 6 (treated HEPG2FR); lanes 7 to 9 (untreated controls for HEPG2CR, HEPG2 and HEPGFR respectively); and lane 10 (molecular marker).

5.3.4: Analysis of cell invasion and migration after TG2 down-regulation

Following the result that both parental and drug-resistant HEPG2 clones have invasive and migration potential as shown in the results previously presented in section 5.3.1, it is therefore, pertinent to establish the possible involvement of TG2 in facilitation of the invasive and migration features of the cells. Consequently, TG2 down-regulation was carried out as previously reported in section 5.2.5, followed by the assessment of the invasive and migration behaviours of the different cell line under investigation. The results revealed that TG2 down-regulation has a considerable effect on the invasive and migration capabilities of the cells. A comparison of the results shown in figure 5.5 with the results of invasion and migration assay shown in figure 5.2 (section 5.3.1), revealed a reduced percentage of cell invasion and migration after TG2 down-regulation. However, TG2 down-regulation has an obvious minimal effect on the invasiveness of 5-FU-resistant (HEPG2FR) cells, where only 11% reduction was achieved relative to parental HEPG2 (21%) and cisplatin-resistant (HEPG2CR) (19%) cells respectively (figure 5.5).

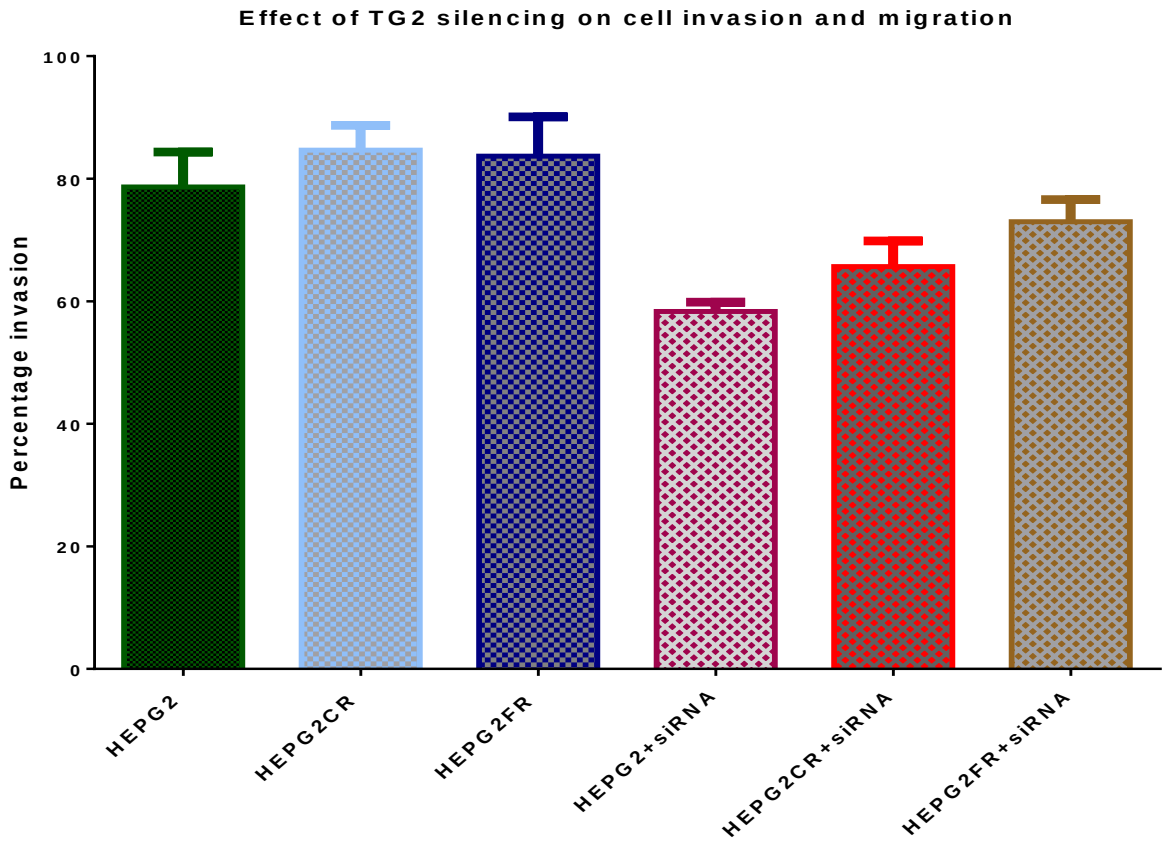


Figure 5.5: Comparison of percentage invasion and migration of parental and drug-resistant HEPG2 clones on matrigel-coated surface before and after TG2 down-regulation. Reduction in cell invasion and migration was recorded in all the cell lines relative to the results recorded before TG2 silencing. Statistical analysis using one-way ANOVA shows that the invasiveness of both parental and drug-resistant cells was significantly reduced following TG2 protein down-regulation by siRNA, with a p value of 0.0001 and strength of statistical significance (***) as shown in appendix 4, table A4.23. A further statistical analysis of the invasive ability of each HEPG2 clone with and without siRNA was done using student t-test. For HEPG2 vs HEPG2+siRNA, p value = 0.0339; HEPG2CR vs HEPG2CR+siRNA, p value = 0.0094; and HEPG2FR vs HEPG2FR+siRNA, p value = 0.0332; all of which indicate that the siRNA down-regulation of TG2 protein expression significantly reduced cellular invasiveness at 95% confidence interval and where $p < 0.05$ equals statistical significance (see appendix 4, tables A4.24, A4.25 and A4.26 respectively, and appendix 2, table A2.4.2).

5.3.5: Assay of cellular susceptibility to chemotherapy after TG2 down-regulation

After TG2 down-regulation by siRNA interference, it is important to investigate if the causal relationship between TG2 and cancer drug resistance is dependent on its expression. Consequently, the susceptibility of parental and drug-resistant HEPG2 clones to cisplatin or 5-FU was evaluated in the presence of reduced TG2 expression. The results obtained from these experiments relative to the results previously presented in chapter two, section 2.3.3 (figure 2.3 and figure 2.4) showed that both parental and drug-resistance cells were less susceptible to chemotherapy-induced death after TG2 down-regulation. Statistical analysis of HEPG2 and HEPG2CR susceptibility to cisplatin-induced death after down-regulation of TG2 protein expression by siRNA shows that HEPG2 cells are significantly more to cisplatin following TG2 down-regulation, with p value of 0.0167 (figure 5.6). However, a student t-test evaluation of the effect of cisplatin on HEPG2CR vs HEPG2CR+siRNA shows that TG2 down-regulation has no significant effect on the susceptibility of HEPG2CR cells to cisplatin-induced death (p value = 0.3103 > 0.05 at 95% confidence interval) (figure 5.6). On the other hand, analysis of the susceptibility of HEPG2 and HEPG2FR to 5-FU following TG2 down-regulation by siRNA using one-way ANOVA shows that TG2 down-regulation has no significant effect on the susceptibility of both parental and 5-FU-resistant HEPG2 clones to 5-FU treatment, with p value of 0.0774 which is greater than 0.05 at 95% confidence interval (figure 5.7). A student t-test of the effect of TG2 down-regulation on the susceptibility of HEPG2 clones to 5-FU-induced death further confirmed that it has insignificant effect on both HEPG2 and HEPG2FR, where the p value for HEPG2 vs HEPG2+siRNA is 0.1144 and the p value for HEPG2FR vs HEPG2FR+siRNA is 0.3198, all of which are greater than 0.05 at 95% confidence interval (figure 5.7).

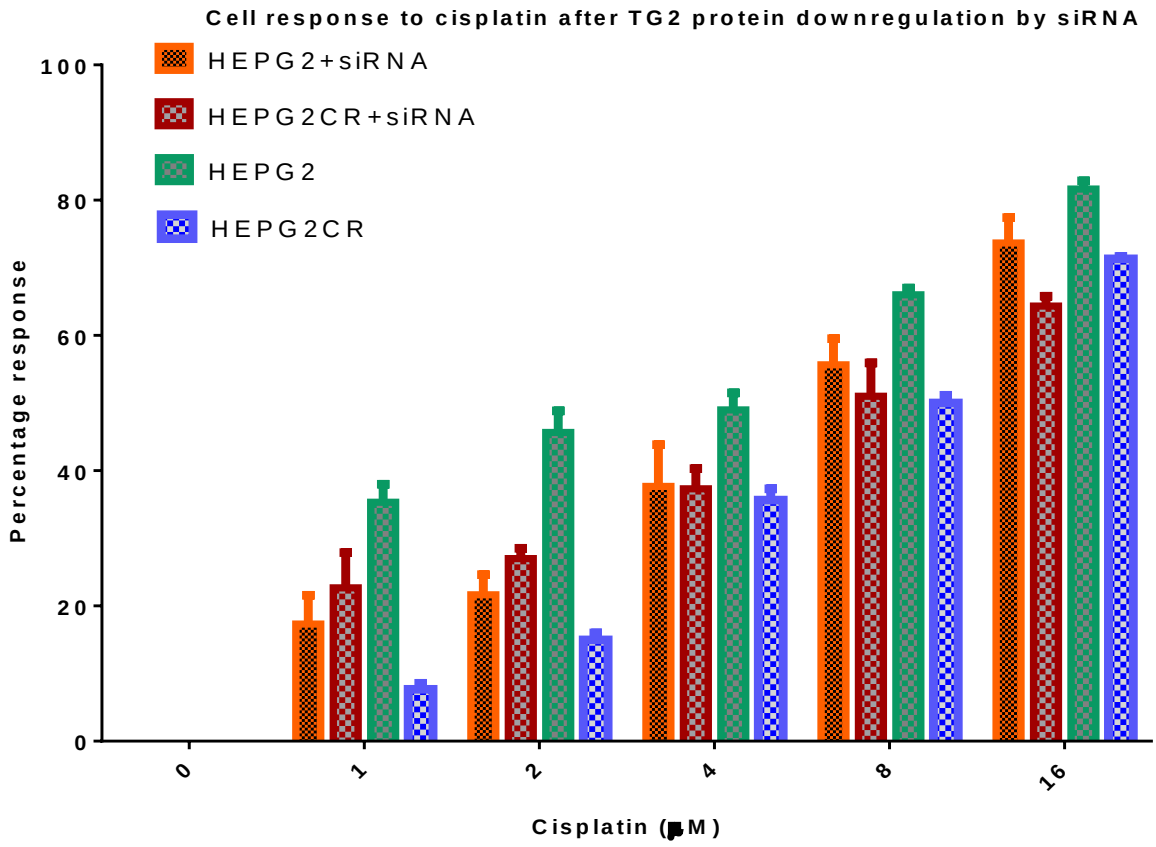


Figure 5.6: Assessment of HEPG2 and HEPG2CR response to cisplatin-induced death after down-regulation of TG2 protein expression by siRNA, showing significant increase in sensitivity of HEPG2 cells to cisplatin following TG2 down-regulation, with p value of 0.0167 after a student t-test (appendix 4, table A4.27). However, a student paired t-test evaluation of the effect of cisplatin on HEPG2CR with and without siRNA (HEPG2CR vs HEPG2CR+siRNA), shows that TG2 down-regulation has no significant effect on the sensitivity of HEPG2CR cells to cisplatin-induced death (p value = 0.3103 > 0.05 at 95% confidence interval) (appendix 4, table A4.28). Additionally, a further statistical analysis by one-way ANOVA shows that TG2 down-regulation by siRNA significantly increased cell death due to cisplatin; p value = 0.0103, where p value < 0.05 defines statistical significance at 95% confidence interval (see appendix 4, table A4.29 and appendix 2, table A2.4.4).

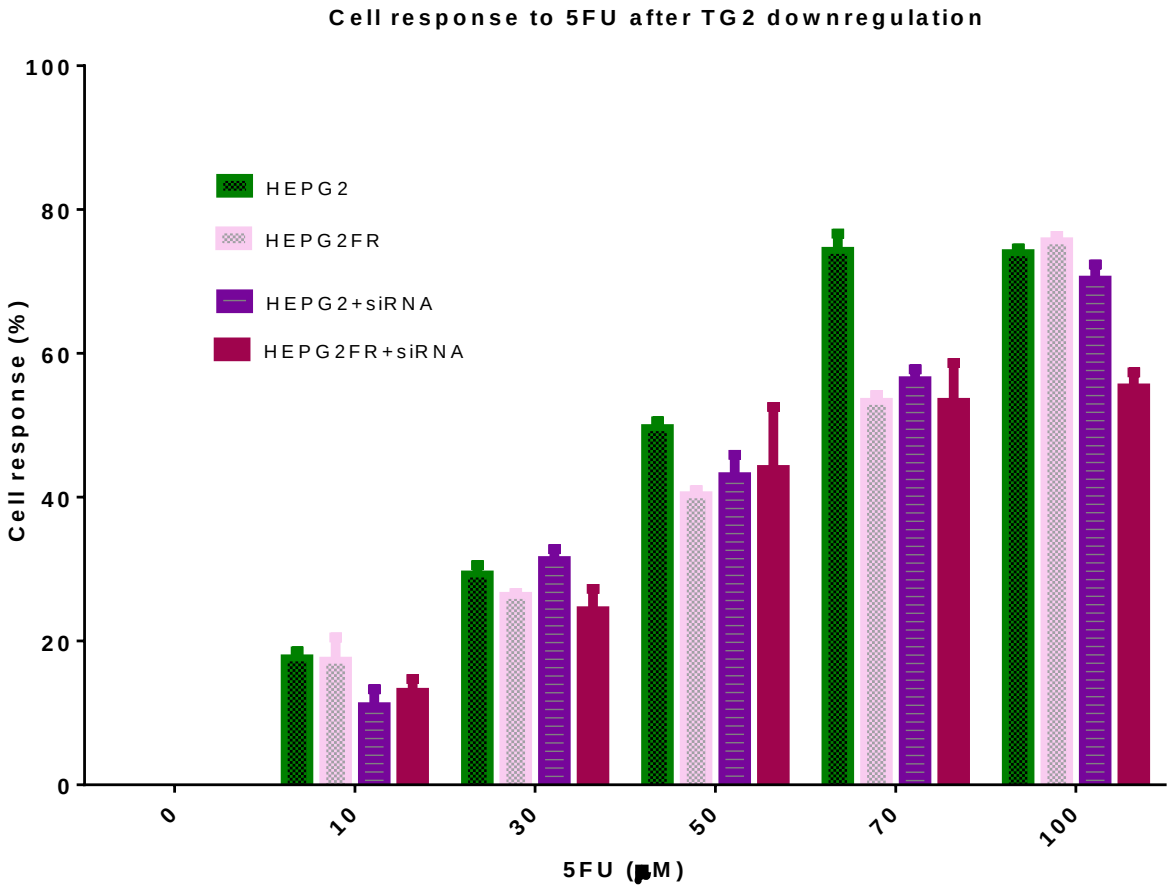


Figure 5.7: A representation of the susceptibility of HEPG2 and HEPG2FR to 5-FU following TG2 down-regulation by siRNA. Statistical analysis by one-way ANOVA shows that TG2 down-regulation has no significant effect on the susceptibility of both parental and 5-FU-resistant HEPG2 clones to 5-FU treatment, with p value of 0.0774 which is greater than 0.05 at 95% confidence interval (see appendix 4, table A4.30). A student paired t-test of the effect of TG2 down-regulation on the susceptibility of HEPG2 clones to 5-FU-induced death confirmed that it has insignificant effect, where the p value for HEPG2 vs HEPG2+siRNA is 0.1144 and the p value for HEPG2FR vs HEPG2FR+siRNA is 0.3198, all of which are greater than 0.05 at 95% confidence interval (appendix 4, tables A4.31 and A4.32 respectively, and appendix 2, table A2.4.5).

5.3.6: Flow cytometric analysis of drug-induced cell death after TG2 down-regulation

After the downregulation of TG2 protein expression by siRNA interference, flow cytometric assay of cellular susceptibility to drug-induced death revealed that there is no significant improvement in the susceptibility of HEPG2CR or HEPG2FR to cisplatin or 5-FU treatment, respectively. A student paired t-test of the susceptibility HEPG2+siRNA vs HEPG2 to cisplatin-induced death shows that the downregulation of TG2 protein expression has significant effect on the susceptibility of the parental cells to cisplatin with p value of 0.0399, which is less than 0.05 at which statistical significance is defined (figure 5.8). However, a student paired t-test of HEPG2CR+siRNA vs HEPG2CR shows that the downregulation of TG2 protein expression has no significant effect on the susceptibility of HEPG2CR to cisplatin-induced death, with p value of $0.1887 > 0.05$, which defines statistical significance at 95% confidence interval (figure 5.8). In the case of 5-FU, both HEPG2 and HEPG2FR cells show no improvement in their susceptibility to 5-FU-induced death after TG2 protein downregulation, as indicated by a p value of 0.2484 for HEPG2+siRNA vs HEPG2, and a p value of 0.0703 for HEPG2FR+siRNA vs HEPG2FR, where $p < 0.05$ is statistically significant at 95% confidence interval (figure 5.9).

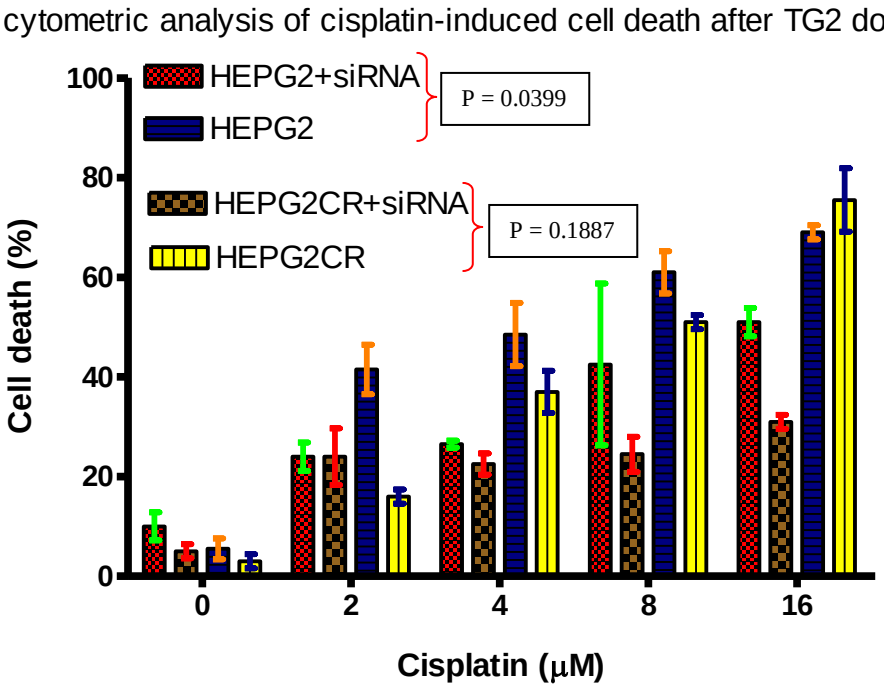


Figure 5.8: Flow cytometric analysis of the susceptibility of HEPG2 clones to cisplatin-induced death after the downregulation of TG2 protein expression by siRNA interference. Student paired t-test of the susceptibility of HEPG2+siRNA vs HEPG2 to cisplatin-induced death shows a significant increase in HEPG2 cells' death due to cisplatin, with a p value of 0.0399, where $p < 0.05$ is statistically significant at 95% confidence interval (appendix 4, table A4.61). Conversely, a student paired t-test of death response of HEPG2CR+siRNA vs HEPG2CR to cisplatin shows no significant improvement in HEPG2CR susceptibility to cisplatin, with p value of 0.1887 (see appendix 4, table A4.62).

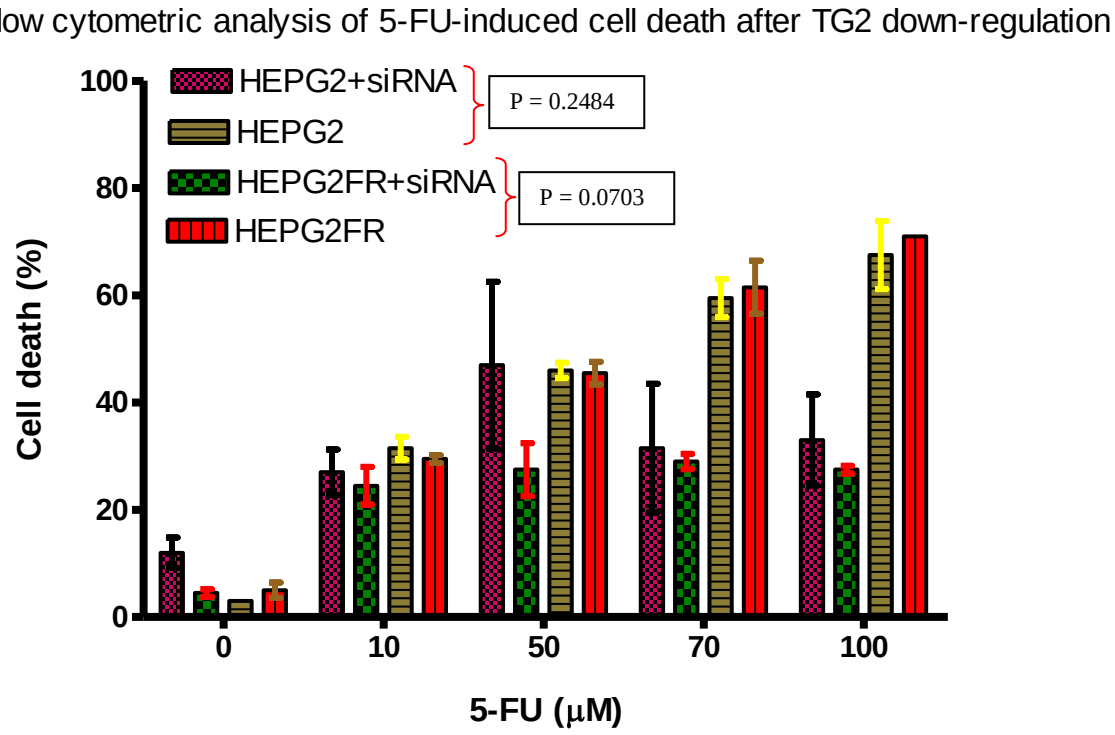


Figure 5.9: Flow cytometric analysis of the susceptibility of parental and 5-FU-resistant HEPG2 cells to 5-FU-induced death after the downregulation of TG2 protein expression by siRNA interference. Student paired t-test of the susceptibility of HEPG2+siRNA vs HEPG2 to death due to 5-FU shows that downregulation of TG2 protein expression has no significant effect on the susceptibility of HEPG2 cells to 5-FU-induced death, with a p value of 0.2484, where $p < 0.05$ is statistically significant at 95% confidence interval (appendix 4, table A4.63). Similarly, a student paired t-test of death response of HEPG2FR+siRNA vs HEPG2FR to 5-FU shows no significant improvement in HEPG2FR susceptibility to 5-FU-induced death, with p value of 0.0703 (see appendix 4, table A4.64).

5.3.7: Analysis of TG2 activity after inhibition by cystamine

To establish the optimum concentration of cystamine at which TG2 activity was inhibited, without affecting the viability of the cells, varying concentrations of cystamine, ranging from 0mM to 4mM were applied to the different cell lines and the resulting lysates were

subsequently analysed for TG2-specific activity. The results showed a steady decline in TG2 activity as cystamine concentration increased, with lowest activity of TG2 recorded at highest concentration (2.5mM) for all the cell lines (figure 5.10). Prior to cell lysis, a fraction of the cells were with trypan blue and examined under the microscope to ascertain their viability, and the cells looked healthy across the concentration range tested.

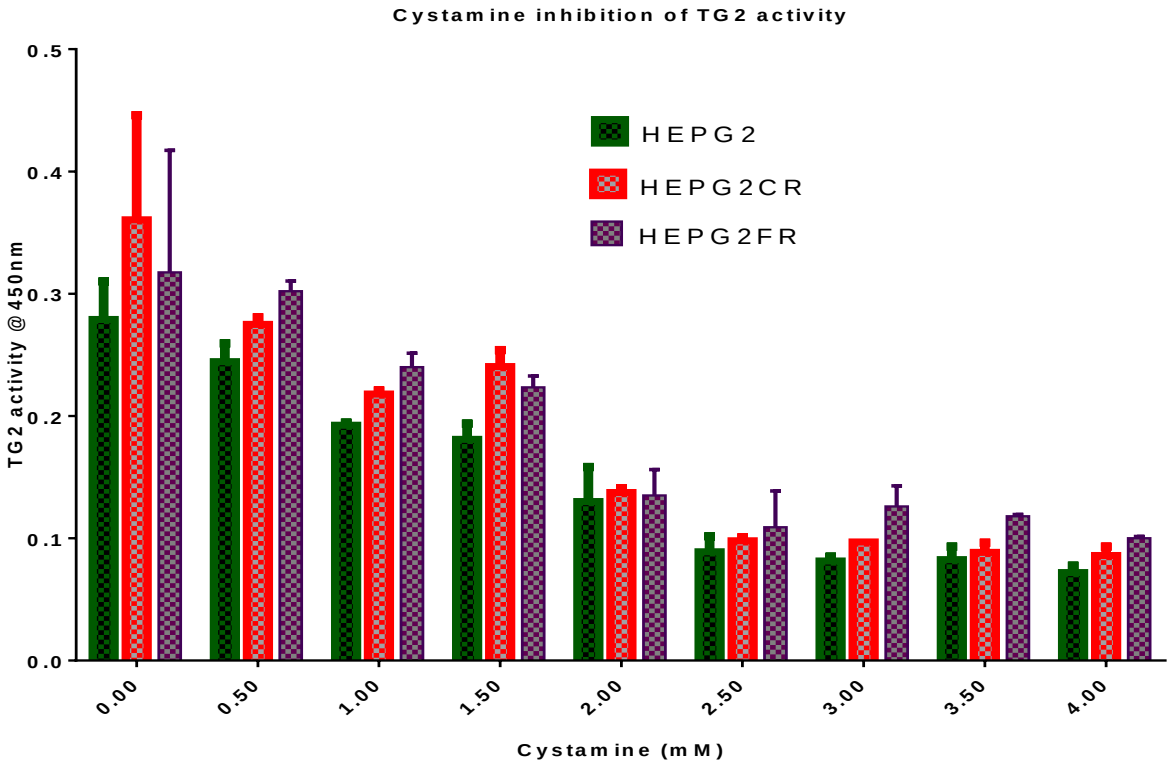


Figure 5.10: Inhibition of TG2 activity using different cystamine concentrations, ranging from 0mM to 4mM; to establish the best concentration of cystamine at which TG2 activity will be optimally inhibited without affecting the viability of the cells. Statistical analysis by One-way ANOVA shows that cystamine significantly reduced the activity of TG2 in all HEPG2 clones, with p value of 0.0018 at $p < 0.05$ = statistical significance (see appendix 4, table A4.34, and appendix 2, table A2.4.6).

5.3.8: Evaluation of cell invasion and migration after the inhibition of TG2 activity

After establishing that 2.5mM concentration of cystamine is the optimal concentration needed to inhibit TG2 activity, without adversely affecting cell viability, the three clones of HEPG2 cells under investigation were treated with 2.5mM of cystamine and evaluated for their invasive and migration capabilities. The results obtained from this experiment revealed that the ability of the cells to invade matrigel-coated surface and migrate to distant site was significantly reduced in comparison with the results obtained from similar experiment (without inhibition of TG2 activity) earlier presented in section 5.3.1, figure 5.2. From figure 5.11 below, it is evident that the percentage of invasion and migration recorded in all the cell lines decreased markedly. Furthermore, comparing the percentage of cell invasion and migration after TG2 down-regulation (section 5.3.4, figure 5.5) and the post-TG2 inhibition percentage of invasion and migration (figure 5.11), showed that the invasive and migration behaviours of parental and drug-resistant HEPG2 cells were reduced in both cases. However, inhibition of TG2 activity has more effect on cellular invasion and migration relative to TG2 down-regulation, whilst HEPG2FR cell line was less affected in both circumstances, relative to HEPG2 and HEPG2CR cells.

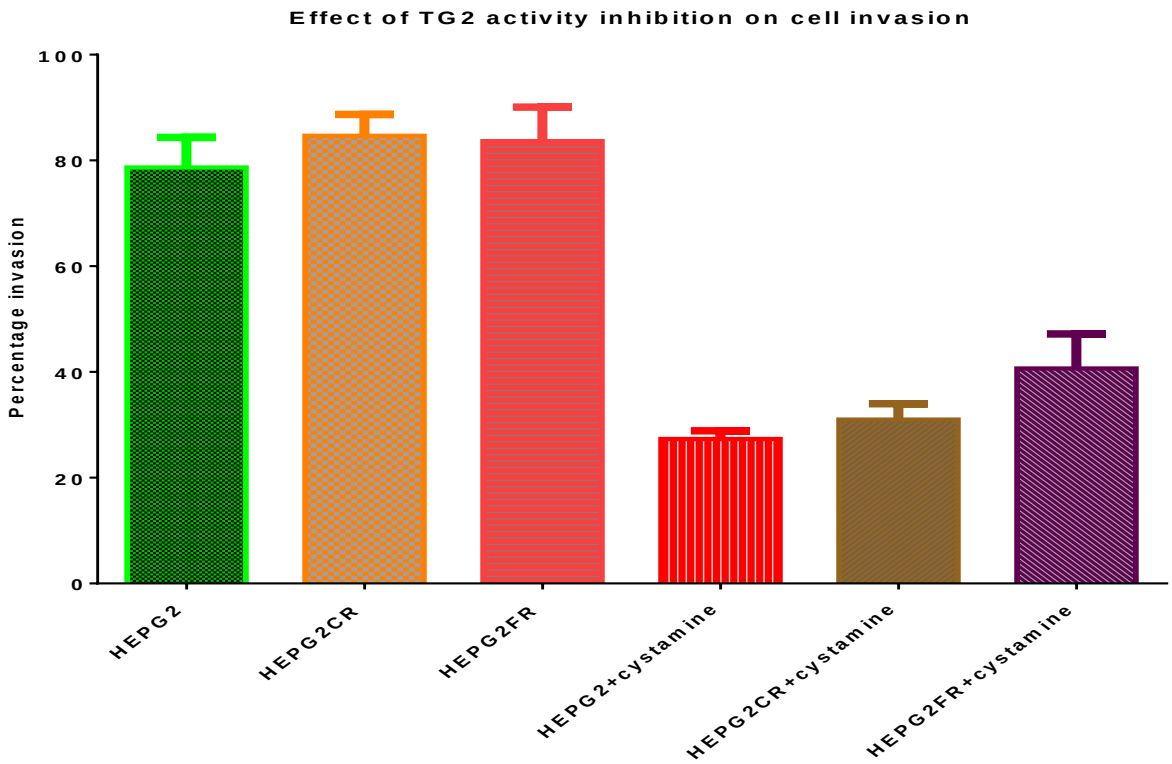


Figure 5.11: A representation of percentage invasion and migration of parental and drug-resistant HEPG2 clones on matrigel-coated surface before and after the inhibition of TG2 activity with 2.5mM cystamine. A marked reduction in cell invasion and migration was recorded in all the cell lines relative to the results recorded before TG2 activity inhibition. Again, the invasiveness of HEPGFR was relatively affected less. Statistical analysis of the effect of inhibition of TG2 activity on the invasive capacity of parental and drug-resistant HEPG2 clones by One-way ANOVA shows that cellular invasiveness was significantly reduced following TG2 activity inhibition, with a p value of 0.0009 at $p < 0.05$ = statistical significance as shown in appendix 4, table A4.35. A t-test of each clone of HEPG2 with cystamine against same clone without cystamine further confirmed that the inhibition of TG2 activity significantly inhibits the invasive ability of both parental and drug-resistant cells. For HEPG2 vs HEPG2+cystamine, p value = 0.0063; HEPG2CR vs HEPG2CR+cystamine, p value is 0.0019; and HEPG2FR vs HEPG2FR+cystamine, p value is 0.0013, all of which are statistically significant with p values < 0.05 at 95% confidence interval (appendix 4, table A4.36, A4.37 and A4.38 respectively, and appendix 2, table A2.4.3).

5.3.9: Assessment of cellular sensitivity to chemotherapeutic drugs after the inhibition of TG2 activity

Following the inhibition of TG2 activity, it is pertinent to establish if the causal relationship between TG2 and cancer drug resistance is determined by its activity. Consequently, parental and drug-resistant HEPG2 cells were tested for their susceptibilities to cisplatin or 5-FU after the inhibition of TG2 activity. Comparing the results with the results previously reported in chapter two, section 2.3.3 (figure 2.3 and 2.4), both parental HEPG2 cell line and drug-resistant clones show an increased susceptibility to both cisplatin and 5-FU toxicity, with highest susceptibility recorded in parental cells relative to the drug-resistant cells. One-way ANOVA analysis of the susceptibility of HEPG2 and HEPG2CR cells to cisplatin-induced death with or without cystamine inhibition of TG2 activity shows signigicant increase in the susceptibility of the cells to cisplatin, with p value of 0.0106 (figure 5.12). Interestingly, a student t-test of the susceptibility of HEPG2 vs HEPG2+cystamine shows that HEPG2 cells are highly susceptible to cisplatin-induced death but the inhibition of TG2 activity makes no significant difference in cells' response to cisplatin, with p value of 0.4165 (figure 5.12). However, for HEPG2CR vs HEPG2CR+cystamine, inhibition of TG2 activity significantly improved cellular susceptibility to cisplatin-induecd death, with a p value of 0.0188, following a student t-test (figure 5.12) .

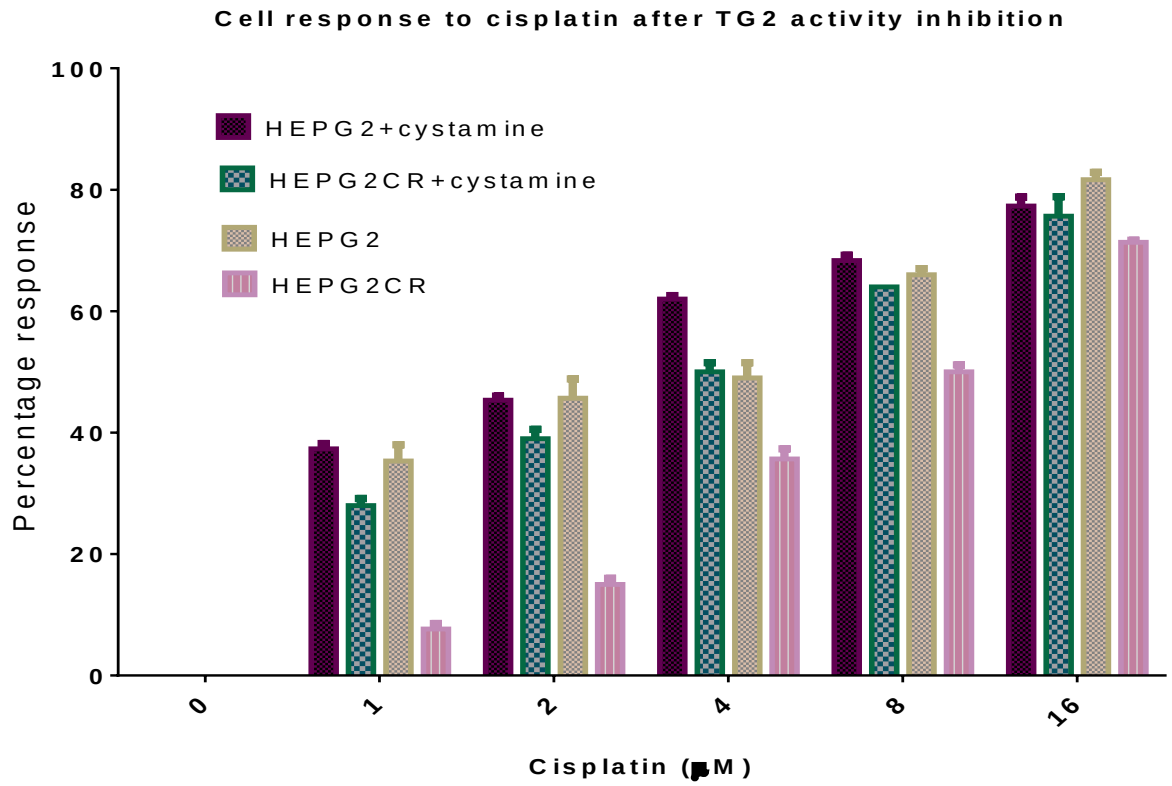


Figure 5.12: Percentage respone of HEPG2 and HEPG2CR cells to cisplatin treatment after the inhibition of TG2 activity with 2.5mM of cystamine. One-way ANOVA analysis of the susceptibility of HEPG2 and HEPG2CR cells to cisplatin-induced death with or without cystamine inhibition of TG2 activity shows signigicant increase in the sensivity of the cells to cisplatin following inhibition of TG2 activity, with p value of 0.0106 which is less than 0.05 at which statistical significance was defined (appendix 4, table A4.39). Interestingly, a student paired t-test of the susceptiblity of HEPG2 vs HEPG2+cystamine shows that HEPG2 cells are highly sensitive to cisplatin-induced death and the inhibition of TG2 activity makes no significant difference in cells' response to cisplatin, with p value of 0.4165 which is greater than 0.05 (appendix 4, table A4.40). However, for HEPG2CR vs HEPG2CR+cystamine, inhibition of TG2 activity significantly improved cell response to cisplatin-induecd death, with a p value of 0.0188 after a student t-test at 95% confidence interval and p value < 0.05 = statistical significance (see appendix 4, table A4.41 and appendix 2, table A2.4.7).

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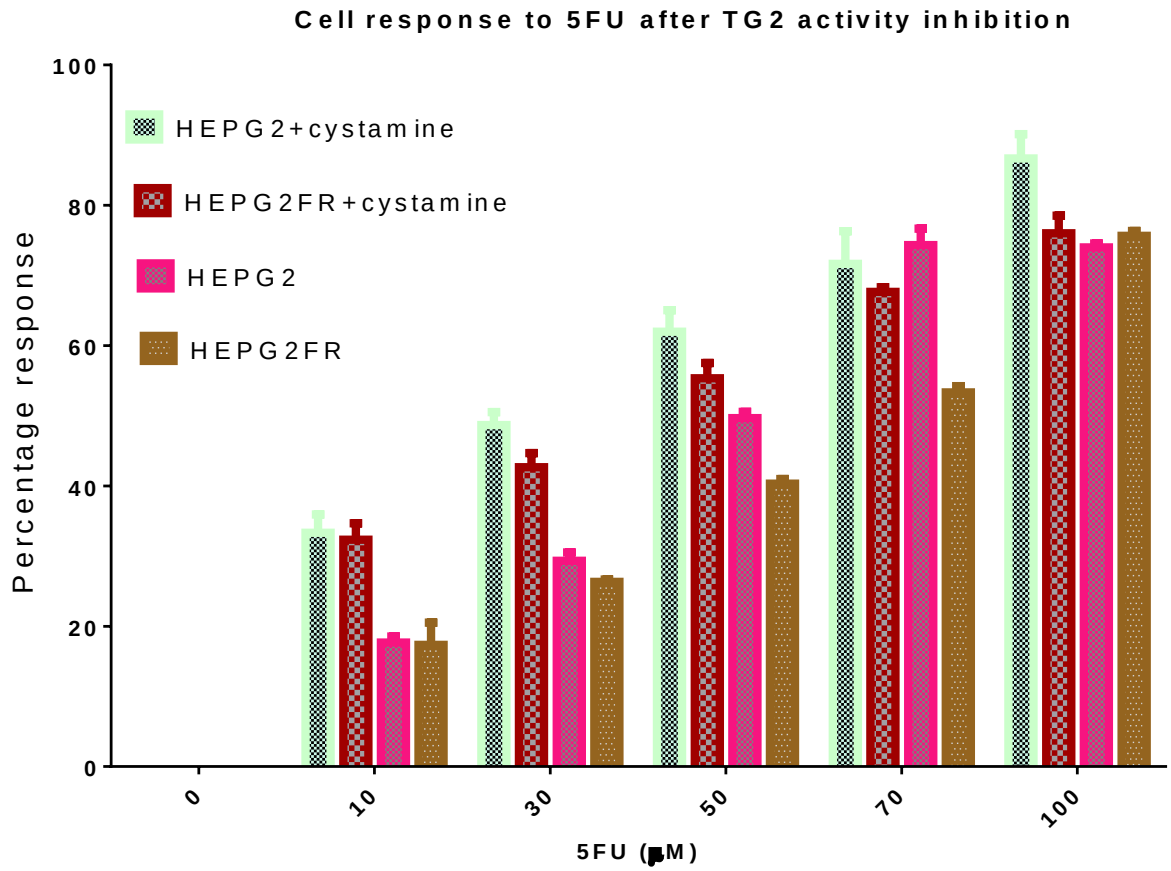

Figure 5.13: Susceptibility of HEPG2 and HEPG2FR to 5-FU-induced death following the inhibition of TG2 activity with 2.5mM of cystamine. Statistical analysis of the results by one-way ANOVA shows that cystamine inhibition of TG2 activity significantly increased the susceptibility of HEPG2 and HEPG2FR cells to 5-FU-induced death, with p value of 0.0054, where p value < 0.05 = statistical significance (see appendix 4, table A4.42). The increased sensitivity of HEPG2 clones to 5-FU-induced death following inhibition of TG2 activity was further confirmed for both HEPG2 and HEPG2FR cells using student paired t-test. For HEPG2 vs HEPG2+cystamine, p value is 0.0456 (appendix 4, table A4.43), while HEPG2FR vs HEPG2FR+cystamine, p value is 0.0239 (appendix 4, table A4.44). These p values are less than 0.05, hence, statistically significant at 95% confidence interval (see also appendix 2, table A2.4.8).

5.3.10: Comparison of the effects of TG2 protein silencing and TG2 activity inhibition on cellular sensitivity to cisplatin and 5-FU

Direct comparison of the implications of TG2 protein expression down-regulation and TG2 activity inhibition in parental and drug-resistant HEPG2 cells revealed that the enzyme activity of TG2 is more important in the definition of its role in drug resistance than its expression. As shown in figure 5.14 and figure 5.15, when TG2 protein is present and its enzymatic activity is absent due to cystamine inhibition, both parental and drug-resistant HEPG2 cells were markedly sensitive to cisplatin and 5-FU treatment. Conversely, when the TG2 protein expression is down-regulated with residual enzymatic activity present (though not measured), HEPG2 cells were less sensitive to cisplatin and 5-FU drug therapy.

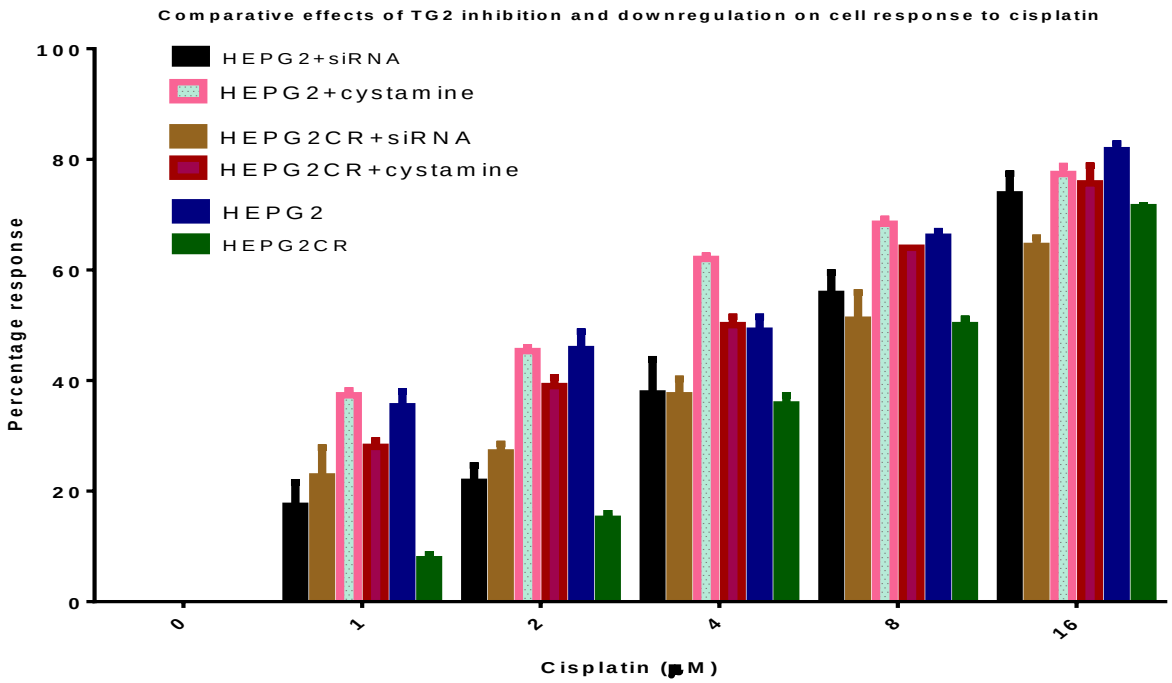

Figure 5.14: Comparison of the effects of TG2 expression down-regulation and TG2 activity inhibition on the susceptiblity of parental and cisplatin-resistant HEPG2 cells to cisplatin therapy. The expression of TG2 protein without its activity in parental and cisplatin-resistant cells (HEPG2+cystamine and HEPG2CR+cystamine) resulted in pronouced sensitivity of the cells to cisplatin treatment. However, with the down-regulation of TG2 protein expression and residual enzymatic activity (HEPG2+siRNA and HEPG2CR+siRNA) the cells were comapratively less susceptible to cisplatin-induced death. One-way ANOVA analysis of the results shows that there is significant difference in cellular response to cisplatin-induced death following the inhibition of TG2 activity and the down-regulation of its expression, with p value of 0.0056 as shown in appendix 4, table A4.45. Further statistical comparison of HEPG2+cystamine vs HEPG2+siRNA by student paired t-test shows that response to HEPG2 to cisplatin-induced death significantly increased when TG2 activity was inhibited compared to when its expression was down-regulated, with a p value of 0.0212, where $p < 0.05$ = statistical significance at 95% confidence interval (appendix 4, table A4.46). Similarly, HEPG2CR+cystamine was more susceptible to cisplatin-induced death compared to HEPG2CR+siRNA, with a p value of 0.0083 as shown in appendix 4, table A4.47.

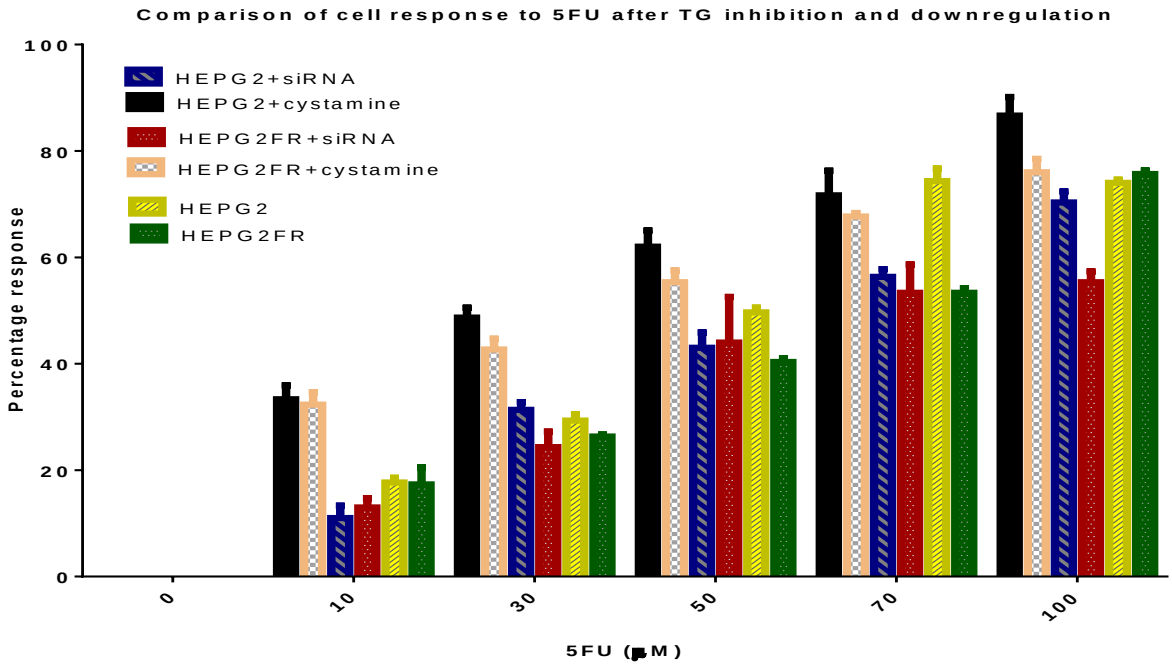

Figure 5.15: Comparison of the effects of TG2 expression down-regulation and TG2 activity inhibition on the susceptibility of parental and 5-FU-resistant HEPG2 cells to 5-FU-induced death. The expression of TG2 protein without its activity in parental and 5-FU-resistant cells (HEPG2+cystamine and HEPG2FR+cystamine) resulted in an increased susceptibilit of the cells to 5-FU therapy. However, with the down-regulation of TG2 protein expression and residual enzymatic activity (HEPG2+siRNA and HEPG2FR+siRNA) the cells were relatively less susceptible to 5-FU-induced death. One-way ANOVA analysis of the results shows that there is significant difference in cellular response to 5-FU therapy following the inhibition of TG2 activity and the down-regulation of its expression, with p value of 0.0005 as shown in appendix 4, table A4.48. Furthermore, comparison of HEPG2+cystamine vs HEPG2+siRNA by student t-test shows that response to HEPG2 to 5-FU-induced death significantly increased when TG2 activity was inhibited compared to when its expression was down-regulated, with a p value of 0.0051, where $p < 0.05$ = statistical significance at 95% confidence interval (appendix 4, table A4.49). Similarly, HEPG2FR+cystamine was more susceptible to 5-FU-induced death compared to HEPG2FR+siRNA, with a p value of 0.0066 as shown in appendix 4, table A4.50.

5.3.11: Flow cytometric analysis of drug-induced cell death after inhibition of TG2 activity

After the inhibition of TG2 activity using cystamine, both parental and drug-resistant HEPG2 clones became significantly susceptible to chemotherapy-induced death. Student paired t-test of the susceptibility of HEPG2+cystamine and HEPG2 to cisplatin-induced death shows that TG2 activity inhibition significantly increased HEPG2 death due to cisplatin, with a p value of 0.0486, where $p < 0.05$ is statistically significant at 95% confidence interval (figure 5.14). Similarly, a student paired t-test of death response of HEPG2CR+cystamine vs HEPG2CR to cisplatin shows that HEPG2CR susceptibility to cisplatin was significantly increased following TG2 activity inhibition, with p value of 0.0435 (figure 5.16). These results are in conformity with the results of the CCK8 post-inhibition susceptibility assays previously reported in section 5.3.9, figure 5.12 and 5.13. For 5-FU, both parental and 5-FU-resistant cells were shown to be more susceptible to 5-FU-induced death after TG2 activity inhibition, as indicated by a p value of 0.0489 for HEPG2 vs HEPG2+cystamine, and a p value of 0.0399 for HEPG2FR vs HEPG2FR+cystamine, where $p < 0.05$ is statistically significant at 95% confidence interval (figure 5.17).

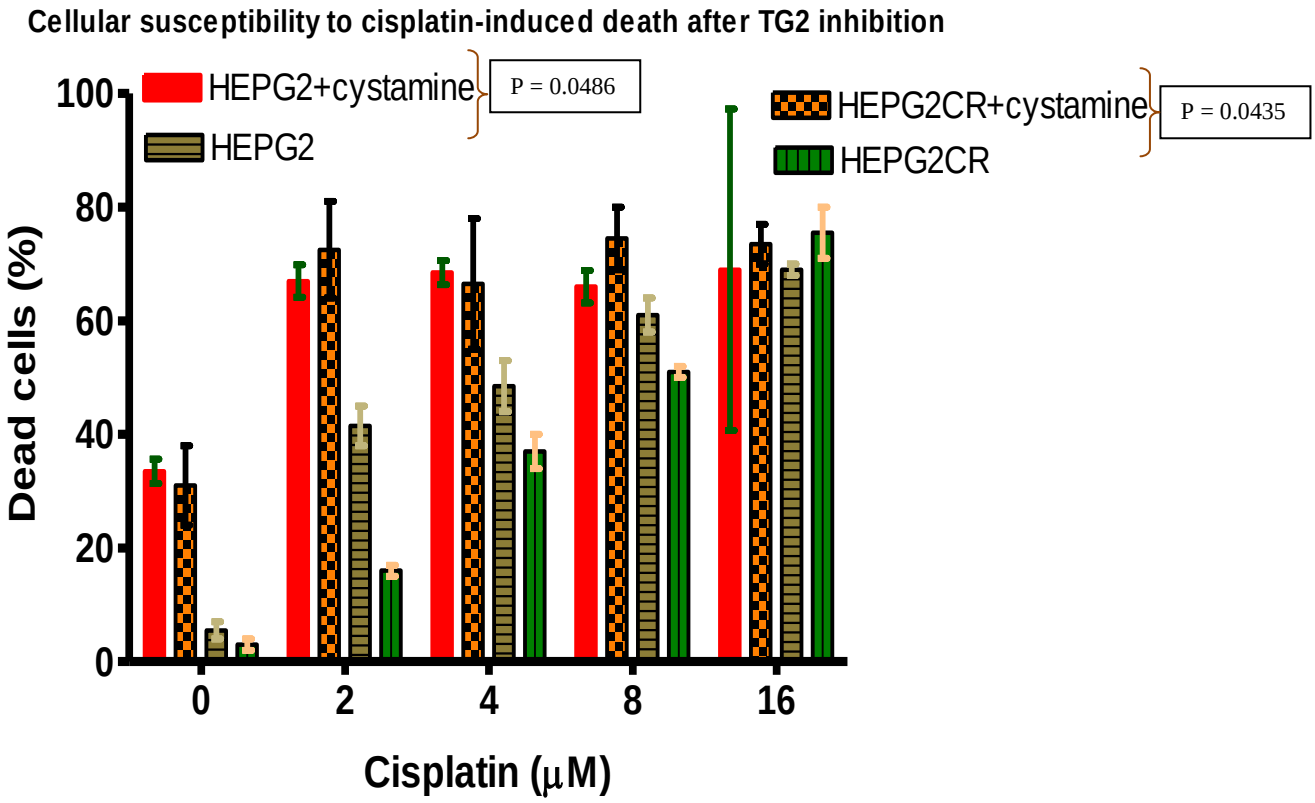

Figure 5.16: Flow cytometric analysis of the susceptibility of parental and cisplatin-resistant cells to cisplatin-induced death following TG2 activity inhibition with cystamine. Student t-test of the susceptibility of HEPG2+cystamine and HEPG2 to cisplatin-induced death shows that TG2 activity inhibition significantly increased HEPG2 death due to cisplatin, with a p value of 0.0486, where $p < 0.05$ is statistically significant at 95% confidence interval (appendix 4, table A4.57). Similarly, a student paired t-test of death response of HEPG2CR+cystamine vs HEPG2CR to cisplatin shows that HEPG2CR susceptibility to cisplatin was significantly increased following TG2 activity inhibition, with p value of 0.0435 (see appendix 4, table A4.58).

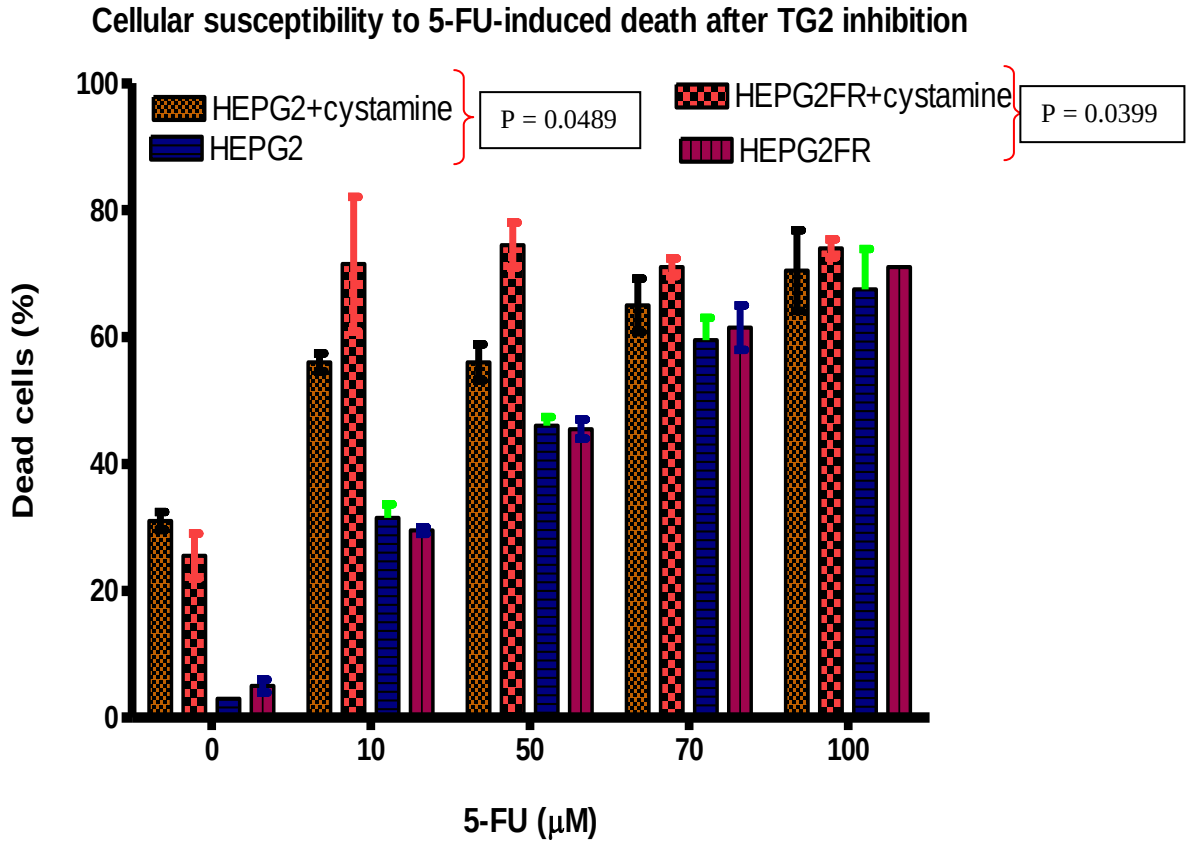

Figure 5.17: Flow cytometric analysis of the susceptibility of parental and 5-FU-resistant cells to 5-FU-induced death following TG2 activity inhibition with cystamine. Student t-test of the susceptibility of HEPG2+cystamine and HEPG2 to 5-FU-induced death reveals that TG2 activity inhibition significantly increased HEPG2 death due to 5-FU, with a p value of 0.0489, where $p < 0.05$ is statistically significant at 95% confidence interval (appendix 6, table A6.59). Similarly, a student paired t-test of the susceptibility of HEPG2FR+cystamine vs HEPG2FR to 5-FU shows that HEPG2FR susceptibility to 5-FU was significantly increased following TG2 activity inhibition, with p value of 0.0399 (see appendix 4, table A4.60).

5.4: DISCUSSION

Evasion of apoptosis is a common feature of advanced cancer cells, which gives the tumour cells the ability to develop drug-resistant phenotype and metastasize to distant sites (Lundin *et al*. 2003). Characteristically, drug resistance and metastasis are intertwined and share many commonalities. For instance, Mehta *et al.* (2010) suggested that cancer cells selected for drug resistance *in vitro,* show more aggressive metastatic potential *in vivo*; and metastatic tumour cells show more resistance to anticancer agents than the primary tumour cells. The results presented in this thesis suggest that both parental and drug-resistant HEPG2 cells have moderately increased metastatic potential, as evidenced in their ability to invade and migrate through the matrigel-coated transmembrane inserts. However, there is no significant difference in the invasiveness of drug-resistant cells relative to the parental cells (figure 5.2). Thus, with reference to the views of Mehta *et al* (2010), it is rational to argue that the ability of drug-resistant cells to show more invasiveness than their parental counterparts is not a common denominator of all drug-resistant cancer cells but dependent on the cancer type from which the cancer cells were derived.

The results presented in chapter four, section 4.3.2 (figure 4.5, 4.6 and 4.7) showed higher expression level of TG2 in drug-resistant clones of HEPG2 relative to the parental cell line; aligning with previous reports that TG2 was one of the eleven metastasis-associated proteins that were selectively elevated in metastatic cancer cells (Jiang *et al* 2003; Mangala *et al.* 2007; Yakubov *et al.* 2013). In the light of the above findings and in accordance with the report that the selective knockdown of TG2 protein using siRNA reduced the adherence, survival and invasion capabilities of cancer cells on matrigel-coated plates (Mangala *et al.* 2007); this thesis investigated the relationship between TG2 expression and the metastasis ability of HEPG2 clones. Analysis of invasion and migration of the three HEPG2 cell lines on

matrigel-coated transmembrane inserts after the down-regulation of TG2 expression by siRNA showed a reduced invasive and migration potential in all the cell lines as shown in figure 5.5. However, the level of reduction in the invasive and migration potential of the HEPG2 cell lines was not as profound as reported in breast cancer cells (Mangala *et al.* 2007), ovarian cancer (Satpathy *et al.* 2007), and malignant melanoma (Fok *et al.* 2006). Hence, it is rational to argue that the relationship between TG2 expression and cancer metastasis may be dependent on cell type.

Flow cytometric analyses of cellular susceptibility to drug-induced death following the downregulation of TG2 protein expression by siRNA interference show no significant improvement in the susceptibility of drug-resistant cells to cisplatin or 5-FU treatment. Although, the downregulation of TG2 protein expression has significant effect on the susceptibility of the parental cells to cisplatin with p value of 0.0399 (figure 5.8); the susceptibility of HEPG2CR to cisplatin-induced death was not improved, with p value of 0.1887 > 0.05 (figure 5.8). In the case of 5-FU, both parental and 5-FU-resistant cells show no improvement in their susceptibility to 5-FU-induced death after TG2 protein downregulation, as indicated by a p value of 0.2484 for parental cells, and a p value of 0.0703 for HEPG2FR cells (figure 5.9). These results distinctly oppose the various reports in other cell types that down-regulation of TG2 protein expression by siRNA interference sensitizes drug-resistant cancer cells to chemotherapy-induced death both *in vitro* and *in vivo* (Verma & Mehta, 2007; Hwang *et al.* 2008; Verma *et al.* 2008; Mangala *et al.* 2007; Yakubov *et al.* 2013).

This thesis is the first to evaluate the implication of TG2 down-regulation in hepatocellular carcinoma and the results presented herein, may be an indication that the relationship

between TG2 and cancer drug-resistance is characteristic of cancer cells derived from hepatocytes alone. Furthermore, the decrease in cellular susceptiblity to anticancer drugs reported here highlights the importance of TG2 as a major pro-survival protein and points to the possibility that HEPG2 cell line may possess a distinct, alternative, strong survival signalling protein that might not be present in other cell types previously reported. Consequently, the reduced expression of TG2 following the introduction of siRNA may upset the survival signalling network, triggering the "backup" protein as an augmentative defence strategy. This may be the reason behind the suggestion that the absence of TG2 can be compensated by other transglutaminase family members, when it is a possible case of functional substitution by a different survival signalling protein.

Conversely, investigation of the causal relationship between TG2 activity and cellular invasion and migration revealed a direct link between the enzyme activity and metastasis. Following the inhibition of TG2 activity using cystamine, a further analysis of invasion and migration of the three HEPG2 cell lines on matrigel-coated plates, cell invasion and migration were strongly attenuated in all the cell lines (figure 5.11). Similarly, both parental and drug-resistant cells were markedly susceptible to drug-induced death when TG2 activity was inhibited (figures 5.12, 5.13, 5.16, and 5.17). The results presented in this thesis are the first to suggest a causal relationship between TG2 enzyme activity and cancer drug resistance and metastasis. Additionally, direct comparison of the effects of down-regulation of TG2 protein expression and inhibition its activity on the susceptibility of parental and drug-resistant HEPG2 cells to cisplatin and 5-FU therapy (figure 5.14 and figure 5.15), showed that TG2's crosslinking activity is more important in defining its role in liver cancer drug resistance than its expression. Interestingly, TG2's role in cancer drug resistance and metastasis has been suggested to be dependent on TG2-mediated integrin-FN interaction,

which is non-enzymatic and independent of TG2 transamidation and cross-linking activities as reviewed by Odii & Coussons (2014). However, these results indicate that activation of cellular survival signaling pathway and consequent drug resistance and metastasis may require the ability of TG2 to cross-link and post-translationally modify some intracellular and extracellular proteins. Hence, the inhibition of TG2 activity may render such proteins ineffective and latent, with resultant increase in cellular susceptibility to chemotherapy-induced death and decrease in invasive and migration potential of the cells.

CHAPTER SIX

GENERAL SUMMARY, FUTURE DIRECTIONS AND CONCLUSIONS

6.1: GENERAL SUMMARY

In order to study the cellular and molecular mechanisms of drug resistance for any given cancer type, development of good models of drug-resistant cells is indispensable to the success of such studies. This is because such scenario could mimic what happens *in vivo*, and could as well be useful for testing new therapeutic agents. The high mortality rate associated with advanced HCC calls for a probe into its mechanism of resistance to chemotherapy. Consequently, drug-resistant HEPG2 sub-clones were produced from the parental cell line. The protocol reported herein, serves as a simplified method of selection of drug-resistant hepatocellular carcinoma cells from human hepatocellular carcinoma (HEPG2) cell line using pharmacologic agents and mimicking clinical treatment pattern. The stepwise method of selection as outlined, can serve as a first-hand guide for the selection of drug-resistant cell line needed for any liver cancer-related drug-resistance studies; and serves as a protocol for the establishment of drug-resistant cell line models of other cancer types.

Following the assessment of dose-dependent toxicity of cisplatin to HEPG2 cells and the observation that the drug is highly toxic to the cells, in agreement with many clinical reports of wide range of cisplatin-induced toxicities, this thesis investigated the kinetics of cisplatin toxicity to hepatocellular carcinoma cells. This was done with a view to understanding how the drug action leads to tissue/organ damage and suggesting a model for further test. The study shows that the optimal concentration of cisplatin for HEPG2 cell line is 4μM, and the optimal treatment duration is 12 hours. It has also shown that the shorter the treatment time, the less the cellular toxicity and the less the damage to organ system. The study has furthered

the importance of exploiting response to cisplatin in attempts to understand its cytotoxic kinetics and establish the optimal treatment conditions.

To understand the expression patterns of TGM2 gene and TG2 protein, as well as the enzyme activity in parental and drug-resistant HEPG2 cells, the profiles of TGM2 gene and TG2 protein expression, and the enzyme activity were investigated. The results showed that the expression patterns of TGM2 and its gene product, TG2, in drug-resistant hepatocellular carcinoma cell lines are in agreement with earlier reports of elevated TG2 expression in drug-resistant and metastatic cells derived from other cancer types (Mehta *et al.* 2010). However, any variation in expression or activity of the enzyme could be due to the differences in the mechanisms of actions of the induction stimuli. Furthermore, the observation that TG2 is the predominant member of the Tgase family in liver cells is particularly fascinating. This thesis has shown the specific patterns of expression of TG2 in liver carcinogenesis by providing for the first time, first-hand information on the pattern of expression and activity of the enzyme in a hepatocellular carcinoma cell line; hence, providing insights into the involvement of TG2 in liver cancer drug resistance.

Additionally, a comparison of TG2 activity profiles with apoptosis profiles for parental and drug-resistant HEPG2 cell lines revealed an apparent relationship between TG2 activity and cell death progression. This is because decreased TG2 activity is directly proportional to increased cell death in both parental and drug-resistant clones of HEPG2. Though, for 5-FU-treated cells, TG2 protein expression and enzyme activity continued to increase with increase in drug concentration, explaining why HEPG2 cells are less susceptible to 5-FU than cisplatin. It is therefore, reasonable to conclude that increase in TG2 protein expression and

enzyme activity may be pro-survival signalling strategies employed by the cells to evade drug-induced death.

Following the observation that drug-resistant HEPG2 clones express higher levels of TG2 protein relative to the parental cell line (figures 4.4 to 4.7), the causal relationship between TG2 protein and cancer drug resistance and metastasis was investigated. The results show that siRNA down-regulation of TG2 expression enhances cellular susceptibility to anticancer agents, and leads to reduced invasion and migration potential of parental and drug-resistant cells on matrigel-coated surface. The inhibition of TG2 activity using cystamine profoundly increased chemosensitivity of parental and drug-resistant cells and attenuated their potential to invade and migrate through matrigel-coated surface.

Interestingly, the role of TG2 protein in cancer drug resistance and metastasis has been suggested to be dependent on TG2-mediated integrin-FN interaction, which is non-enzymatic as reviewed by Odii & Coussons (2014). However, this thesis reports for the first time, that activation of cellular survival signaling pathway and consequent drug resistance and metastasis may require the ability of TG2 to cross-link and/or post-translationally modify certain specific intracellular and extracellular proteins. Thus, the roles of TG2 protein in liver cancer drug resistance and metastasis are determined by its enzymatic activity rather than protein – protein interactions. Even though protein – protein interactions may be contributory to TG2's functions in liver cancer drug resistance and metastasis, the crosslinking activity of TG2 is necessary to modify the proteins and activate such interactions. Furthermore, the roles of TG2 in cancer drug resistance and metastasis may not be compensated for by any other member of the transglutaminase family because they lack specialized structural conformations needed for such functions in agreement with Odii and Coussons, (2014).

6.2: FUTURE DIRECTIONS

It is important to test cisplatin on 5-FU-resistant cells and vice versa; development of cell line model resistant to cisplatin/5-FU combination is hereby suggested. This will enable the study of the susceptibility of drug-resistant HEPG2 cells to the combined therapy of cisplatin and 5-FU.

To gain more insights into the kinetics of cisplatin toxicity in the clinic, the work reported in chapter three of this thesis should be repeated in normal/non-cancerous cells and perhaps eventually extended to animal models to enable the establishment of a clinical optimal treatment time for cisplatin. Thus, this thesis suggests animal model experiments at optimal treatment time of cisplatin using an appreciable cisplatin dosage, followed by the removal of the drug by plasmapheresis (Guenter *et al.* 2006) or haemodialysis (Lagrange *et al.* 1994).

The striking observation in liver cancer cells that the inhibition of TG2 activity is more potent in promoting the susceptibility of the cells to anticancer drugs and reducing their invasion and migration potential relative to the down-regulation of TG2 expression requires further investigation in cancer cells derived from other cancer types. It is also important to investigate the protein – protein interactions that define TG2 roles in liver cancer drug-resistance and metastasis whilst identifying the set of *in vivo* substrates of TG2 that determine its functions in liver cancer cells. This will help to unmask the survival signalling proteins that may be activated to substitute for TG2 absence following its down-regulation, thus, accounting for the surprising increase in cell survival observed after TG2 down-regulation. This may provide further answers to the proposition of compensation for TG2 function by other members of the transglutaminase family in the event of absence, when it may be a case of functional substitution by a different survival signalling protein.

It is important to measure the residual activity of TG2 after the down-regulation of its expression. This will enable us to understand whether the poor susceptibility of parental and drug-resistant HEPG2 cells to cisplatin and 5-FU therapy following TG2 silencing was due to residual TG2 enzymatic activity. Additionally, there is the need to investigate the effects of TG2 activity inhibition on the sensitivity of normal cells (hepatocytes) to drug therapy. This is to ensure such feature is only characteristic of cancer cells and not of normal cells, and the incorporation of TG2 activity inhibitors in anticancer drug therapies will not affect non-target normal cells. Subsequently, animal model experiments investigating the role of TG2 enzyme activity in cancer drug resistance and metastasis will be envisaged.

Statistically, student paired t-test and one-way AVOVA were used throughout the thesis to establish statistical significance. Generalised Linear Model (GLM) would be necessary to define statistical differences between concentrations, particularly in chapter four; however, this could not be applied because the experiments in chapter four were done in duplicates. Future experiments will therefore, require GLM, hence, should be done in triplicates to give the data enough power.

The suggestion that in the event of TG2 absence its biological functions could be successfully compensated for by other members of the transglutaminase family (reviewed by Odii and Coussons, 2014) has been made without recourse to the distinguishing features of TG2 among the transglutaminase family. It is therefore, necessary to carry out further investigations to ascertain the main reasons why TG2 knockout is not embryonic lethal; instead of relying on the assertion that its functions are compensated for by other transglutaminase enzymes. Furthermore, a systematic investigation should be carried out to

establish with certainty, the possibility of and premise for the replacement of TG2 function by any other member of the transglutaminase family.

6.3: CONCLUSIONS

Gene regulation determines enzyme availability and level of activity. Consequently, increase in TG2 expression leads to increase in the enzyme activity, with resultant increase in cancer drug resistance and metastasis; depending on cell type and stimulation. From the results presented in this thesis, it is tempting to conclude that the causal relationship between TG2 and cancer drug resistance and metastasis may be dependent on both TG2 expression (availability) and enzymatic activity. However, either TG2 protein expression or its enzymatic activity or both, can lead to drug resistance and metastasis depending on cell type and the conditions of treatment (type of stimulation). Additionally, the surprising increase in cell survival and reinforcement of resistance by the HEPG2 cells after the down-regulation of TG2 expression are indications of inherent, strong survival signalling network that might be responsible for the characteristic drug resistance and refractory properties of advanced hepatocellular carcinoma. It may also be due to the residual activity of TG2 after silencing. Consequently, modulation of TG2 expression and activity may serve as useful therapeutic target for liver cancer treatment.

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APPENDIX ONE

Publication and poster presentations

A1.1: Publications

1. Odii, B. O and Coussons, P. 2012. Pharmacological isolation of experimental models of drug-resistant hepatocellular carcinoma cell line. *Journal of Cancer Therapy*, **3** (4) 216-221. doi: 10.4236/jct.2012.34031.

2. Odii, B. O and Coussons, P. 2014. Biological Functionalities of Transglutaminase 2 and the Possibility of its Compensation by other Members of the Transglutaminase Family. *The Scientific World Journal*. *http://dx.doi.org/10.1155/2014/714561*

A1.2: Poster and abstract presentations

1. Odii, B. O. & Coussons, P. J. 2014. Roles of transglutaminase 2 (TG2) in development of drug resistance and metastasis by cancer cells. *Abstract for oral presentation at the 8th Annual Research Student Conference, June 2014, Chelmsford.*
2. Odii, B. O. & Coussons, P. J. 2012. Untangling the molecular basis of cisplatin/5-fluourouracil combination therapy of liver cancer. *Faculty of Science and Technology, 2nd Annual Research and Scholarship Conference, Anglia Ruskin University, 23 May 2012. Chelmsford.*
3. Odii, B. O. 2012. Isolation of Drug-Resistant HEPG2 Cells from Hepatocellular Carcinoma Cell Line. *6th Annual Research student conference, Anglia Ruskin University, 6 June 2012. Cambridge.*

APPENDIX TWO

RESULT TABLES

A2.1: Chapter two result tables

Table A2.1.1: Analysis of HEPG2 cell viability using cell counting kit-8 (CCK8) after treatment with cisplatin for twelve hours

	Replicates			% Viability	0.267			
Cisplatin (µM)	A	B	C	A	B	C	Mean (%)	STDEV
0	0.68	0.673	0.686	100	100	100	100	0
1	0.538	0.549	0.536	66	69	64	66	2.720
2	0.529	0.521	0.53	63	63	63	63	0.458
4	0.48	0.473	0.478	52	51	50	51	0.622
8	0.421	0.433	0.428	37	41	38	39	1.839
16	0.355	0.352	0.349	21	21	20	21	0.915

Table A2.1.2: Analysis of HEPG2 cell viability using cell counting kit-8 (CCK8) after treatment with cisplatin for twenty four hours

	Replicates			% Viability	0.263			
Cisplatin (µM)	A	B	C	A	B	C	Mean (%)	STDEV
0	0.547	0.559	0.571	100	100	100	100	0
1	0.438	0.469	0.453	62	70	62	64	4.585
2	0.402	0.442	0.428	49	60	54	54	5.802
4	0.4	0.409	0.434	48	49	56	51	3.928
6	0.389	0.411	0.377	44	50	37	44	6.512
8	0.357	0.371	0.365	33	36	33	34	1.951
10	0.337	0.345	0.351	26	28	29	27	1.277
12	0.329	0.317	0.319	23	18	18	20	2.902
14	0.326	0.314	0.322	22	17	19	20	2.497
16	0.319	0.318	0.311	20	19	16	18	2.136
18	0.306	0.314	0.309	15	17	15	16	0.884
20	0.299	0.302	0.307	13	13	14	13	0.824

Table A2.1.3: Assessment of level of resistance in HEPG2 cells selected for resistance against cisplatin

	Replicates			% Viability	0.277			
Cisplatin (µM)	A	B	C	A	B	C	Mean (%)	STDEV
0	0.608	0.59	0.606	100	100	100	100	0
1	0.58	0.579	0.576	92	96	91	93	3.063
2	0.555	0.549	0.552	84	87	84	85	1.809
4	0.48	0.485	0.489	61	66	64	64	2.582
6	0.479	0.472	0.476	61	62	60	61	0.931
8	0.436	0.439	0.44	48	52	50	50	1.872
10	0.392	0.401	0.392	35	40	35	36	2.754
12	0.388	0.379	0.382	34	33	32	33	0.814
14	0.382	0.38	0.377	32	33	30	32	1.257
16	0.37	0.368	0.371	28	29	29	29	0.488
18	0.374	0.369	0.365	29	28	27	28	1.279
20	0.36	0.364	0.364	25	28	26	26	1.360

Table A2.1.4: CCK8 analysis of HEPG2 cell viability after treatment with various concentrations of 5-FU for twenty four hours

	Replicates			% Viability	0.262			
5-FU (µM)	A	B	C	A	B	C	Mean (%)	STDEV
0	0.539	0.535	0.542	100	100	100	100	0
10	0.489	0.491	0.489	82	84	81	82	1.438
20	0.476	0.488	0.489	77	83	81	80	2.829
30	0.453	0.46	0.457	69	73	70	70	1.896
40	0.43	0.426	0.421	61	60	57	59	2.085
50	0.397	0.405	0.402	49	52	50	50	1.851
60	0.391	0.376	0.383	47	42	43	44	2.468
70	0.344	0.358	0.351	30	35	32	32	2.802
80	0.342	0.346	0.346	29	31	30	30	0.949
90	0.341	0.334	0.336	29	26	26	27	1.224
100	0.333	0.331	0.337	26	25	27	26	0.789

Table A2.1.5: Assessment of resistance level of HEPG2 cells selected for resistance against 5-FU

	Replicates			% Viability	0.231			
5-FU (µM)	A	B	C	A	B	C	Mean (%)	STDEV
0	1.013	0.998	1.021	100	100	100	100	0
10	0.923	0.819	0.89	88	77	83	83	5.934
20	0.82	0.811	0.801	75	76	72	74	1.921
30	0.807	0.798	0.809	74	74	73	74	0.385
40	0.792	0.8	0.786	72	74	70	72	1.985
50	0.695	0.697	0.696	59	61	59	60	0.986
60	0.636	0.674	0.655	52	58	54	54	3.051
70	0.599	0.599	0.587	47	48	45	47	1.491
80	0.537	0.521	0.529	39	38	38	38	0.789
90	0.432	0.418	0.438	26	24	26	25	0.941
100	0.427	0.422	0.409	25	25	23	24	1.418

A2.2: Chapter three result tables

Table A2.2.1: Cell death analysis by flow cytometry after twelve hours treatment with cisplatin (figure 3.1)

Cisplatin (µM)	A	B	AVG	Stdev
0	79.02	76.05	77.535	2.100
1	75.01	69.84	72.425	3.656
2	67.45	60.05	63.75	5.232
4	50.77	51.72	51.245	0.672
8	34.73	31.6	33.165	2.213
16	28.65	31.08	29.865	1.718

Table A2.2.2: Analysis of cell death by flow cytometry following twenty four hours treatment with cisplatin (figure 3.1)

Cisplatin (µM)	A	B	AVG	Stdev
0	72.99	72.08	72.535	0.643
1	69.84	70.49	70.165	0.459
2	59.4	59.58	59.49	0.127
4	50.99	50.52	50.755	0.332
8	26.74	29.53	28.135	1.973
16	17.65	26.53	22.09	6.279

Table A2.2.3: Flow cytometric analysis of HEPG2 cell death after forty eight hours treatment with various concentrations of cisplatin (figure 3.1)

Cisplatin (µM)	A	B	AVG	Stdev
0	94.46	88.76	91.61	4.031
1	68.99	65.33	67.16	2.588
2	63.92	57.95	60.935	4.221
4	40.19	48	44.095	5.523
8	23.1	21.44	22.27	1.174
16	19.38	17.37	18.375	1.421

A2.3: Chapter four result tables

Table A2.3.1: TG2 expression levels in HEPG2 cells after twenty four hours treatment with various concentrations of cisplatin (figure 4.1)

	TG2 level			
Cisplatin (µM)	A	B	Mean	Stdev
0	1	1	1	0
1	1.204	1.342	1.273	0.098
2	1.467	1.414	1.441	0.037
4	1.576	1.469	1.523	0.076
8	1.264	1.268	1.266	0.003
16	1.164	1.242	1.203	0.055

Table A2.3.2: TG2 expression levels in HEPG2CR after twenty four hours treatment with various concentrations of cisplatin (figure 4.1)

	TG2 level			
Cisplatin (µM)	A	B	Mean	Stdev
0	1	1	1	0
1	1.5	1.486	1.493	0.01
2	1.717	1.787	1.752	0.049
4	2.833	2.759	2.796	0.052
8	1.5	1.333	1.4165	0.118
16	1.164	1.275	1.2195	0.078

Table A2.3.3: TG2 expression levels in HEPG2 cell line after 5-FU treatment for twenty four hours (figure 4.2)

	TG2 Level			
5FU (µM)	A	B	Mean	Stdev
0	1	1	1	0
10	1.87	1.48	1.675	0.276
30	1.26	1.24	1.25	0.014
50	1.22	0.87	1.045	0.247
70	0.43	0.87	0.65	0.311
90	0.35	0.41	0.38	0.042

Table A2.3.4: TG2 expression levels in HEPG2FR cell line after 5-FU treatment for twenty four hours (figure 4.2)

	TG2 Level			
5FU (µM)	A	B	Mean	Stdev
0	1	1	1	0
10	0.639	0.333	0.486	0.216
30	0.389	0.5	0.4445	0.078
50	0.42	0.383	0.4015	0.026
70	0.319	0.306	0.3125	0.009
90	0.308	0.417	0.3625	0.077

Table A2.3.5: Tgase activity in HEPG2 cell line after twenty four hours treatment with various concentrations of cisplatin (figure 4.9)

Cisplatin (µM)	A	B	Mean	Stdev
0	0.1	0.101	0.1005	0.001
1	0.107	0.104	0.1055	0.002
2	0.161	0.158	0.1595	0.002
4	0.177	0.179	0.178	0.001
8	0.15	0.155	0.1525	0.004
16	0.122	0.122	0.122	0

Table A2.3.6: Tgase activity in HEPG2CR cells after twenty four hours treatment with various concentrations of cisplatin (figure 4.9)

Cisplatin (µM)	A	B	Mean	Stdev
0	0.102	0.106	0.104	0.003
1	0.112	0.112	0.112	0
2	0.174	0.17	0.172	0.003
4	0.193	0.183	0.188	0.007
8	0.167	0.174	0.1705	0.005
16	0.135	0.138	0.1365	0.002

Table A2.3.7: Tgase activity in HEPG2 cells after twenty four hours 5-FU treatment (figure 4.10)

5-FU(µM)	A	B	Mean	Stdev
0	0.065	0.071	0.068	0.004
10	0.128	0.127	0.1275	0.001
30	0.166	0.169	0.1675	0.002
50	0.172	0.171	0.1715	0.001
70	0.181	0.177	0.179	0.003
100	0.193	0.19	0.1915	0.002

Table A2.3.8: Tgase activity in HEPG2FR cells after twenty four hours 5-FU treatment (figure 4.10)

5-FU(µM)	A	B	Mean	Stdev
0	0.082	0.078	0.08	0.003
10	0.131	0.133	0.132	0.001
30	0.174	0.17	0.172	0.003
50	0.186	0.188	0.187	0.001
70	0.191	0.187	0.189	0.003
100	0.213	0.211	0.212	0.001
TG	0.46	0.44	0.45	0.014

Table A2.3.9: TG2-sepecific activity in HEPG2 and HEPG2CR cells after twenty four hours cisplatin treatment (figure 4.11)

Cisplatin (µM)	HEPG2		AVG	STDEV	CR		AVG	STDEV
0	0.16	0.134	0.147	0.019	0.196	0.196	0.196	0
2	0.161	0.157	0.159	0.003	0.195	0.194	0.195	0.001
4	0.199	0.156	0.178	0.030	0.202	0.199	0.201	0.002
8	0.167	0.133	0.15	0.024	0.178	0.166	0.172	0.008
16	0.144	0.137	0.141	0.005	0.112	0.112	0.112	0
rTG2	3.498	3.278	3.388	0.156	3.498	3.278	3.388	0.156

Table A2.3.10: TG2-sepecific activity in HEPG2 and HEPG2FR cells after twenty four hours 5-FU treatment (figure 4.12)

5FU (µM)	Hep		AVG	STDEV	FR		AVG	STDEV
0	0.16	0.134	0.147	0.018	0.203	0.186	0.195	0.012
10	0.122	0.173	0.1475	0.036	0.188	0.155	0.172	0.023
30	0.169	0.166	0.1675	0.002	0.137	0.169	0.153	0.022
50	0.127	0.116	0.1215	0.008	0.173	0.193	0.183	0.014
100	0.13	0.163	0.1465	0.023	0.206	0.198	0.202	0.006
rTG2	3.498	3.278	3.388	0.156	3.498	3.278	3.388	0.156

A2.4: Chapter five result tables

Table A2.4.1: Percentage invasion and migration of parental and drug-resistant hepatocarcinoma cell lines on matrigel-coated surface (figure 5.2).

Cell type	% invasion				
	A	B	C	AVG	STDEV
HEPG2	77	74	85	79	5.686
HEPG2CR	89	81	84	85	4.041
HEPG2FR	79	81	91	84	6.429

Table A2.4.2: Percentage invasion and migration of parental and drug-resistant HEPG2 clones on matrigel-coated surface after TG2 down-regulation by siRNA (figure 5.5).

Cell type	% invasion A	B	C	Avg	STDEV
HEPG2	60	58	57	58	1.528
HEPG2CR	69	67	61	66	4.163
HEPG2FR	76	74	69	73	3.606

Table A2.4.3: Percentage invasion and migration of parental and drug-resistant HEPG2 clones on matrigel-coated surface following the inhibition of TG2 activity with 2.5mM cystamine (figure 5.11).

Cell type	% invasion A	B	C	Avg	STDEV
HEPG2	27	29	26	27.3333	1.528
HEPG2CR	31	28	34	31	3
HEPG2FR	34	41	47	40.6667	6.506

Table A2.4.4: Susceptibility of HEPG2 and HEPG2CR cells to cisplatin-induced death after TG2 down-regulation by siRNA (figure 5.6).

Cisplatin (μM)	HEPG2 +siRNA (%)			HEPG2CR +siRNA (%)			HEPG2 (%)			HEPG2CR (%)		
0	0	0	0	0	0	0	0	0	0	0	0	0
1	20	23	9	14	22	32	38	30	38	8	6	9
2	26	16	23	24	29	28	51	40	46	16	13	16
4	50	31	32	33	43	36	52	51	44	39	34	34
8	63	54	50	42	52	59	67	64	67	52	48	50
16	74	80	67	64	67	62	80	81	84	72	71	71

Table A2.4.5: Percentage susceptibility of HEPG2 and HEPG2FR cells to 5-FU-induced death after TG2 down-regulation by siRNA (figure 5.7).

5-FU (μM)	HEPG2 (%)			HEPG2FR (%)			HEPG2 +siRNA (%)			HEPG2FR +siRNA (%)		
0	0	0	0	0	0	0	0	0	0	0	0	0
10	18	16	19	12	23	17	15	11	7	10	16	13
30	31	27	30	26	26	27	34	31	29	19	29	25
50	51	48	50	41	39	41	43	38	48	34	37	61
70	70	75	78	53	52	55	54	56	59	48	64	48
100	74	75	73	75	75	77	67	74	70	59	55	52

Table A2.4.6: Inhibition of TG2 activity in the three cell lines using different cystamine concentrations (0mM to 4mM) (figure 5.10).

Cystamine	HEPG2		HEPG2CR		HEPG2FR	
0.00	0.301	0.257	0.421	0.300	0.388	0.247
0.50	0.255	0.234	0.271	0.279	0.296	0.308
1.00	0.194	0.191	0.220	0.216	0.248	0.232
1.50	0.172	0.190	0.231	0.250	0.230	0.217
2.00	0.110	0.150	0.140	0.135	0.120	0.150
2.50	0.080	0.098	0.100	0.096	0.130	0.088
3.00	0.079	0.084	0.097	0.097	0.114	0.138
3.50	0.075	0.090	0.083	0.094	0.117	0.119
4.00	0.068	0.076	0.091	0.081	0.101	0.099

Table A2.4.7: Susceptibility of HEPG2 and HEPG2CR to cisplatin-induced death after the inhibition of TG2 activity with 2.5mM of cystamine (figure 5.12).

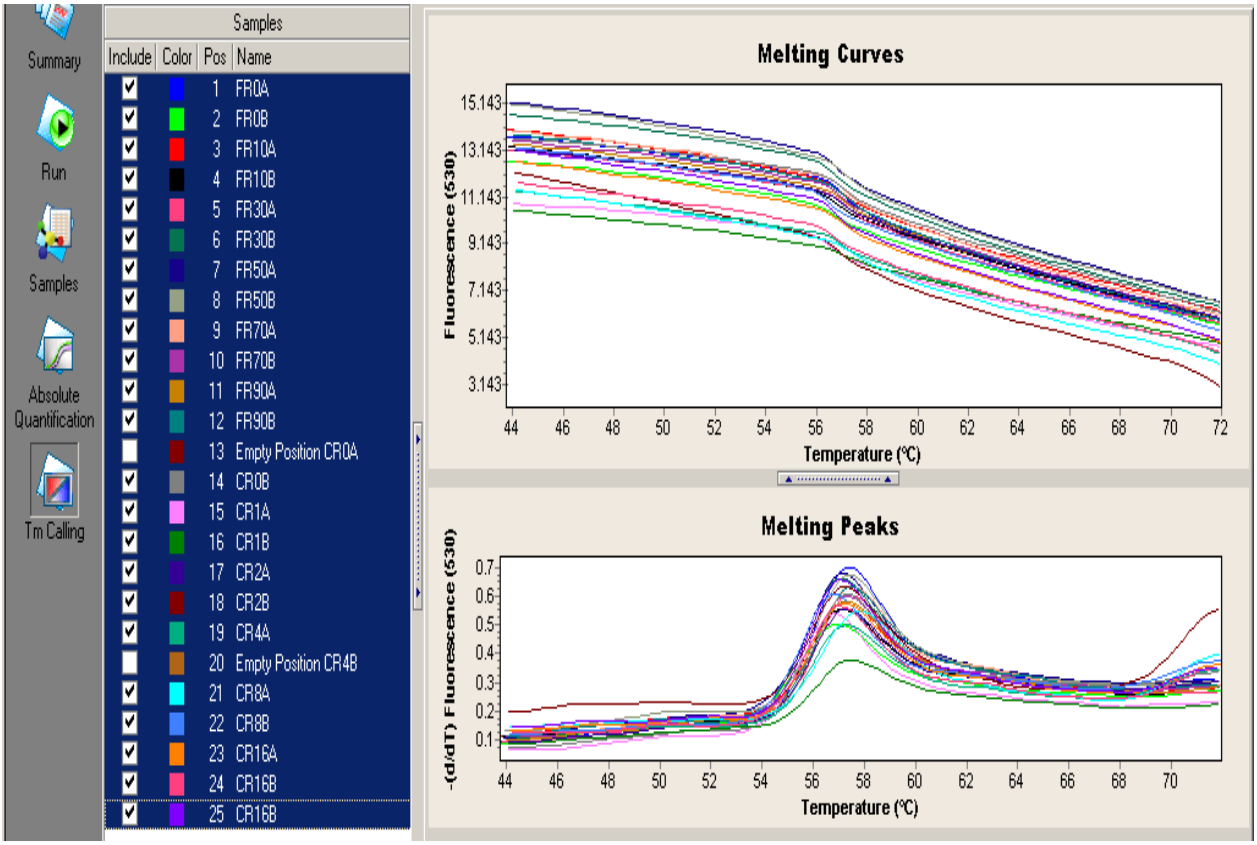
Cisplatin (µM)	HEPG2 +cystamine (%)			HEPG2CR +cystamine (%)			HEPG2 (%)			HEPG2CR (%)		
0	0	0	0	0	0	0	0	0	0	0	0	0
1	37	36	39	28	30	26	38	30	38	8	6	9
2	46	46	44	40	41	36	51	40	46	16	13	16
4	62	63	61	49	53	48	52	51	44	39	34	34
8	68	67	70	64	64	64	67	64	67	52	48	50
16	80	77	75	70	81	76	80	81	84	72	71	71

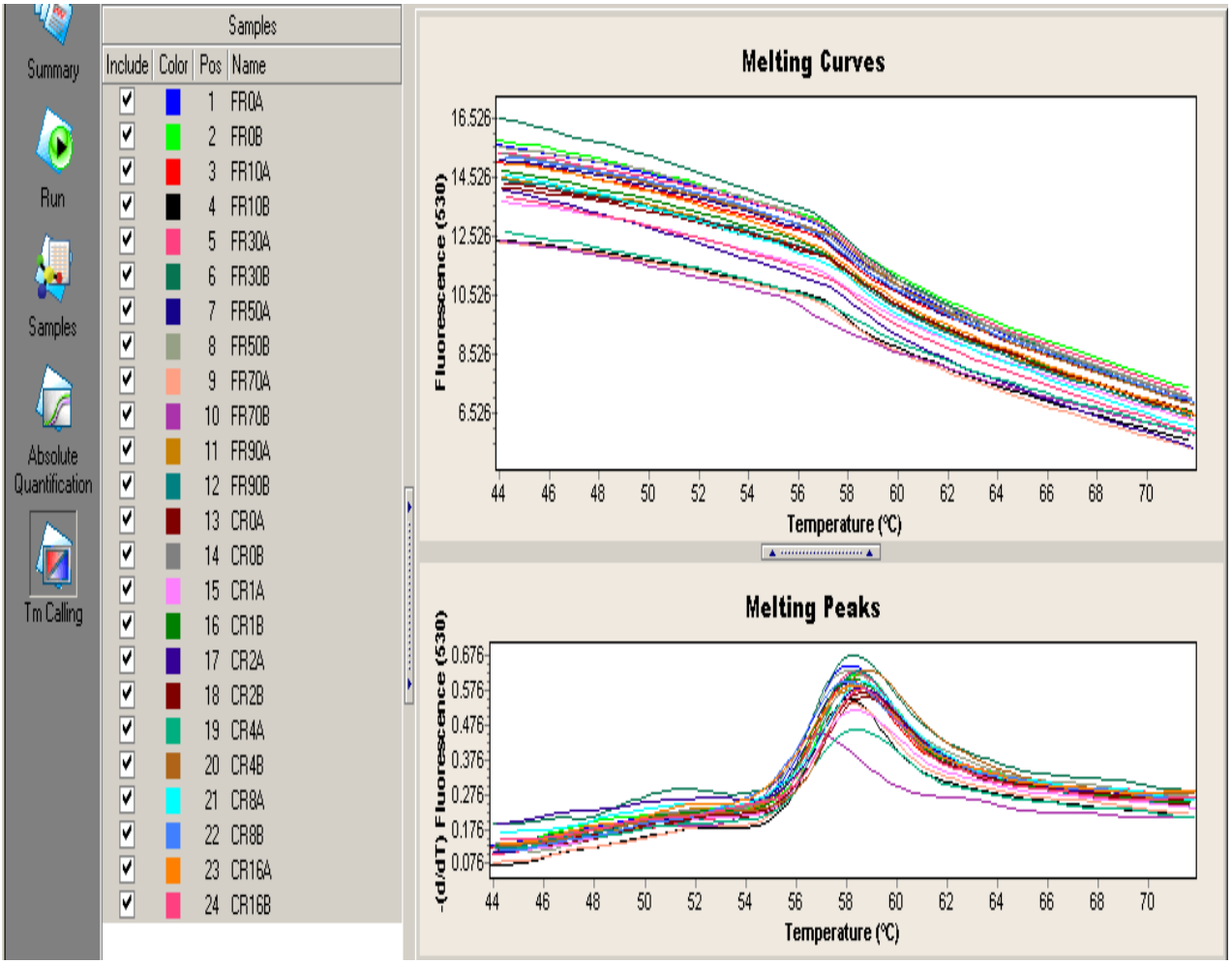
Table A2.4.8: Susceptibility of HEPG2 and HEPG2FR to 5-FU-induced death following the inhibition of TG2 activity with 2.5mM of cystamine (figure 5.13)

5-FU(µM)	HEPG2 +cystamine (%)			HEPG2FR +cystamine (%)			HEPG2 (%)			HEPG2FR (%)		
0	0	0	0	0	0	0	0	0	0	0	0	
10	33	29	38	28	36	33	18	16	19	12	23	17
30	45	51	50	46	39	43	31	27	30	26	26	27
50	60	58	68	58	51	57	51	48	50	41	39	41
70	80	64	71	67	69	67	70	75	78	53	52	55
100	81	86	93	73	74	81	74	75	73	75	75	77

APPENDIX THREE

Examples of RT-PCR melting & amplification curves (see section 4.2.2)

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Tm Calling
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3 FR10A
4 FR10B
5 FR30A
6 FR30B
7 FR50A
8 FR50B
9 FR70A
10 FR70B
11 FR90A
12 FR90B
13 CR0A
14 CR0B
15 CR1A
16 CR1B
17 CR2A
18 CR2B
19 CR4A
20 CR4B
21 CR8A
22 CR8B
23 CR16A
24 CR16B
Melting Curves
Fluorescence (530)
16.526
14.526
12.526
10.526
8.526
6.526
44 46 48 50 52 54 56 58 60 62 64 66 68 70
Temperature (°C)
Melting Peaks
-(d/dT) Fluorescence (530)
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0.476
0.376
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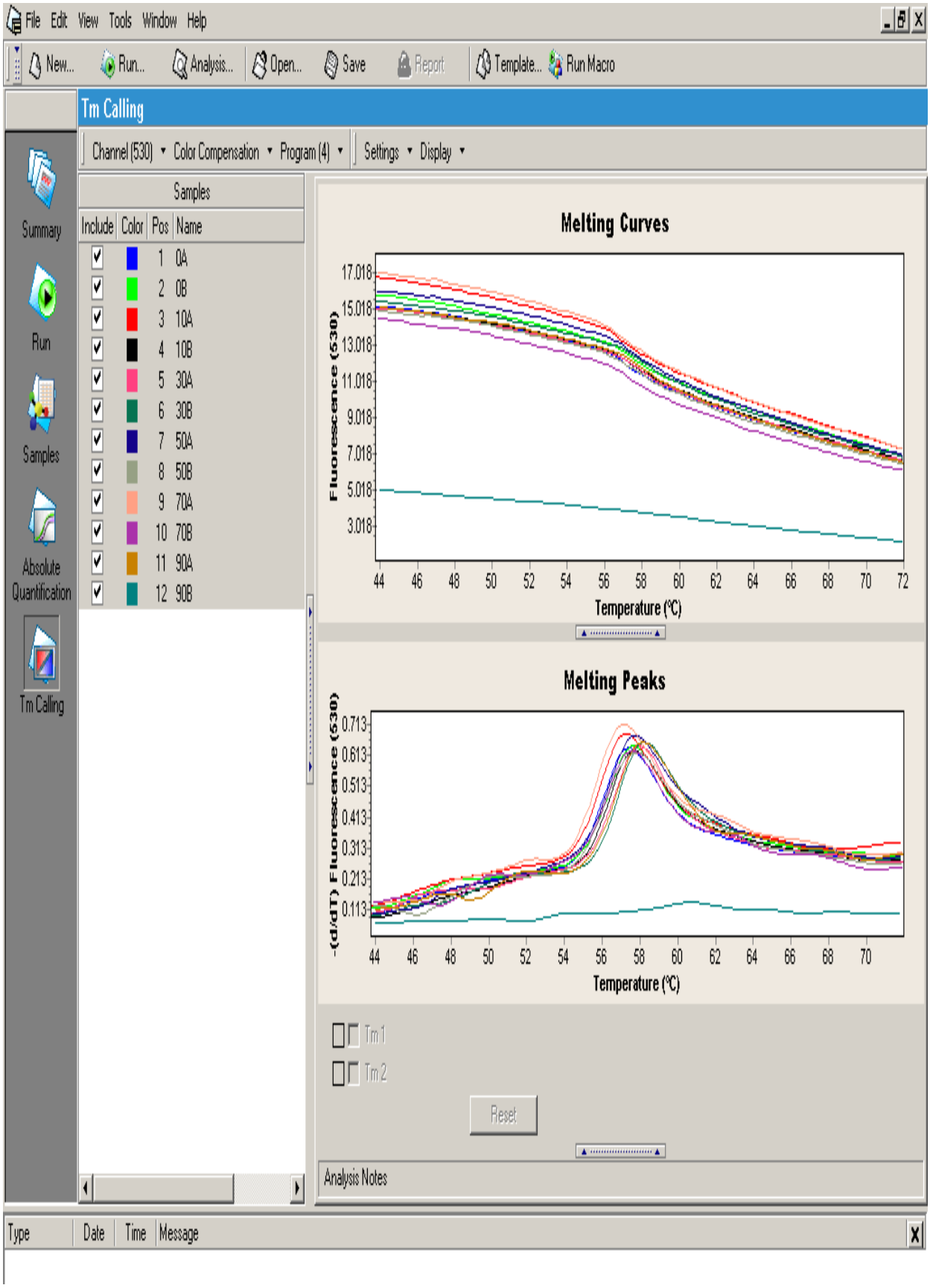
0.076
44 46 48 50 52 54 56 58 60 62 64 66 68 70
Temperature (°C)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Summary
Run
Samples
Absolute Quantification
Tm Calling
Samples
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2 0B
3 10A
4 10B
5 30A
6 30B
7 50A
8 50B
9 70A
10 70B
11 90A
12 90B
Melting Curves
Fluorescence (530)
17.018
15.018
13.018
11.018
9.018
7.018
5.018
3.018
44 46 48 50 52 54 56 58 60 62 64 66 68 70 72
Temperature (°C)
Melting Peaks
-(d/dT) Fluorescence (530)
0.713
0.613
0.513
0.413
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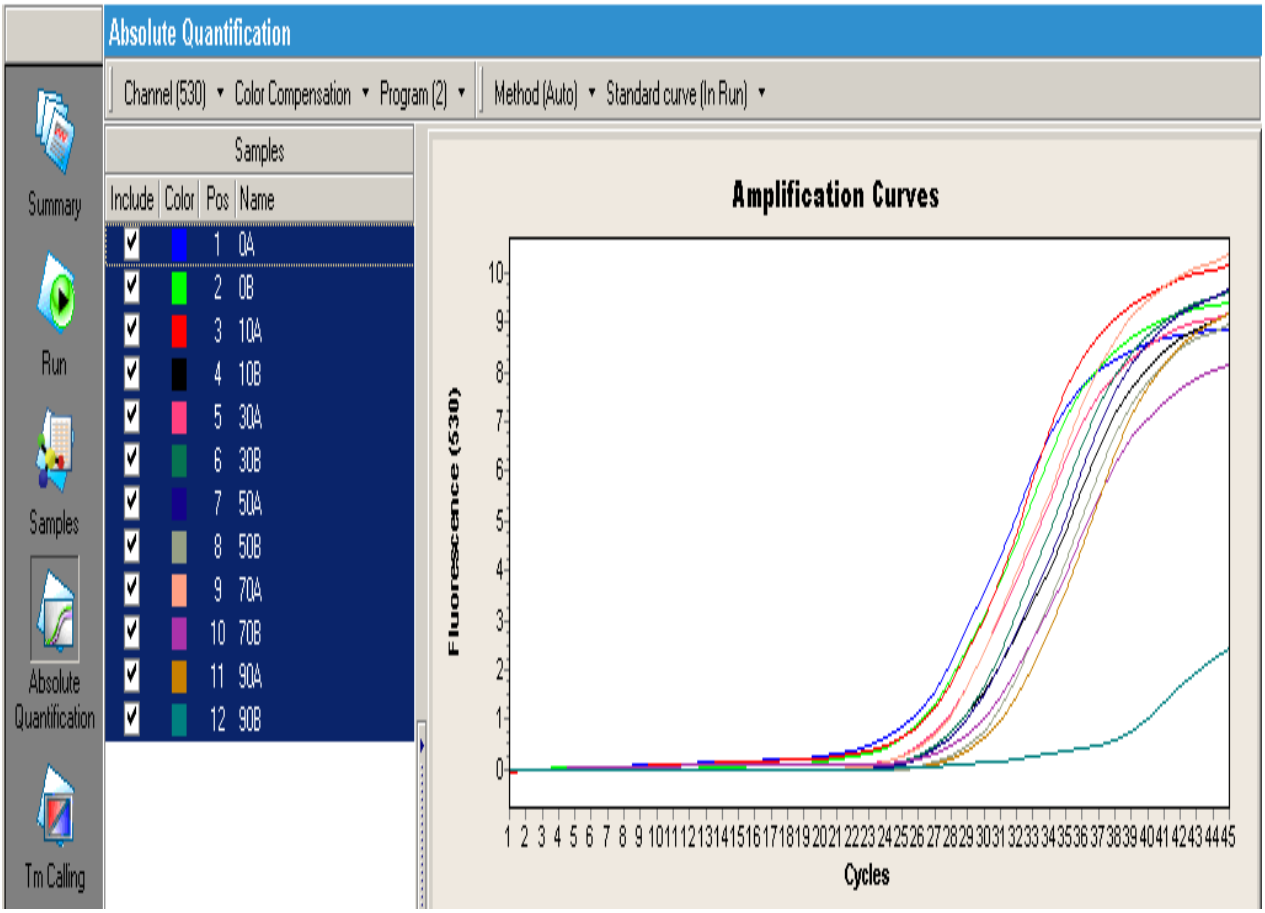
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Samples
Absolute Quantification
Tm Calling
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Include
Color
Pos
Name
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2 0B
3 10A
4 10B
5 30A
6 30B
7 50A
8 50B
9 70A
10 70B
11 90A
12 90B
Amplification Curves
Fluorescence (530)
Cycles

APPENDIX FOUR
STATISTICAL DATA EXTRACTS

Table A4.0: HEPG2 response to cisplatin after 12 to 24 hours treatment (figure 2.1)

Cisplatin (μM)	12 hours			24 hours			
0.00	0.00	0.00	0.00	0.00	0.00	0.00	50.00
1.00	34.00	31.00	36.00	38.00	30.00	38.00	50.00
2.00	37.00	37.00	37.00	51.00	40.00	46.00	50.00
4.00	48.00	49.00	50.00	52.00	51.00	44.00	50.00
8.00	63.00	59.00	62.00	67.00	64.00	67.00	50.00
16.00	79.00	79.00	80.00	80.00	81.00	84.00	50.00

Table A4.1: Student paired t-test for figure 2.1

Table Analyzed	Cisplatin dose response curves
Column A	12 hours
vs.	vs.
Column B	24 hours
Paired t test	
P value	0.0861
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.133 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-2.889
SD of differences	3.318
SEM of differences	1.354
95% confidence interval	-6.371 to 0.5929
R squared	0.4764
How effective was the pairing?	
Correlation coefficient (r)	0.9933
P value (one tailed)	< 0.0001
P value summary	****
Was the pairing significantly effective?	Yes

Table A4.2: 5-FU dose response table

5-FU (μM)	HEPG2			
0.00	0.00	0.00	0.00	50.00
10.00	18.00	16.00	19.00	50.00
20.00	23.00	17.00	19.00	50.00
30.00	31.00	27.00	30.00	50.00
40.00	39.00	40.00	43.00	50.00
50.00	51.00	48.00	50.00	50.00
60.00	53.00	58.00	57.00	50.00
70.00	70.00	65.00	68.00	50.00
80.00	71.00	69.00	70.00	50.00
90.00	71.00	74.00	74.00	50.00
100.00	74.00	75.00	73.00	50.00

Table A4.3: Comparison of HEPG2 and HEPG2CR cells' response to cisplatin (figure 2.3)

Cisplatin (μM)	HEPG2CR			HEPG2			
0.00	0.00	0.00	0.00	0.00	0.00	0.00	50.00
1.00	8.00	6.00	9.00	38.00	30.00	38.00	50.00
2.00	16.00	13.00	16.00	51.00	40.00	46.00	50.00
4.00	39.00	34.00	34.00	52.00	51.00	44.00	50.00
6.00	39.00	38.00	40.00	56.00	50.00	63.00	50.00
8.00	52.00	48.00	50.00	67.00	64.00	67.00	50.00
10.00	65.00	60.00	65.00	74.00	72.00	71.00	50.00
12.00	66.00	67.00	68.00	77.00	82.00	82.00	50.00
14.00	68.00	67.00	70.00	78.00	83.00	81.00	50.00
16.00	72.00	71.00	71.00	80.00	81.00	84.00	50.00
18.00	71.00	72.00	73.00	85.00	83.00	85.00	50.00
20.00	75.00	72.00	74.00	87.00	87.00	86.00	50.00

Table A4.4: A student paired t-test for figure 2.3

Column B	HEPG2
vs.	vs.
Column A	HEPG2CR
Paired t test	
P value	< 0.0001
P value summary	****
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.269 df=11
Number of pairs	12
How big is the difference?	
Mean of differences	14.61
SD of differences	8.074
SEM of differences	2.331
95% confidence interval	9.481 to 19.74
R squared	0.7813
How effective was the pairing?	
Correlation coefficient (r)	0.9543
P value (one tailed)	< 0.0001
P value summary	****
Was the pairing significantly effective?	Yes

Table A4.5: Comparison of HEPG2 and HEPG2FR cells' response to 5-FU (figure 2.4)

5FU (μM)	HEPG2			HEPG2FR			
0.00	0.00	0.00	0.00	0.00	0.00	0.00	50.00
10.00	18.00	16.00	19.00	12.00	23.00	17.00	50.00
20.00	23.00	17.00	19.00	25.00	24.00	28.00	50.00
30.00	31.00	27.00	30.00	27.00	26.00	27.00	50.00
40.00	39.00	40.00	43.00	28.00	26.00	30.00	50.00
50.00	51.00	48.00	50.00	41.00	39.00	41.00	50.00
60.00	53.00	58.00	57.00	58.00	42.00	46.00	50.00
70.00	70.00	65.00	68.00	53.00	52.00	55.00	50.00
80.00	71.00	69.00	70.00	61.00	62.00	62.00	50.00
90.00	71.00	74.00	74.00	74.00	76.00	74.00	50.00
100.00	74.00	75.00	73.00	75.00	75.00	77.00	50.00

Table A4.6: A student paired t-test for figure 2.4

Table Analyzed	5FU dose-dependent response
Column A	HEPG2
vs.	vs.
Column B	HEPG2FR
Paired t test	
P value	0.0317
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=2.087 df=10
Number of pairs	11
How big is the difference?	
Mean of differences	4.152
SD of differences	6.599
SEM of differences	1.990
95% confidence interval	-0.2815 to 8.585
R squared	0.3033
How effective was the pairing?	
Correlation coefficient (r)	0.9667
P value (one tailed)	< 0.0001
P value summary	****
Was the pairing significantly effective?	Yes

Table A4.7: Expression of TGM2 in HEPG2 and HEPG2CR after cisplatin treatment (figure 4.1)

Cisplatin (µM)	HEPG2		HEPG2CR	
0	1.000	1.000	1.000	1.000
1	1.204	1.342	1.500	1.486
2	1.467	1.414	1.717	1.787
4	1.576	1.469	2.833	2.759
8	1.264	1.268	1.500	1.333
16	1.164	1.242	1.164	1.275

Table A4.8: t-test analysis for figure 4.1

Table Analyzed	Expression of TGM2 gene in HEPG2 and HEPG2CR cells
Column A	HEPG2
vs.	vs.
Column B	HEPG2CR
Wilcoxon matched-pairs signed rank test	
P value	0.0313
Exact or approximate P value?	Exact
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of positive, negative ranks	0.0 , -15.00
Sum of signed ranks (W)	-15.00
Number of pairs	6
Median of differences	
Median	-0.1853
How effective was the pairing?	
rs (Spearman)	1.000
P value (one tailed)	0.0014
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.9: Expression of TGM2 gene in HEPG2 and HEPG2FR cells after 5-FU treatment (figure 4.2)

5-FU (µM)	HEPG2		HEPG2FR	
0	1.00	1.00	1.000	1.000
10	1.87	1.48	0.639	0.333
30	1.26	1.24	0.389	0.500
50	1.22	0.87	0.420	0.383
70	0.43	0.87	0.319	0.306
90	0.35	0.41	0.308	0.417

Table A4.10: A student t-test of TGM2 gene expression in HEPG2 and HEPG2FR cells after 5-FU treatment (figure 4.2)

Table Analyzed	Expression of TGM2 gene in HEPG2 and HEPG2FR cells
Column A	HEPG2
vs.	vs.
Column B	HEPG2FR
Paired t test	
P value	0.0478
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.608 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	0.4988
SD of differences	0.4686
SEM of differences	0.1913
95% confidence interval	0.007086 to 0.9906
R squared	0.5763
How effective was the pairing?	
Correlation coefficient (r)	0.2158
P value (one tailed)	0.3407
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.11: Effect of cisplatin on Tgase activity in HEPG2 and HEPG2CR cells (figure 4.9)

Cisplatin (µM)	HEPG2		HEPG2CR	
0	0.100	0.101	0.102	0.106
1	0.107	0.104	0.112	0.112
2	0.161	0.158	0.174	0.170
4	0.177	0.179	0.193	0.183
8	0.150	0.155	0.167	0.174
16	0.122	0.122	0.135	0.138

Table A4.12: A student t-test of Tgase activity in HEPG2 and HEPG2CR cells after cisplatin treatment (figure 4.9)

Table Analyzed	CBZ-Hydroxamate assay of Tgase activity after cisplatin treatment
Column A	HEPG2
vs.	vs.
Column B	HEPG2CR
Paired t test	
P value	0.0041
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.000 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-0.01083
SD of differences	0.005307
SEM of differences	0.002167
95% confidence interval	-0.01640 to -0.005264
R squared	0.8333
How effective was the pairing?	
Correlation coefficient (r)	0.9919
P value (one tailed)	< 0.0001
P value summary	****
Was the pairing significantly effective?	Yes

Table A4.13: Effect of 5-FU on Tgase activity in HEPG2 and HEPG2FR cells (figure 4.10)

5-FU (µM)	HEPG2		HEPG2FR	
0	0.065	0.071	0.082	0.078
10	0.128	0.127	0.131	0.133
30	0.166	0.169	0.174	0.170
50	0.172	0.171	0.186	0.188
70	0.181	0.177	0.191	0.187
100	0.193	0.190	0.213	0.211

Table A4.14: A student t-test of Tgase activity in HEPG2 and HEPG2FR cells after 5-FU treatment (figure 4.10)

Table Analyzed	Effect of 5-FU on Tgase activity
Column A	HEPG2
vs.	vs.
Column B	HEPG2FR
Paired t test	
P value	0.0073
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.359 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-0.01117
SD of differences	0.006274
SEM of differences	0.002561
95% confidence interval	-0.01775 to -0.004582
R squared	0.7917
How effective was the pairing?	
Correlation coefficient (r)	0.9922
P value (one tailed)	< 0.0001
P value summary	****
Was the pairing significantly effective?	Yes

Table A4.15: TG2 activity in HEPG2 and HEPG2CR cells after cisplatin treatment (figure 4.11)

Cisplatin (µM)	HEPG2		HEPG2CR	
0	0.160	0.134	0.196	0.196
2	0.161	0.157	0.195	0.194
4	0.199	0.156	0.202	0.199
8	0.167	0.133	0.178	0.166
16	0.144	0.137	0.112	0.112

Table A4.16: Student t-test of TG2 activity in HEPG2 and HEPG2CR cells shown in figure 4.11

Table Analyzed	TG2-specific activity in HEPG2 and HEPG2CR cells
Column A	HEPG2
vs.	vs.
Column B	HEPG2CR
Paired t test	
P value	0.1987
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.539 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	-0.0202
SD of differences	0.02935
SEM of differences	0.01313
95% confidence interval	-0.05665 to 0.01625
R squared	0.3719
How effective was the pairing?	
Correlation coefficient (r)	0.6675
P value (one tailed)	0.1091
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.17: TG2 activity in HEPG2 and HEPG2FR cells after 5-FU treatment as shown in figure 4.12

5-FU (µM)	HEPG2		HEPG2FR	
0	0.160	0.134	0.203	0.186
10	0.122	0.173	0.188	0.155
30	0.169	0.166	0.137	0.169
50	0.127	0.116	0.173	0.193
100	0.130	0.163	0.206	0.198

Table A4.18: Student t-test of TG2 activity in HEPG2 and HEPG2FR cells after 5-FU treatment (figure 4.12)

Table Analyzed	TG2-specific activity in HEPG2 and HEPG2FR cells
Column A	HEPG2
vs.	vs.
Column B	HEPG2FR
Paired t test	
P value	0.0331
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=2.508 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	-0.0348
SD of differences	0.03102
SEM of differences	0.01387
95% confidence interval	-0.07332 to 0.003721
R squared	0.6113
How effective was the pairing?	
Correlation coefficient (r)	-0.5069
P value (one tailed)	0.1917
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.19: One-way ANOVA analysis of the invasiveness of parental and drug-resistant HEPG2 clones as shown in figure 5.2

Table Analyzed	Metastasis assay				
ANOVA summary					
F	1.033				
P value	0.4115				
P value summary	Ns				
Are differences among means statistically significant? (P < 0.05)	No				
R square	0.2562				
Brown-Forsythe test					
F (DFn, DFd)	0.08553 (2, 6)				
P value	0.9191				
P value summary	Ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Significantly different standard deviations? (P < 0.05)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	62.00	2	31.00	F (2, 6) = 1.033	P = 0.4115
Residual (within columns)	180.0	6	30.00		
Total	242.0	8			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	9				

Table A4.20: A t-test of the invasive abilities of HEPG2 vs HEPG2CR (figure 5.2)

Table Analyzed	Metastasis assay
Column A	HEPG2
vs.	vs.
Column B	HEPG2CR
Paired t test	
P value	0.2539
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.585 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-6.000
SD of differences	6.557
SEM of differences	3.786
95% confidence interval	-22.29 to 10.29
R squared	0.5567
How effective was the pairing?	
Correlation coefficient (r)	0.1233
P value (one tailed)	0.4607
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.21: Student t-test of the invasiveness of HEPG2 vs HEPG2FR (figure 5.2)

Table Analyzed	Metastasis assay
Column A	HEPG2
vs.	vs.
Column C	HEPG2FR
Paired t test	
P value	0.0820
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=3.273 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-5.000
SD of differences	2.646
SEM of differences	1.528
95% confidence interval	-11.57 to 1.572
R squared	0.8427
How effective was the pairing?	
Correlation coefficient (r)	0.9118
P value (one tailed)	0.1347
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.22: A student t-test of the invasiveness of HEG2CR vs HEPG2FR (figure 5.2)

Table Analyzed	Metastasis assay
Column C	HEPG2FR
vs.	vs.
Column B	HEPG2CR
Paired t test	
P value	0.8581
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.2027 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-1.000
SD of differences	8.544
SEM of differences	4.933
95% confidence interval	-22.22 to 20.22
R squared	0.02013
How effective was the pairing?	
Correlation coefficient (r)	-0.2951
P value (one tailed)	0.4047
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.23: One-way ANOVA of the invasiveness of HEPG2 clones with and without siRNA interference with TG2 protein expression as shown in figure 5.5

Table Analyzed	Effect of TG2 silencing on cell invasion and migration				
ANOVA summary					
F	15.99				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.8695				
Brown-Forsythe test					
F (DFn, DFd)	0.3020 (5, 12)				
P value	0.9024				
P value summary	Ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Significantly different standard deviations? (P < 0.05)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1635	5	326.9	F (5, 12) = 15.99	P < 0.0001
Residual (within columns)	245.3	12	20.44		
Total	1880	17			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	18				

Table A4.24: A student t-test of the invasiveness of HEPG2 cells on matrigel-coated plates with and without siRNA (figure 5.5)

Table Analyzed	Effect of TG2 silencing on cell invasion and migration
Column A	HEPG2
vs.	vs.
Column D	HEPG2+siRNA
Paired t test	
P value	0.0339
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.289 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	20.33
SD of differences	6.658
SEM of differences	3.844
95% confidence interval	3.793 to 36.87
R squared	0.9333
How effective was the pairing?	
Correlation coefficient (r)	-0.5565
P value (one tailed)	0.3122
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.25: A student t-test of the invasiveness of HEPG2CR cells on matrigel-coated plates with and without siRNA (figure 5.5)

Table Analyzed	Effect of TG2 silencing on cell invasion and migration
Column B	HEPG2CR
vs.	vs.
Column E	HEPG2CR+siRNA
Paired t test	
P value	0.0094
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=7.181 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	19.00
SD of differences	4.583
SEM of differences	2.646
95% confidence interval	7.616 to 30.38
R squared	0.9627
How effective was the pairing?	
Correlation coefficient (r)	0.3764
P value (one tailed)	0.3772
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.26: A student t-test of the invasiveness of HEPG2FR cells on matrigel-coated plates with and without siRNA (figure 5.5)

Table Analyzed	Effect of TG2 silencing on cell invasion and migration
Column C	HEPG2FR
vs.	vs.
Column F	HEPG2FR+siRNA
Unpaired t test	
P value	0.0332
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=2.506 df=4
How big is the difference?	
Mean ± SEM of column C	83.67 ± 3.712, n=3
Mean ± SEM of column F	73.00 ± 2.082, n=3
Difference between means	10.67 ± 4.256
95% confidence interval	-1.149 to 22.48
R squared	0.6110
F test to compare variances	
F,DFn, Dfd	3.179, 2, 2
P value	0.4785
P value summary	Ns
Significantly different? (P < 0.05)	No

Table A4.27: A t-test of HEPG2 cells' response to cisplatin-induced death following TG2 protein down-regulation by siRNA as shown in figure 5.6

Table Analyzed	Cell response to cisplatin after TG2 protein downregulation by siRNA
Column C	HEPG2
vs.	vs.
Column A	HEPG2+siRNA
Paired t test	
P value	0.0167
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.531 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	11.94
SD of differences	8.285
SEM of differences	3.382
95% confidence interval	3.250 to 20.64
R squared	0.7138
How effective was the pairing?	
Correlation coefficient (r)	0.9552
P value (one tailed)	0.0015
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.28: A student t-test of HEPG2CR sensitivity to cisplatin-induced death following TG2 protein down-regulation by siRNA as shown in figure 5.6

Table Analyzed	Cell response to cisplatin after TG2 protein downregulation by siRNA
Column D	HEPG2CR
vs.	vs.
Column B	HEPG2CR+siRNA
Paired t test	
P value	0.3103
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.129 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-3.778
SD of differences	8.199
SEM of differences	3.347
95% confidence interval	-12.38 to 4.827
R squared	0.2030
How effective was the pairing?	
Correlation coefficient (r)	0.9647
P value (one tailed)	0.0009
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.29: One-way ANOVA of the sensitivity of parental and cisplatin-resistant HEPG2 clone to cisplatin-induced death following TG2 down-regulation (figure 5.6)

Table Analyzed	Cell response to cisplatin after TG2 protein downregulation by siRNA				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	10.14				
P value	0.0103				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4880				
R square	0.6699				
Was the matching effective?					
F	90.65				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.9089				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	901.5	3	300.5	F (1.464, 7.319) = 10.14	P = 0.0103
Individual (between rows)	13425	5	2685	F (5, 15) = 90.65	P < 0.0001
Residual (random)	444.3	15	29.62		
Total	14771	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Table A4.30: One-way ANOVA of the sensitivity of parental and 5-FU-resistant HEPG2 clone to 5-FU-induced death following TG2 down-regulation (figure 5.7)

Table Analyzed	Cell response to 5FU after TG2 downregulation				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	3.087				
P value	0.0774				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.7874				
R square	0.3817				
Was the matching effective?					
F	101.3				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.9543				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	256.3	3	85.44	F (2.362, 11.81) = 3.087	P = 0.0774
Individual (between rows)	14019	5	2804	F (5, 15) = 101.3	P < 0.0001
Residual (random)	415.2	15	27.68		
Total	14690	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Table A4.31: A t-test of HEPG2 response to 5-FU-induced death following TG2 protein down-regulation by siRNA as shown in figure 5.7

Table Analyzed	Cell response to 5FU after TG2 downregulation
Column A	HEPG2
vs.	vs.
Column C	HEPG2+siRNA
Paired t test	
P value	0.1144
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.910 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	5.500
SD of differences	7.055
SEM of differences	2.880
95% confidence interval	-1.903 to 12.90
R squared	0.4218
How effective was the pairing?	
Correlation coefficient (r)	0.9778
P value (one tailed)	0.0004
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.32: A student t-test of HEPG2FR response to 5-FU-induced death following TG2 protein down-regulation by siRNA as shown in figure 5.7

Table Analyzed	Cell response to 5FU after TG2 downregulation
Column B	HEPG2FR
vs.	vs.
Column D	HEPG2FR+siRNA
Paired t test	
P value	0.3198
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.104 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	3.833
SD of differences	8.503
SEM of differences	3.471
95% confidence interval	-5.090 to 12.76
R squared	0.1961
How effective was the pairing?	
Correlation coefficient (r)	0.9554
P value (one tailed)	0.0015
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.33: Effect of cystamine on TG2 activity in parental and drug-resistant HEPG2 clones (figure 5.10)

cystamiine	HEPG2		HEPG2CR		HEPG2FR	
0.00	0.301	0.257	0.421	0.300	0.388	0.247
0.50	0.255	0.234	0.271	0.279	0.296	0.308
1.00	0.194	0.191	0.220	0.216	0.248	0.232
1.50	0.172	0.190	0.231	0.250	0.230	0.217
2.00	0.110	0.150	0.140	0.135	0.120	0.150
2.50	0.080	0.098	0.100	0.096	0.130	0.088
3.00	0.079	0.084	0.097	0.097	0.114	0.138
3.50	0.075	0.090	0.083	0.094	0.117	0.119
4.00	0.068	0.076	0.091	0.081	0.101	0.099

Table A4.34: One-way ANOVA of the effect of cystamine on TG2 activity in HEPG2 clones as shown in figure 5.10

Table Analyzed	Cystamine inhibition of TG2 activity				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	12.17				
P value	0.0018				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.7857				
R square	0.6034				
Was the matching effective?					
F	88.75				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.9462				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.006247	2	0.003123	F (1.571, 12.57) = 12.17	P = 0.0018
Individual (between rows)	0.1822	8	0.02278	F (8, 16) = 88.75	P < 0.0001
Residual (random)	0.004106	16	0.0002566		
Total	0.1926	26			
Data summary					
Number of treatments (columns)	3				
Number of subjects (rows)	9				

Table A4.35: One-way ANOVA of the effect of inhibition of TG2 activity on the invasive abilities of HEPG2 clones as shown in figure 5.11

Table Analyzed	Effect of TG2 activity inhibition on cell invasion				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	128.2				
P value	0.0009				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.3234				
R square	0.9846				
Was the matching effective?					
F	3.147				
P value	0.0870				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.009581				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	11299	5	2260	F (1.617, 3.234) = 128.2	P = 0.0009
Individual (between rows)	111.0	2	55.50	F (2, 10) = 3.147	P = 0.0870
Residual (random)	176.3	10	17.63		
Total	11586	17			
Data summary					
Number of treatments (columns)	6				
Number of subjects (rows)	3				

Table A4.36: Student t-test of the invasiveness of HEPG2 vs HEPG2+cystamine as shown in figure 5.11

Table Analyzed	Effect of TG2 activity inhibition on cell invasion
Column A	HEPG2
vs.	vs.
Column D	HEPG2+cystamine
Paired t test	
P value	0.0063
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=12.53 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	51.33
SD of differences	7.095
SEM of differences	4.096
95% confidence interval	33.71 to 68.96
R squared	0.9874
How effective was the pairing?	
Correlation coefficient (r)	-0.9018
P value (one tailed)	0.1422
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.37: Student t-test of the invasiveness of HEPG2CR vs HEPG2CR+cystamine as shown in figure 5.11

Table Analyzed	Effect of TG2 activity inhibition on cell invasion
Column B	HEPG2CR
vs.	vs.
Column E	HEPG2CR+cystamine
Paired t test	
P value	0.0019
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=23.00 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	53.67
SD of differences	4.041
SEM of differences	2.333
95% confidence interval	43.63 to 63.71
R squared	0.9962
How effective was the pairing?	
Correlation coefficient (r)	0.3712
P value (one tailed)	0.3790
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.38: Student t-test of the invasiveness of HEPG2FR vs HEPG2FR+cystamine as shown in figure 5.11

Table Analyzed	Effect of TG2 activity inhibition on cell invasion
Column C	HEPG2FR
vs.	vs.
Column F	HEPG2FR+cystamine
Paired t test	
P value	0.0013
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=28.15 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	43.00
SD of differences	2.646
SEM of differences	1.528
95% confidence interval	36.43 to 49.57
R squared	0.9975
How effective was the pairing?	
Correlation coefficient (r)	0.9164
P value (one tailed)	0.1311
P value summary	Ns
Was the pairing significantly effective?	No

Table A4.39: One-way ANOVA of the effect of inhibition of TG2 activity on the susceptibility of HEPG2 clones to cisplatin-induced death as shown in figure 5.12

Table Analyzed	Cell response to cisplatin after TG2 activity inhibition				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	11.02				
P value	0.0106				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4484				
R square	0.6879				
Was the matching effective?					
F	78.80				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.8913				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1230	3	409.9	F (1.345, 6.726) = 11.02	P = 0.0106
Individual (between rows)	14658	5	2932	F (5, 15) = 78.80	P < 0.0001
Residual (random)	558.0	15	37.20		
Total	16445	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Table A4.40: Student t-test of the susceptibility of HEPG2 vs HEPG2+cystamine to cisplatin-induced death as shown in figure 5.12

Table Analyzed	Cell response to cisplatin after TG2 activity inhibition
Column C	HEPG2
vs.	vs.
Column A	HEPG2+cystamine
Paired t test	
P value	0.4165
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8853 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-2.111
SD of differences	5.841
SEM of differences	2.385
95% confidence interval	-8.241 to 4.019
R squared	0.1355
How effective was the pairing?	
Correlation coefficient (r)	0.9781
P value (one tailed)	0.0004
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.41: Student t-test of the susceptibility of HEPG2CR vs HEPG2CR+cystamine to cisplatin-induced death as shown in figure 5.12

Table Analyzed	Cell response to cisplatin after TG2 activity inhibition
Column D	HEPG2CR
vs.	vs.
Column B	HEPG2CR+cystamine
Paired t test	
P value	0.0188
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.424 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-12.83
SD of differences	9.182
SEM of differences	3.748
95% confidence interval	-22.47 to -3.198
R squared	0.7010
How effective was the pairing?	
Correlation coefficient (r)	0.9432
P value (one tailed)	0.0024
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.42: One-way ANOVA of the susceptibility of HEPG2 clones to 5-FU-induced death following cystamine inhibition of TG2 activity as shown in figure 5.13

Table Analyzed	Cell response to 5FU after TG2 activity inhibition				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	8.064				
P value	0.0054				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.7637				
R square	0.6173				
Was the matching effective?					
F	107.3				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.9319				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	735.7	3	245.2	F (2.291, 11.46) = 8.064	P = 0.0054
Individual (between rows)	16316	5	3263	F (5, 15) = 107.3	P < 0.0001
Residual (random)	456.1	15	30.41		
Total	17508	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Table A4.43: Student t-test of the susceptibility of HEPG2 vs HEPG2+cystamine to 5-FU-induced death as shown in figure 5.13

Table Analyzed	Cell response to 5FU after TG2 activity inhibition
Column C	HEPG2
vs.	vs.
Column A	HEPG2+cystamine
Paired t test	
P value	0.0456
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.647 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-9.556
SD of differences	8.843
SEM of differences	3.610
95% confidence interval	-18.84 to -0.2749
R squared	0.5835
How effective was the pairing?	
Correlation coefficient (r)	0.9583
P value (one tailed)	0.0013
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.44: Student t-test of the susceptibility of HEPG2FR vs HEPG2FR+cystamine to 5-FU-induced death as shown in figure 5.13

Table Analyzed	Cell response to 5FU after TG2 activity inhibition
Column D	HEPG2FR
vs.	vs.
Column B	HEPG2FR+cystamine
Paired t test	
P value	0.0239
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.203 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-10.17
SD of differences	7.774
SEM of differences	3.174
95% confidence interval	-18.32 to -2.008
R squared	0.6724
How effective was the pairing?	
Correlation coefficient (r)	0.9593
P value (one tailed)	0.0012
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.45: ANOVA comparison of the effects of TG2 down-regulation and activity inhibition on the susceptibility of HEPG2 clones to cisplatin-induced death (figure 5.14)

Table Analyzed	Comparative effects of TG2 inhibition and downregulation on cell response to cisplatin				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	11.23				
P value	0.0056				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.3275				
R square	0.6920				
Was the matching effective?					
F	134.8				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.8925				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1720	5	344.0	F (1.637, 8.186) = 11.23	P = 0.0056
Individual (between rows)	20645	5	4129	F (5, 25) = 134.8	P < 0.0001
Residual (random)	765.5	25	30.62		
Total	23130	35			
Data summary					
Number of treatments (columns)	6				
Number of subjects (rows)	6				

Table A4.46: Student t-test of the susceptibility of HEPG2+cystamine vs HEPG2+siRNA to cisplatin-induced death as shown in figure 5.14

Table Analyzed	Comparative effects of TG2 inhibition and downregulation on cellresponse to cis
Column C	HEPG2+cystamine
vs.	vs.
Column A	HEPG2+siRNA
Paired t test	
P value	0.0212
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.311 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	14.06
SD of differences	10.40
SEM of differences	4.246
95% confidence interval	3.142 to 24.97
R squared	0.6867
How effective was the pairing?	
Correlation coefficient (r)	0.9288
P value (one tailed)	0.0037
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.47: Student t-test of the susceptibility of HEPG2CR+cystamine vs HEPG2CR+siRNA to cisplatin-induced death as shown in figure 5.14

Table Analyzed	Comparative effects of TG2 inhibition and downregulation on cell response to cisplatin
Column D	HEPG2CR+cystamine
vs.	vs.
Column B	HEPG2CR+siRNA
Paired t test	
P value	0.0083
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.217 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	9.056
SD of differences	5.260
SEM of differences	2.147
95% confidence interval	3.536 to 14.58
R squared	0.7806
How effective was the pairing?	
Correlation coefficient (r)	0.9933
P value (one tailed)	< 0.0001
P value summary	****
Was the pairing significantly effective?	Yes

Table A4.48: ANOVA comparison of the effects of TG2 down-regulation and activity inhibition on the susceptibility of HEPG2 clones to 5-FU treatment (figure 5.15)

Table Analyzed	Comparison of cell response to 5FU after TG inhibition and Downregulation				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	10.06				
P value	0.0005				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.6416				
R square	0.6679				
Was the matching effective?					
F	147.5				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.9074				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1513	5	302.6	F (3.208, 16.04) = 10.06	P = 0.0005
Individual (between rows)	22188	5	4438	F (5, 25) = 147.5	P < 0.0001
Residual (random)	752.1	25	30.08		
Total	24453	35			
Data summary					
Number of treatments (columns)	6				
Number of subjects (rows)	6				

Table A4.49: Student t-test of the susceptibility of HEPG2+cystamine vs HEPG2+siRNA to 5-FU-induced death as shown in figure 5.15

Table Analyzed	Comparison of cell response to 5FU after TG inhibition and downregulation
Column A	HEPG2+cystamine
vs.	vs.
Column C	HEPG2+siRNA
Paired t test	
P value	0.0051
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.744 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	15.06
SD of differences	7.773
SEM of differences	3.173
95% confidence interval	6.898 to 23.21
R squared	0.8182
How effective was the pairing?	
Correlation coefficient (r)	0.9731
P value (one tailed)	0.0005
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.50: Student t-test of the susceptibility of HEPG2FR+cystamine vs HEPG2FR+siRNA to 5-FU-induced death as shown in figure 5.15

Table Analyzed	Comparison of cell response to 5FU after TG inhibition and downregulation
Column D	HEPG2FR+siRNA
vs.	vs.
Column B	HEPG2FR+cystamine
Paired t test	
P value	0.0066
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.465 df=5
Number of pairs	6
How big is the difference?	
Mean of differences	-14.00
SD of differences	7.680
SEM of differences	3.135
95% confidence interval	-22.06 to -5.941
R squared	0.7995
How effective was the pairing?	
Correlation coefficient (r)	0.9706
P value (one tailed)	0.0006
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.51: Assessment of the stability of HEPG2CR in drug-free conditions (as shown in figure 2.5)

Cisplatin (μM)	2 weeks			1 month			3 months			
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
1.00	10.00	10.00	9.00	8.00	9.00	9.00	8.00	6.00	9.00	50.00
2.00	16.00	19.00	18.00	16.00	18.00	14.00	16.00	13.00	16.00	50.00
4.00	35.00	37.00	35.00	39.00	36.00	34.00	39.00	34.00	34.00	50.00
8.00	47.00	55.00	50.00	50.00	44.00	54.00	52.00	48.00	50.00	50.00
16.00	74.00	71.00	70.00	69.00	78.00	68.00	72.00	71.00	71.00	50.00

Table A4.52: One-way ANOVA of HEPG2CR stability in drug-free medium as shown in figure 2.5

Table Analyzed	HEPG2CR stability test				
ANOVA summary					
F	0.001823				
P value	0.9982				
P value summary	ns				
Are differences among means statistically significant? (P < 0.05)	No				
R square	0.0002431				
Brown-Forsythe test					
F (DFn, DFd)	0.003152 (2, 15)				
P value	0.9969				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)	0.0009865				
P value	0.9995				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2.704	2	1.352	F (2, 15) = 0.001823	P = 0.9982
Residual (within columns)	11121	15	741.4		
Total	11124	17			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	18				

Table A4.53: One-way ANOVA multiple comparison of HEPG2CR stability in drug-free medium after 2 weeks, 1 month, and three months (figure 2.5)

Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary				
2 weeks vs. 1 month	0.5556	-40.28 to 41.39	No	Ns		A-B		
2 weeks vs. 3 months	0.9444	-39.89 to 41.78	No	Ns		A-C		
1 month vs. 3 months	0.3889	-40.44 to 41.22	No	Ns		B-C		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
2 weeks vs. 1 month	30.89	30.33	0.5556	15.72	6	6	0.04998	15
2 weeks vs. 3 months	30.89	29.94	0.9444	15.72	6	6	0.08496	15
1 month vs. 3 months	30.33	29.94	0.3889	15.72	6	6	0.03498	15

Table A4.54: Assessment of the stability of HEPG2FR in drug-free conditions (as shown in figure 2.6)

5-FU (μM)	2 weeks			1 month			3 months			
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	50.00
10.00	19.00	19.00	16.00	14.00	20.00	21.00	12.00	23.00	17.00	50.00
30.00	28.00	29.00	24.00	24.00	26.00	29.00	27.00	26.00	27.00	50.00
50.00	42.00	41.00	45.00	43.00	39.00	39.00	41.00	39.00	41.00	50.00
70.00	53.00	50.00	56.00	56.00	48.00	53.00	53.00	52.00	55.00	50.00
100.00	80.00	74.00	71.00	75.00	77.00	72.00	75.00	75.00	77.00	50.00

Table A4.55: One-way ANOVA of HEPG2FR stability in drug-free medium as shown in figure 2.6

Table Analyzed	HEPG2FR stability in drug-free medium				
ANOVA summary					
F	0.0008079				
P value	0.9992				
P value summary	Ns				
Are differences among means statistically significant? (P < 0.05)	No				
R square	0.0001077				
Brown-Forsythe test					
F (DFn, DFd)	0.002286 (2, 15)				
P value	0.9977				
P value summary	Ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)	0.001876				
P value	0.9991				
P value summary	Ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.148	2	0.5741	F (2, 15) = 0.0008079	P = 0.9992
Residual (within columns)	10658	15	710.5		
Total	10659	17			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	18				

Table A4.56: One-way ANOVA multiple comparison of HEPG2FR stability in drug-free medium after 2 weeks, 1 month, and three months (figure 2.6)

Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary				
2 weeks vs. 1 month	0.6111	-39.36 to 40.59	No	Ns		A-B		
2 weeks vs. 3 months	0.3889	-39.59 to 40.36	No	Ns		A-C		
1 month vs. 3 months	-0.2222	-40.20 to 39.75	No	Ns		B-C		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
2 weeks vs. 1 month	35.94	35.33	0.6111	15.39	6	6	0.05616	15
2 weeks vs. 3 months	35.94	35.56	0.3889	15.39	6	6	0.03574	15
1 month vs. 3 months	35.33	35.56	-0.2222	15.39	6	6	0.02042	15

Table A4.57: Student t-test of the susceptibility of HEPG2+cystamine vs HEPG2 to cisplatin-induced death as shown in figure 5.16

Table Analyzed	Data 1
Column A	HEPG2+cystamine
Vs	Vs
Column C	HEPG2
Paired t test	
P value	0.0486
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.804 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	15.70
95% confidence interval	0.1594 to 31.24
R squared	0.6629
How effective was the pairing?	
Correlation coefficient (r)	0.8977
P Value (one tailed)	0.0002
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.58: Student t-test of the susceptibility of HEPG2CR+cystamine vs HEPG2CR to cisplatin-induced death as shown in figure 5.16

Table Analyzed	Data 1
Column B	HEPG2CR+cystamine
Vs	Vs
Column D	HEPG2CR
Paired t test	
P value	0.0435
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.913 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	27.10
95% confidence interval	1.276 to 52.92
R squared	0.6797
How effective was the pairing?	
Correlation coefficient (r)	0.6845
P Value (one tailed)	0.0145
P value summary	*
Was the pairing significantly effective?	Yes

Table A4.59: Student t-test of the susceptibility of HEPG2+cystamine vs HEPG2 to 5-FU-induced death as shown in figure 5.17

Table Analyzed	Data 1
Column A	HEPG2+cystamine
Vs	Vs
Column C	HEPG2
Paired t test	
P value	0.0489
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.798 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	14.20
95% confidence interval	0.1092 to 28.29
R squared	0.6618
How effective was the pairing?	
Correlation coefficient (r)	0.9671
P Value (one tailed)	P<0.0001
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.60: Student t-test of the susceptibility of HEPG2FR+cystamine vs HEPG2FR to 5-FU-induced death as shown in figure 5.17

Parameter	Value
Table Analyzed	Data 1
Column B	HEPG2FR+cystamine
Vs	Vs
Column D	HEPG2FR
Paired t test	
P value	0.0399
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.000 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	20.80
95% confidence interval	1.554 to 40.05
R squared	0.6923
How effective was the pairing?	
Correlation coefficient (r)	0.8048
P Value (one tailed)	0.0025
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.61: Student t-test of the susceptibility of HEPG2+siRNA vs HEPG2 to cisplatin-induced death as shown in figure 5.8

Column A	HEPG2+siRNA
Vs	Vs
Column C	HEPG2
Paired t test	
P value	0.0399
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.000 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	-14.30
95% confidence interval	-27.53 to -1.070
R squared	0.6924
How effective was the pairing?	
Correlation coefficient (r)	0.9379
P Value (one tailed)	P<0.0001
P value summary	***
Was the pairing significantly effective?	Yes

Table A4.62: Student t-test of the susceptibility of HEPG2CR+siRNA vs HEPG2CR to cisplatin-induced death as shown in figure 5.8

Column B	HEPG2CR+siRNA
Vs	Vs
Column D	HEPG2CR
Paired t test	
P value	0.1887
P value summary	Ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.583 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	-15.10
95% confidence interval	-41.59 to 11.39
R squared	0.3851
How effective was the pairing?	
Correlation coefficient (r)	0.8186
P Value (one tailed)	0.0019
P value summary	**
Was the pairing significantly effective?	Yes

Table A4.63: Student t-test of the susceptibility of HEPG2+siRNA vs HEPG2 to 5-FU-induced death as shown in figure 5.9

Column A	HEPG2+siRNA
Vs	Vs
Column C	HEPG2
Paired t test	
P value	0.2484
P value summary	Ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.350 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	-11.40
95% confidence interval	-34.85 to 12.05
R squared	0.3129
How effective was the pairing?	
Correlation coefficient (r)	0.7006
P Value (one tailed)	0.0120
P value summary	*
Was the pairing significantly effective?	Yes

Table A4.63: Student t-test of the susceptibility of HEPG2FR+siRNA vs HEPG2FR to 5-FU-induced death as shown in figure 5.9

Column B	HEPG2FR+siRNA
vs	Vs
Column D	HEPG2FR
Paired t test	
P value	0.0703
P value summary	Ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.452 df=4
Number of pairs	5
How big is the difference?	
Mean of differences	-19.90
95% confidence interval	-42.43 to 2.633
R squared	0.6004
How effective was the pairing?	
Correlation coefficient (r)	0.8615
P Value (one tailed)	0.0007
P value summary	***
Was the pairing significantly effective?	Yes