



**AN INVESTIGATION INTO THE ROLE OF  
TRANSGLUTAMINASE 2 IN THE UPTAKE OF  
CISPLATIN IN PARENTAL AND CHEMORESISTANT  
CANCER CELLS**

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## **Dedications**

I dedicate this thesis to my loving parents, Dhekal and Durga Meshram, whose efforts and love brought me to this stage.

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ANGLIA RUSKIN UNIVERSITY

ABSTRACT

FACULTY OF SCIENCE AND TECHNOLOGY

DOCTOR OF PHILOSOPHY

AN INVESTIGATION INTO THE ROLE OF TRANSGLUTAMINASE 2 IN THE  
UPTAKE OF CISPLATIN IN PARENTAL AND CHEMORESISTANT CANCER  
CELLS

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A major problem in the successful treatment of cancer is a loss in the effectiveness of chemotherapeutic drugs, such as cisplatin, due to development of drug resistance by tumour cells. Such resistance necessitates prescription of higher and potentially toxic concentrations of drugs. The side-effects of such treatments therefore both reduces the quality of patients' life, and also contributes to the failure of effective chemotherapy. At present, the mechanisms by which such drug resistance operates are not fully understood. However, candidate markers for drug resistance are emerging, one of which is transglutaminase 2 (TG2).

TG2 is a ubiquitous, multifunctional enzyme that is implicated in several pathological and physiological processes, including the regulation of cell division and cell death. Two TG2 isoforms, TG2-L and TG2-S, have been characterised. The overexpression of TG2 has been observed in various aspects of cancer, including the development of drug-resistance and cell survival. However, the amount to which TG2 contributes to the cellular environment of normal and drug-resistant cancer cells is poorly understood and is still controversial. In order to establish whether TG2 is directly involved in drug resistance mechanisms, TG2 gene expression at the mRNA level and protein levels was modulated in the hepatocarcinoma (HepG2) cell line, and the effects were measured, using RT-PCR, Western blotting, flow cytometry, confocal microscopy, and a range of assays for determination of TG2-specific enzymatic activity.

This study provides evidence that TG2 expression and its associated transamination activity are inhibited during the initiation of apoptosis by cisplatin, an observation that was reversed by increasing the expression of TG2 following the treatment of cells with retinoic acid. Single-dose treatment of HepG2 cells with IC<sub>80</sub> concentrations of cisplatin generated drug-resistant progeny cells that exhibited reduced TG2 expression but increased enzyme activity, and this induced chemoresistance was accompanied by a reduced ability of cells to take up Alexa fluor 546/488-labelled cisplatin. Interestingly, treatment of cells with a classic TG2 inhibitor (cystamine), or treatment by TG2 mRNA silencing (siRNA), both reversed this effect and increased the level of uptake of fluorescent cisplatin in both parental and chemoresistant cancer cells.

In conclusion, these results indicate that the presence of elevated levels of TG2 in hepatocellular carcinoma prevents and/or limits the entry of anti-cancer drugs. Thus, TG2 may represent a novel biomarker and possibly a therapeutic target for overcoming the chemotherapeutic resistance in hepatocarcinoma.

**Key words:** Cancer, Transglutaminase 2, Drug-resistant, Cisplatin, Cellular uptake

## **Publications**

### **Research articles:**

Dipak D Meshram, Claire V S Pike, and Peter J Coussons. "Inhibition of Transglutaminase 2 activity increases cisplatin cytotoxicity in a model of human hepatocarcinoma chemotherapy". *European Journal of Pharmacology*, 2017. In press.

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## **Declaration**

I declare that this work has not previously been submitted for the acceptance of any other degree or diploma.

## **List of commonly used abbreviations**

AD – Alzheimer's disease

ADP - Adenosine diphosphate

AKAP13 - Protein kinase A (PKA) anchor protein 13

Akt – Protein kinase B

AMP - Adenosine monophosphate

APAF-1 - Apoptotic protease activating factor-1

ATP – Adenosine triphosphate

ATPase - Adenosine triphosphatase

Bcl-2 – B-cell lymphoma 2 family of proteins

Bcr - Breakpoint cluster region protein

BH3 – Bcl-2 homology 3

Ca<sup>2+</sup> – Calcium

CBZ- Benzyloxycarbonyl group

DAPI - 4', 6-diamidino-2-phenylindole

DISC - Death-inducing signalling complex

DLK - Dual leucine zipper-bearing kinase

DNA – Deoxynucleic acid

DNAse- Deoxyribonuclease

DNC- Dansylcadaverine

DTT- Dithreothreitol

ECM – Extracellular membrane

EDTA- Ethylenediamine tetraacetic acid

FADD - Fas-associated death domain

GDP- Guanosine diphosphate

GMP- Guanosine monophosphate

GPCR- G Protein Coupled Receptor

GPR 56 – G protein-coupled receptor 56

GTP – Guanosine triphosphate

GTPase – Guanosine triphosphatase

HD – Huntington's disease

HEPG2- Hepatocellular carcinoma cells



HGFL/MSP - Hepatocyte growth factor-like/macrophage-stimulating protein  
 HPLC- High performance liquid chromatography  
 Htt – Huntingtin protein  
 IBD – Inflammatory bowel disease  
 MES- (2-[N-Morpholino]ethane-sulphonic acid)  
 mRNA – messenger RNA  
 p53 – protein 53  
 PAGE- Polyacrylamide gel electrophoresis  
 PD – Parkinson’s disease  
 PKA – Protein kinase A  
 PLCδ1- Phospholipase Cδ1  
 Rac 1 – Ras-related C3 botulinum toxin substrate 1  
 RBC – Red blood cells  
 RNA – Ribonucleic acid  
 RNase- Ribonuclease  
 RON – Recepteur d’Origine Nantais  
 ROS – Reactive oxygen species  
 SDS - Sodium dodecyl sulphate  
 siRNA – small interfering RNA  
 SP1 – Specificity protein 1  
 STAT3 – signal transducer and activator of transcription 3  
 TBST- Tris-buffered saline with Tween-20  
 TG – Transglutaminase  
 TG2 – Transglutaminase 2  
 TGF – Transforming growth factor  
 TIG3 – Tarazotine induced gene-3  
 TNF – Tumour necrosis factor  
 TRADD - TNF receptor-associated death domain  
 TRIS - Tris(hydroxymethyl)amino-methane

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## **CHAPTER 1:**

### **GENERAL INTRODUCTION**

#### **1.1 Background to the current study**

Despite the known roles of transglutaminase 2 in the development of cancer chemotherapeutic drug resistance, there is still more to discover regarding the implications of transglutaminase 2 in such mechanisms. Therefore, the overall aim of this study is to investigate the role(s) of transglutaminase 2 in the cellular uptake of the anti-cancer drug cisplatin in cisplatin-resistant compared to cisplatin-sensitive immortalised hepatocarcinoma (HepG2) cell lines.

The proper equilibrium between cell death and proliferation is crucial for multicellular organisms to maintain healthy life. Cell death occurs in distinctive ways, i.e. through processes such as apoptosis, autophagy-associated cell death, necrosis, Wallerian degeneration, excitotoxicity, mitotic catastrophe, anoikis, and in the case of skin cornification (Green, 2011; Piacentini, et al., 2011). The end result of these processes is ultimately the removal from tissues of dead cells, usually under normal conditions, through the process of phagocytosis, and in this way the human body remains healthy (Green, 2011). Anything that disturbs these processes can make cells more or less responsive to abnormal conditions.

Apoptosis is well-ordered and is achieved through the stimulation of specific intracellular pathways in response to various intracellular or extracellular death stimuli (Green, 2011). This “programmed cell death” performs many critical roles in physiological homeostasis and many diseases. It has important roles in the control of cancer, one of the deadly diseases of uncontrolled cell growth and proliferation, in which apoptosis is known to be deregulated (Hanahan and Weinberg, 2011). Some related mechanisms may cause cancer cells to become resistant to anti-cancer drugs

that are known to normally initiate apoptosis (Holohan, et al., 2013; Housman, et al., 2014). One important component of the mechanisms that control apoptosis involves extensive intracellular protein cross-linking by transglutaminases (Fesus and Piacentini, 2002). The failure of this and other processes that affect plasma membrane and cytoskeletal structures may create cells that become resistant to chemotherapeutic drugs.

Over the past three decades, the development of drug resistance in patients undergoing chemotherapy has been a major clinical challenge in cancer research. Several proteins have been identified that are involved in the mechanisms of drug resistance, and amongst these, transglutaminase 2 (TG2) has been identified as a putative tumour marker that is involved in multiple mechanisms related to cancer development and drug resistance. These include: gene regulation; tumour evasion of apoptosis; inflammation; and epithelial-mesenchymal transition (Mehta, Kumar and Im Kim, 2010; Budillon, Carbone, and D'Gennaro, 2013).

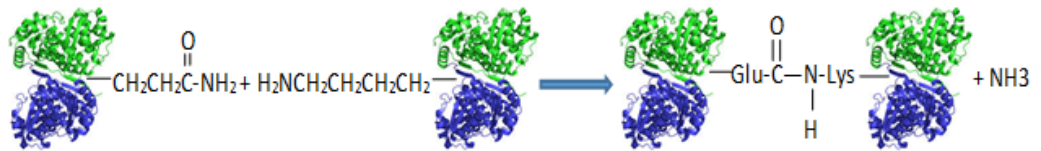
There is limited knowledge regarding the role of TG2 in the cellular uptake/endocytosis of anti-cancer drugs such as cisplatin. Thus, this dissertation is focused on investigating the role of transglutaminase 2 in the cellular uptake of anti-cancer drugs. The project developed drug resistant cells, in which elevated levels of transglutaminase 2 appeared to be associated with a reduced rate of trafficking of cisplatin across the plasma membrane. This study provides the first evidence to support a role for TG2 in the mechanism of cellular uptake of cisplatin. It also reports for the first time that cisplatin can cause direct inhibition of guinea pig liver transglutaminase activity and that TG2 transamination activity plays an important role in the mechanism of cisplatin induced cell death. It is thus hypothesised that cisplatin may act as an inhibitor of TG2 *in vivo*, and in this way may contribute to the induction of apoptosis in cancer cells.

## **1.2 General background to transglutaminase research**

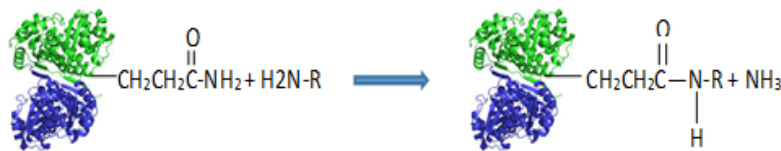
Transglutaminase 2 was first isolated in 1957, from guinea pig liver extract, and characterised as a transamidating enzyme (Clarke, Sarkar and Waelsch, 1957). Following this discovery, it has been subsequently shown that transglutaminases are not only present in mammals, but are present in many other diverse living organisms, e.g., unicellular organisms, invertebrates, plants, amphibians, fish and birds (Grenard, Bates and Aeschlimann, 2001).

The transglutaminases (TGases; EC 2.3.2.13) are a peculiar family of structurally- and functionally-related enzymes that catalyse the intracellular and extracellular post-translational modification of proteins by forming  $\gamma$ -glutamyl- $\epsilon$ -lysyl covalent bonds between free amine groups of protein- or peptide-bound lysine and  $\gamma$ -carboxamide groups of other protein- or peptide-bound glutamines, in a calcium-dependent manner (Mehta and Eckert, 2005; Nurminskaya and Belkin, 2012). TGases also catalyze the covalent incorporation of mono- or polyamines into proteins by deamidation and amine exchange with glutamyl residues of proteins. In the absence of amine substrates, hydrolysis of glutamine results in the elimination of the amino group of glutamyl residues by deamidation, leaving behind a glutamic acid residue (figure 1.1) (Ichikawa, et al., 2004; Nurminskaya and Belkin, 2012). These enzymes are also capable of acting in reverse, and in this way can cleave pre-existing isopeptide bonds through isopeptidase activity, again in a calcium-dependent manner (Parameswaran, et al., 1997). However, in the absence of isopeptide cleavage, cross-links formed by transglutaminases are highly resistant to mechanical and chemical attack and normally can be only destroyed by proteolytic degradation of protein chains (Fesus and Piacentini, 2002). In summary, these enzymes appear to have multiple and important roles, as transglutaminase-mediated modification can change the function, stability, and immunogenicity of substrate proteins and so contributes to different diseases, including importantly with respect to this dissertation, cancer.

1) Protein-protein cross-linking



2) Protein aminylation



3) Protein Deamidation



**Figure 1.1 Transglutaminase catalysed reactions in the presence of calcium.**

(1) Protein cross-linking comes through the formation of  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isopeptide bond between the lysine donor residue of one protein and the acceptor glutamine of another protein, (2) Transamination of amine into one of the active glutamine residue of the acceptor protein, (3) Deamidation of glutamine leaving behind glutamic acid (self generated diagram).

### 1.3 The transglutaminase family of gene products

Transglutaminases are broadly distributed in mammalian tissues and fluids and are categorised into at least five groups, centred on their biochemical properties. Some of the family's genes are specifically expressed in single tissues, e.g., plasma; keratinocytes; prostate; and epidermis (Ichikawa, et al., 2004). In humans, nine transglutaminase genes have been found so far, and it appears likely that these enzymes are evolved from a series of single gene duplications (Eckert, et al., 2014). Eight members are catalytically active and all share an identical amino acid sequence at the active site. The family exception is Band 4.2 (an inactive erythrocyte membrane protein). Despite the presence of potential N-linked glycosylation sites and cysteine residues in these proteins, the members of the transglutaminase family normally lack glycosylation and disulphide bonds (Mehta and Eckert, 2005). All TGs, including those that are secreted (such as factor XIII), and those associated with membrane



(e.g. TG1) lack N-terminal hydrophobic sequences. Similarly, all members except the catalytically-inactive Band 4.2 require calcium for their catalytic activity (Eckert, et al., 2005; 2014).

### **1.3.1 Transglutaminase 1**

Transglutaminase 1 (TG1) is sometimes colloquially termed as 'keratinocyte TGase'. Other names are 'TGk' and 'particulate TG'. It is mainly expressed in the stratified squamous epithelial part of the skin, the upper digestive tract and in the lower female genital tract (Eckert, et al., 2014). The protein is membrane-bound and has a fatty acyl linkage in the NH<sub>2</sub>-terminal cysteine residue (Kim, Chung and Steinert, 1995; Klock and Khosla, 2012). In addition to responding to increased Ca<sup>2+</sup> levels and proteolytic cleavage, interaction with tarazotene-induced gene 3 (TIG3) protein seems to regulate its catalytic activity (Eckert, et al., 2009). In addition, retinoic acid reduces expression, whereas phorbol ester induces TG1 mRNA and protein expression (Eckert, et al., 2005).

The main function of TG1 in skin is cell envelope formation during keratinocyte differentiation (Mehta and Eckert, 2005). One study found that TG1 can covalently link synthetic analogue  $\omega$ -hydroxyceramides to one of the glutaminyl residues of human involucrin by ester bond formation (Nemes, et al., 1999). It was also found that TG1 is involved in renal epithelial cell proliferation via activation of the signal transducer and activator of transcription 3 (STAT3) and protein kinase B (Akt) signalling pathway (Ponnusamy, et al., 2009; Zhang, et al., 2009). Mutations in the TG1-encoding gene leads to the development of lamellar ichthyosis, an autosomal recessive hereditary disorder of cornification (Terrinoni, et al., 2012).

Little is known about TG1 in apoptosis, but unpublished data from this study suggests that TG1 mRNA expression levels are decreased during cisplatin-induced apoptosis (Meshram, 2017).

### 1.3.2 Transglutaminase 2

Transglutaminase 2 (TG2 or “tissue-transglutaminase”) is usually characterised as a multifunctional protein, as in contrast with the other transglutaminases, which appear to have narrower, more tissue-specific functions. It can be involved in diverse events in the living or dying cell (Fesus and Piacentini, 2002). It is also often termed ‘liver transglutaminase’, due to its abundance in hepatic tissue, and is one of the most extensively studied tissue-type transglutaminases, although its physiological role is presently not clearly understood (Mehta and Eckert, 2005). TG2, also referred to as TGc or Gh, is a 78 kDa enzyme, widely distributed in tissues and cell types (Fesus and Piacentini, 2002). It has recently been identified as having four differentially-spliced isoforms in addition to the full length protein, including the short form, TG2-S or TGH, and TGH2 (Antonyak, et al., 2006; Tee, et al., 2010). In comparison to the full length TG2, TG2-S (or TGH) lacks the C-terminal 139 amino acids (amino acids 548-687 are missing) and has altered terminal residues 539-548, whereas TGH2 lacks the C-terminal 337 amino acids (amino acids 350-687 are missing) and has altered terminal residues 287-349. The other two variants, TG2v<sub>1</sub> and TG2v<sub>2</sub>, have 54 and 25 amino acids in the C-terminal divergent form of the full length protein respectively (Lai, et al., 2007; Klock and Khosla, 2012; Kojima, Kuo and Tatsukawa, 2012).

Transglutaminase 2 (TG2) is a unique member of the transglutaminase family of enzymes. Apart from its cross-linking role, TG2 is a GTPase and ATPase - capable of hydrolysis of GTP and ATP respectively - in a calcium-independent manner. It also acts as a G (Gh $\alpha$ ) protein for G Protein Coupled Receptor (GPCR) signalling (Achyuthan and Greenberg, 1987; Singh, Erickson and Cerione, 1995; Chen, et al., 1996; Iismaa, et al., 1997; Baek, et al., 2001; Lorand and Graham, 2003).

In the filarial parasite *dirofilaria immitis*, Chandrashekhar, et al., (1998) revealed the protein disulphide isomerase (PDI) activity of TG2. PDI activity catalyses disulphide bond formation in proteins via cysteine residues.

A recent study by Mishra *et al* showed that TG2 has an intrinsic kinase activity, and is capable of phosphorylating proteins. Later, it was reported that TG2 kinase activity has roles in the nucleus, where it contributes to apoptosis by phosphorylating p53 and retinoblastoma proteins. It also helps in regulating chromatin structure and functions by modifying histones (Mishra and Murphy, 2004; 2006; Mishra, et al., 2006; Mishra, Melino and Murphy, 2007).

Transglutaminase 2 has non-enzymatic activities e.g. TG2 works as an adaptor protein during cell signalling. In the nucleus, TG2 interacts with  $\alpha$ 3-importin, whereas in the cytoplasm TG2 interacts with Phospholipase C $\delta$ 1 (PLC $\delta$ 1), protein kinase A (PKA) anchor protein 13 (AKAP13), 14-3-3 proteins, breakpoint cluster region protein (Bcr), and ras-related C3 botulinum substrate 1 (Rac1). In the extracellular membrane (ECM) TG2 interacts with fibronectin, and angiocidin and endostatin, and on the cell surface TG2 not only interacts with transmembrane receptors, like integrins, GPCR, G protein-coupled receptor 56 (GPR56), platelet-derived growth factor receptor (PDGFR), syndecan-4, low density lipoprotein receptor-related proteins (LRP)-1 and 5/6, but also directly binds to matrix metalloproteinase 2 (MMP2). TG2 also directly interacts with milk fat globulin EGF factor 8 (MFG-E8) protein during bridge formation between apoptotic target cells and macrophage  $\beta$ 3 integrins (see review of Nurminskaya and Belkin, 2012). It seems that TG2 contributes in many biological processes, much more than the other transglutaminases and is the focus of this study.

### **1.3.3 Transglutaminase 3**

Transglutaminase 3 (TG3) is a 77 kDa enzyme, located on 20q11-12 chromosome, and is also known as epidermal transglutaminase. It is mostly present in the epidermis, hair follicle and brain. Other names are 'TGE', 'callus TG', 'Hair follicle TG'

and 'bovine snout TG'. Increased  $\text{Ca}^{2+}$  levels regulate its catalytic activity, and TG3 mRNA expression (Klock and Khosla, 2012; Eckert, et al., 2014), and like TG2, TG3 also has the potential to hydrolyse GTP. TG3 cross-links trichohyalin and keratin intermediate filaments to harden the inner sheath of hair, which is needed for hair fibre morphogenesis. The main function of TG3 in the human body is to form the cell envelope during the latter stages of differentiation in the epidermis and hair follicle, in order to protect tissues from dehydration, abrasion and infection. However, the exact mechanism for cell envelope formation is not clearly understood (Mehta and Eckert, 2005; Eckert, et al., 2009; 2014).

A recent study in male "*Anopheles gambiae*" mosquitoes by Le *et al.*, showed that TG3 contributes to the coagulation of seminal fluids by cross-linking "Plugin" protein (Le, et al., 2013). Dermatitis herpetiformis, a skin disease, is caused by TG3 autoantibodies (Sardy, et al., 2002). TG3 expression is found to be down-regulated in head and neck squamous cell carcinoma and in laryngeal carcinoma (Mehta and Eckert, 2005).

#### **1.3.4 Transglutaminase 4**

Transglutaminase 4 (Tgase-4, TG4), is also known as 'prostate transglutaminase', because of its unique expression in the prostate gland. It is also found in prostatic fluid and seminal plasma. Recently, its presence was found in human saliva, and in the vena cava and aortic smooth muscle of mice (Johnson, et al., 2012; Perez, et al., 2013; Eckert, et al., 2014). The TG4 variant has been found in four different isoforms: TG4-L; -M (-M1 and -M2); and -S, in benign prostate hyperplasia and prostate cancer (Cho, 2010a; 2010b). In combination, retinoic acid and androgen treatment regulate the transcriptional expression of TG4 in human prostate cancer cells (Fujimoto, et al., 2009; Rivera-Gonzalez, et al., 2012).

The main role of TG4 in rodent biology is the copulatory plug formation in the female genital tract, and in suppression of sperm antigenicity of male rodents. The TGM4

knockout mouse study shows that presence of TGM4 is necessary for normal male fertility (Cho, 2010b; Dean, 2013). Some evidence is available which suggests that TG4-expression is intrinsically linked to the activation of hepatocyte growth factor-like/macrophage-stimulating protein (HGF-L/MSP) receptor [also known as RON (Recepteur d'Origine Nantais)], which contributes to the increased cell motility in TG4-expressing cells (Jiang, et al., 2010). The enzyme is also involved in cell-matrix adhesion, epithelial-mesenchymal transition and invasiveness in prostate cancer cells (Davies, et al., 2007; Jiang, et al., 2009; 2013; Cao, et al., 2013).

### **1.3.5 Transglutaminase 5**

Transglutaminase5 (TG5) is mainly expressed in foreskin keratinocytes, the epithelial barrier lining, and skeletal muscle cells. This variant has been shown to exist in three alternatively spliced isoforms: delta-3; delta-11; and delta3delta11 (Candi, et al., 2001). Acetylation of TG5 occurs at the N-terminal end (Candi, et al., 2004). Phorbol ester treatment activates endogeneous TG5 and causes it to localise with vimentin intermediate filaments (Rufini, et al., 2004). GTP and ATP both inhibit TG5, whereas calcium activates its protein cross-linking activity. Full length Transglutaminase 5 specifically cross-links loricrin, involucrin, and small proline-rich proteins in the epidermis (Candi, et al., 2001). Apart from the cornified cell envelope formation of keratinocytes, TG5 overexpression has been shown to be involved in Hek293 cell death (Cadot, et al., 2004). Missense mutations in TGM5 gene, at T109M and G113C, lead to the formation of the acral peeling skin syndrome (Cassidy, et al., 2005).

### **1.3.6 Transglutaminase 6**

Transglutaminase 6 (TG6) is mostly expressed in testis and lung and to some extent in the brain of mice and is also called transglutaminase Y, TGy. It is also expressed in a human carcinoma cell lines with neuronal characteristics, where it is catalytically activated after proteolytic cleavage of the proenzyme (Eckert et al., 2014). Like TG3 the main function of TG6 appears to be in cell envelope formation in the epidermis

and the hair follicle. Recently, TG6 antibodies have been found in the autoimmune disease familial-ataxia (Thomas, et al., 2013).

### **1.3.7 Transglutaminase 7**

Little research has been carried out on Transglutaminase 7 (TG7), but it appears that it is found in the testes, lungs, and brain (Mehta and Eckert, 2005). In breast cancer patients, normally low TG7 mRNA expression level was seen to be increased with severity of disease suggesting a relationship with node involvement and patient outcome (Jiang, et al., 2003).

### **1.3.8 Factor XIIIa**

Factor XIIIa is an important component of the blood coagulation system, found in blood plasma. It is also found in astrocytes, platelets, macrophages, the placenta, synovial fluid, the eyes, the heart, chondrocytes, dermal dendritic cells, and the osteoblast lineage. It is activated from a zymogen form, as the A subunit of a heterotetramer. Transforming growth factor- $\beta$ , interleukin-1 $\beta$  and myeloid-enriched factors (GATA-1 and Ets-1) have all been shown to induce factor XIIIa gene expression (Johnson, et al., 2001; Klock and Khosla, 2012; Eckert, et al., 2014).

The primary role of factor XIIIa catalytic activity is stabilisation of fibrin clots during blood coagulation, through cross-linking of proteins and formation of cross-links between fibrin monomers, in the presence of calcium (Nikolajsen, et al., 2014). Tridegin, on the other hand, inhibits factor XIIIa activity (Finney, et al., 1997).

Factor XIIIa, in addition to TG2, is involved in chondrocyte hypertrophic differentiation, in a manner that is not reliant upon transglutaminase catalytic activity (Johnson, Rose and Terkeltaub, 2008). It is also involved in autocatalytic cell death, inflammation, matrix secretion and deposition of proteins in osteoblasts, bone synthesis, hypertension, vascular permeability and angiogenesis. Mutations in Factor XIIIa or

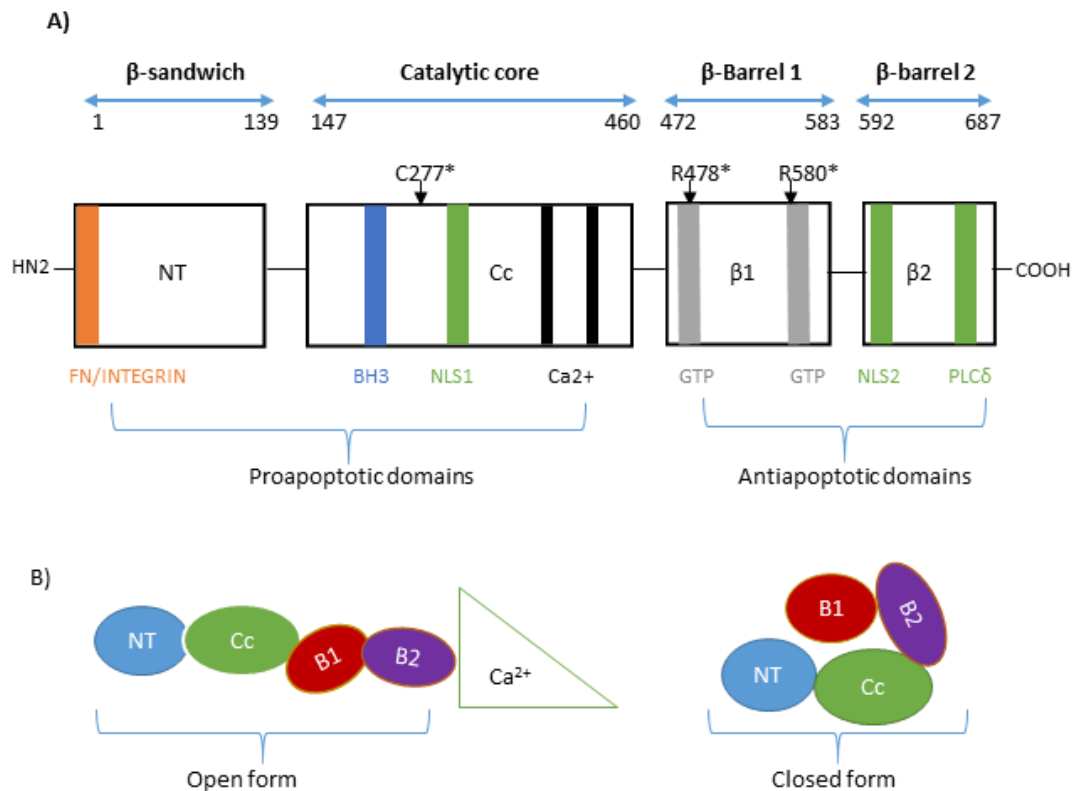
deficiency cause impaired wound healing and bleeding problems (Nurminskaya, et al., 1998; Sane, Kontos and Greenberg, 2007; Jallad, et al., 2011).

### **1.3.9 Erythrocyte membrane protein band 4.2 (Band 4.2)**

Band 4.2, also known as 'ATP-binding erythrocyte membrane protein', is predominantly expressed on the membrane of erythrocytes, foetal liver, bone marrow and spleen. It is catalytically inactive compared to other members of Tgase, because of a Cys → Ala substitution of the catalytically active cysteine in the active site (Mehta and Eckert, 2005; Eckert, et al., 2014). Exon I is known to be alternatively spliced into two isoforms. It is essential for correct assembly of the red blood cell (RBC) membrane skeleton and in maintaining the stability and integrity of RBC by directly interacting with spectrin. Band 4.2 binds to the erythrocyte anion channel and acts as support: as an attachment point between the membrane skeleton and the lipid bilayer. Deficiency of protein band 4.2 in RBCs cause anemia, e.g., a study on Japanese recessive spherocytic elliptocytosis patients suggested that protein band 4.2 expression was completely absent in these patients (Korsgren, et al., 1990; Sung, et al., 1990; 1991; Mandal, Moitra and Basu, 2002), resulting in anemic pathology.

## **1.4 Structural and functional relationship in TG2**

Implications for the multifunctional roles of TG2 can be found by consideration of its primary, secondary and tertiary structure. Structurally, TG2 contains four domains: an NH<sub>2</sub>-terminal  $\beta$ -sandwich; a catalytic core; and two COOH-terminal  $\beta$ -barrels (figure 1.2A) and each domain has a specific role.



### Figure 1.2 Primary structure of TG2

(A) Structural representation of TG2 domains, (B) representation of TG2 domains in open and closed form. Calcium binding opens TG2 domains that makes TG2 catalytically active whereas GTP binding closes and makes TG2 catalytically inactive. NT = amine terminal  $\beta$ -sandwich; Cc = catalytic core;  $\beta 1$  =  $\beta$ -barrel 1;  $\beta 2$  =  $\beta$ -barrel 2 (diagram adapted from Piacentini, et al., 2011).

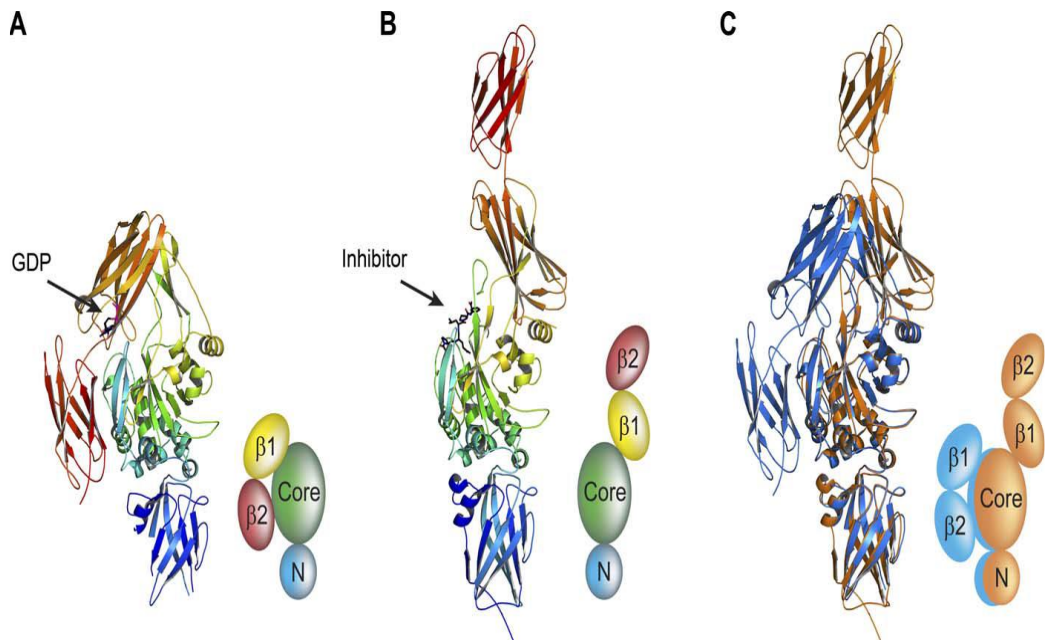
The catalytic core of TG2 contains cysteine proteases (Cys-277, His-335, and Asp-358), and is essential for transglutaminases' catalytic activity, with a binding site for calcium, and two conserved tryptophan residues (W241 and W332), which are necessary for TG2 activity, as these residues stabilize the thiol-intermediates formed during catalysis. The X-ray crystal structure of human TG2 has been solved, at 2 Å resolution, by Pinkas and group (2007) (figure 1.3). In the presence of calcium, TG2 opens its conformation, whereas in the presence of high ratios of GDP/GTP, it closes its catalytic site (figure 1.2B) (Pinkas, et al., 2007). The catalytic core possesses a BH3 (Bcl-2 homology 3) binding domain, which has binding site for the Bcl-2 protein family, involved in cell cycle regulation. The catalytic domain also contains a binding



site for nuclear localisation signals (NLS1), which contribute to its localisation during apoptotic death of cells by cross-linking cellular proteins (Peng, et al., 1999; Liu, Cerione and Clardy, 2002; Murthy, et al., 2002; Rodolfo, et al., 2004; Gundemir, et al., 2012). The binding of cofactors plays crucial roles for TG2, as this determines whether TG2 plays enzymatic or non-enzymatic roles, and like calcium binding, opens the conformation and allows TG2 to perform its catalytic function. On the other hand, binding of GTP/GDP closes TG2 conformation, which allows TG2 to participate in cell signalling pathways (Piacentini, et al., 2011).

The first C-terminal  $\beta$ -barrel contains binding sites for GTP and GDP, and is only found in TG2 and TG3, whereas other members of TG family lack GTP/GDP binding site(s). These two guanine nucleotide cofactors modulate transamidase activity. The second C-terminal  $\beta$ -barrel contains a binding sites for phospholipase C (PLC $\delta$ ), which allows TG2 to function in the pro-inflammatory pathway, and a second binding site for nuclear localization signal (NLS2) (figure 1.2A).

A NH<sub>2</sub>-terminal  $\beta$ -sandwich domain contains a fibronectin/integrin binding site, which is crucial during cell adhesion and extracellular matrix organisation (figure 1.2A). A recent study has shown that TG2 also has a binding site for heparin, which influences the adhesive function of TG2 (Jacob, et al., 2012).



**Figure 1.3 Conformational changes in the structure of transglutaminase 2.** (A) GDP/GTP bound TG2, which represents the closed form (catalytically inactive), (B) Inhibitor bound TG2, which represents the open form but catalytically inactive as the inhibitor is bound to catalytic site, (C) Combination of both (Pinkas, et al., 2007).

### 1.5 Transglutaminase 2 and its substrates

Transglutaminase 2 (TG2) post-translationally modifies proteins; these modified proteins may change their functions and behave in a different manner from their unmodified states. TG2 modifies proteins only if they have specific substrate characteristics around a particular reactive glutamine residue in the primary amino acid sequence; the reactive glutamyl- and lysyl- residues must be accessible and should not be surrounded by certain residues, which are considered to be discouraging factors (Table 1.1) (Coussons, et al., 1992a; Csosz, et al., 2008). However, despite tremendous efforts, the exact molecular selectivity of TG2 towards its substrate is still not fully characterised.

**Table 1.1 Charged side chains that can act as a discouraging factor in the transglutaminase-catalysed reaction.**

The presence of an asterisk (\*) at a position relative to the glutamine (at position 0) indicates that the charged side chain can act as a discouraging feature. Positive charges (Lys/Arg) and Negative charges (Asp/Glu) (adapted from Coussons, et al. 1992a).

| Position          | -5 | -4 | -3 | -2 | -1 | 0 | +1 | +2 | +3 | +4 | +5 |
|-------------------|----|----|----|----|----|---|----|----|----|----|----|
| Positive charges  |    |    |    |    |    |   |    |    |    |    |    |
| Type A substrates | *  |    |    |    |    | Q | *  | *  | *  | *  | *  |
| Type B substrates |    |    |    |    |    | Q | *  |    |    | *  |    |
| Negative charges  |    |    |    |    |    |   |    |    |    |    |    |
| Type A substrates |    | *  |    |    |    | Q |    |    |    | *  | *  |
| Type B substrates |    |    |    | *  |    | Q |    |    |    |    |    |

Many researchers have attempted to characterise TG2 substrates on the basis of amino acid sequences around the glutamine residue in the primary structure of protein substrates and it has been suggested that substrates may be divided broadly into two types (type A – which possess little ordered structure, and type B – which possess ordered structure, around the glutamine residue) (Coussons, et al., 1992a; Csosz, et al., 2008). As a result of such research, the number of potential substrate proteins for TG2 was recently reported to be 155 (see appendix II). The full database of TG2 substrates can be found in the “Transdab wiki database” (Csosz, Mesko and Fesus, 2009).

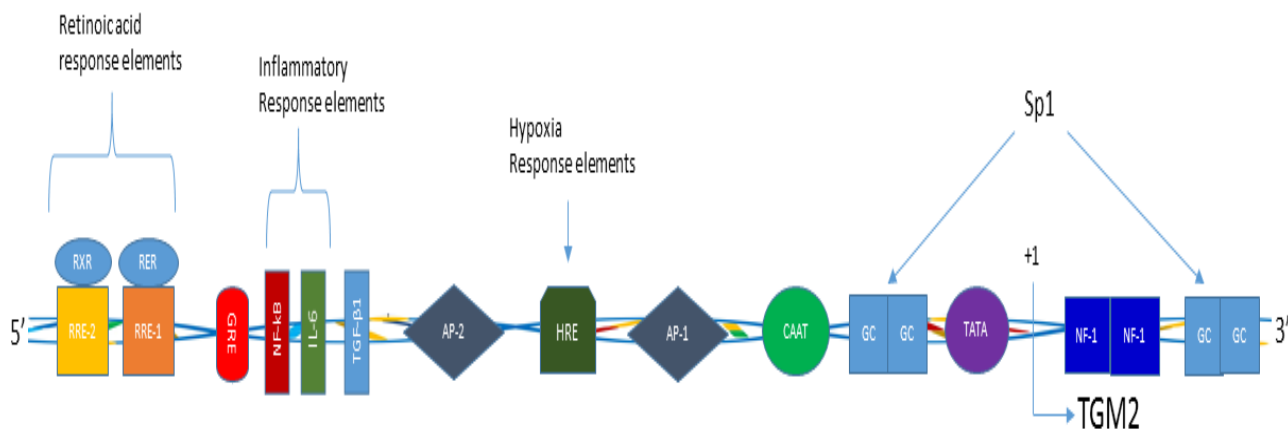
Many proteins are substrates of TG2, e.g., Bcr, Rac1, fibronectin, and angiocidin, where they are enzymatically modified by TG2 (transamidation/cross-linking activity), whereas other proteins that bind noncovalently to TG2 are modulated but not enzymatically modified by TG2, e.g., integrins, PDGFR, MMP2 (Nurminskaya and Belkin, 2012).

## 1.6 The distribution and functions of TG2 in different cell compartments

TG2 was initially thought to only act as a cytoplasmic protein. Later, it was found that TG2 is localised extracellularly, as well as intracellularly. Its occurrence is now known to be spread between the cell surface, plasma membrane, cytosol, nucleus, mitochondria, endolysosomes, and in the extracellular matrix (ECM) (Lorand and Graham, 2003; Zemskov, et al., 2006; Malorni, et al., 2008; Gundemir and Johnson, 2009a; Park, Choi and Ha, 2010; Nurminskaya and Belkin, 2012).

## 1.7 Regulation of TG2 expression and activation

TG2 expression and activation is dependent on cellular physiological conditions. As a multifunctional protein that is present ubiquitously in the extracellular, as well as the intracellular, environment, TG2 expression and activation must be tightly regulated (Gundemir, et al., 2012). Under some physiological conditions, TG2 activation is necessary, whereas in other conditions deactivation is crucial.



**Figure 1.4 Activation and regulation of TG2 gene expression (TGM2) by various factors at different elements**

Regulatory elements of human TG2 gene expression. Elements identified so far that activates TG2 are shown in DNA at various positions. Retinoic acid response elements: RRE-2 (-1731), RRE-1 (-1720), glucocorticoid response element: GRE (-1399), Nuclear factor κB response element: NF-κB (-1338), Interleukin-6 response element: IL-6 (-1190), Tumour growth factor-β1: TGF-β1 (-900), activator protein-2: AP-2 (-634), Hypoxia response element: HRE (-367), activator protein-1: AP-1 (-183), CAAT box (-96), GC box: Sp1 binding motifs (-54, -43, +59, +65), TATA box (-29), Nuclear factor-1: NF-1 (+4, +12). (diagram adapted from Gundemir et al., 2012).

TGM2 gene expression is controlled at the level of transcription, the gene having four Sp1 binding sites that are responsible for human promoter activation (Lu, et al., 1995; Griffin, Casadio and Bergamini, 2002). As it is a multifunctional protein, the TG2 promoter is very sensitive to disturbance in the cell environment and activates very quickly upon binding of various activators (figure 1.4).

One of the best known activator types are retinoids (i.e., retinoic acid and its derivatives). In the mouse, retinoid-dependent TG2 gene expression is mediated through a versatile tripartite retinoid response element, which is located in the promoter, 1.7 kb upstream from the transcription start site. The same position is responsible for human TG2 gene activation (Nagy, et al., 1996). Other activators are transforming growth factor-beta1 (TGF- $\beta$ 1), nuclear factor-kappaB (NF-kappaB), and hypoxia-inducible factor-1 (HIF-1) (Mirza, et al., 1997; Ritter and Davies, 1998; Jang, et al., 2010). Tumour necrosis factor and inflammatory cytokines, such as interleukin-1 $\beta$  and interleukin-6, can also activate TG2 (Suto, Ikura and Sasaki, 1993; Chen, et al., 2000). Recently, it was found that interferon- $\gamma$  (IFN- $\gamma$ ) activates extracellular TG2 in the coeliac intestine via a phosphatidylinositol-3-kinase dependent pathway (DiRaimondo, Klock and Khosla, 2012). Another study showed that bone morphogenic proteins (BMP2 and BMP4) inhibit tissue transglutaminase gene expression in MC3T3 E1 cells (Ritter and Davies, 1998).

## **1.8 Cellular and physiological functions of transglutaminase 2 (TG2)**

To elucidate the physiological roles of transglutaminase at the molecular level, it is clearly important to identify its physiological protein substrates and to clarify the relationship between transglutaminase modification of *in vivo* protein substrates and biological responses (Ichikawa, et al., 2004). Several transglutaminase substrates are involved in normal cellular functions, such as cell motility, extracellular matrix formation, cell adhesion, cell cycle, apoptosis, metabolism, cytoskeleton, signal

transduction, endocytosis, exocytosis, adhesion and migration, cell differentiation, cell growth and proliferation, and autophagy. On the other hand, these modified proteins may lead to abnormal activity and diseases (including coeliac sprue, neurodegenerative diseases, autoimmune diseases, cancer, metabolic and endocrinologic diseases) (Esposito and Caputo, 2004; Facchiano, Facchiano and Facchiano, 2006; Csosz, et al., 2008; Facchiano and Facchiano, 2009). TG2's roles in these processes are described below.

### **1.8.1 Role of TG2 in endocytosis (cellular uptake).**

Entry of many molecules into cells is often not passive, but can be dependent on specific mechanisms of membrane-associated cellular transport, including receptor mediated endocytosis (RME). Endocytosis can control an array of activities, including apoptotic clearance of dead cells, cell signalling, cell-cell interaction and antigen presentation, neuronal transmission and most importantly nutrient uptake (Mellman, 1996). During endocytosis, several proteins are expressed on the surface of cell membranes, which assist in the process of membrane-transport; one such candidate protein is TG2. Initial recognition of TG2 as endocytic protein was made by Davies in 1980 and subsequently, several lines of evidence have been reported to support a role for TG2 involvement in RME of  $\alpha$ 2-microglobulin, polypeptide hormones, and interleukin-8, in mouse peritoneal macrophages, and human erythrocytes (Davies, et al., 1980; Schrier and Junga, 1981; Ray and Samantha, 1996; Abe, et al., 2000).

TG2 works as an ATPase, and this activity may have a role in transporting molecules across the plasma membrane. Some alterations in RME or other functions of TG2-ATPase may also have implications for the reduced uptake of anticancer drugs (e.g. cisplatin) by chemoresistant cancer cells. However, more research is required to find out the link between TG2 and drug transporter proteins, where TG2 may act as an activator or deactivator of drug transporters, or may itself act as a drug transporter, or as a direct inhibitor of transport processes.

### **1.8.2 Role of TG2 in cell adhesion and migration**

TG2 works in collaboration with at least two classes of transmembrane cell-ECM adhesion receptor proteins, i.e.,  $\beta 1/ \beta 3/ \beta 5$  integrins and syndecan-4, where its activity modulates cell adhesion via non-covalent binding (Verma and Mehta, 2007). Subsequently, these proteins interact with fibronectin, collagen, and vitronectin, thus strengthening cells' adhesive properties and thus reducing the probability of metastasis in cancer (Nurminskaya and Belkin, 2012).

### **1.8.3 Role of TG2 in cell cycle and proliferation**

The GTPase or ATPase activity of TG2 is one of the requirements for cell cycle progression through S phase to G2/M phase, and overexpression of TG2 has been shown to affect this progression (Nurminskaya and Belkin, 2012). A recent study by the Nadalutti group has shown that intracellular TG2 is required for cell proliferation. They inhibited TG2 gene expression by siRNA and observed that the endothelial cell number was reduced after the arresting cell cycle at G1 phase (Nadalutti, et al., 2011).

TG2's G-protein activity is found to mediate signalling by  $\alpha$ -1 $\beta$ -adrenergic receptor, initiating Fas-induced hepatocyte proliferation in mice (Sarang, et al., 2005).

### **1.8.4 Role of TG2 in autophagy**

Autophagy is a process of degradation of cytoplasmic components (including dead cells) within lysosomes, which is different from the endocytosis-mediated degradation process (Mizushima, 2007). TG2 inhibits autophagy induction by cross-linking beclin 1, one of the key proteins during autophagosome formation (Luciani, et al., 2010; Grosso and Mouradian, 2012). Impaired autophagy promotes pathological conditions, one of which has been suggested to be Alzheimer's disease (Salminen, et al., 2013).

## **1.8.5 Role of transglutaminase 2 in apoptosis and cell survival**

### **1.8.5.1 TG2 and apoptosis**

Apoptosis (programmed cell death) is one of the most important processes in multi-cellular organisms, and is essential in the development and maintenance of the proper equilibrium of cell differentiation and cell death. Apoptosis occurs through the activation of several specific intracellular as well as extracellular pathways, when the cells encounter death stimuli (Green, 2011).

Two main pathways have been characterised for the induction of apoptosis: (1) The external pathway, in which the cell receives and disseminates the signal coming from the external environment, by activating receptor-specific membrane proteins from the tumour necrosis factor (TNF) receptor family (TNFR), which work as “death receptors”. The TNF ligand binds to the receptor TNF-R1, which activates conformational changes in the receptor. TNF then induces the activation of modulator proteins, Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD), which form death-inducing signalling complex (DISC). The DISC complexes then activate caspase proteins, which ultimately execute the apoptotic process (Wajant, 2002). (2) The second main apoptosis pathway is the internal pathway, in which cells receive and disseminate the signal from the internal cellular environment. The most common signals from inside the cells are increased levels of reactive oxygen species (ROS), DNA damage, default protein response, and deprivation of growth factors. Once a cell suffers from these effectors, the mitochondria of the cell become perturbed, causing the release of pro-apoptotic proteins to the cytosol (Green, 2011). The release of pro-apoptotic factors and of antagonists of cytosolic anti-apoptotic proteins causes activation of Apoptotic protease activating factor-1 (APAF-1) complex, which ultimately activates caspase proteins. Caspase proteins activate a series of further caspases, finally disassembling the cell by proteolytic action, and by activating various apoptotic protein substrates,



including DNases (Green, 2011). A specific endonuclease then cleaves DNA into non-repairable fragments, and the cell is shrunk into cross-linked apoptotic body, which is ultimately engulfed and removed without the generation of an inflammatory response, by phagocytosis (Green, 2011).

An early evidence of TG2 activation occurs following cellular depletion of the enzyme glutathione S-transferase (GST), during the early phases of apoptosis. This depletion occurs because of the functional inactivation of GST P1-1 by TG2-dependent polymerisation (Piredda, et al., 1999). For induction of apoptosis, the intracellular localisation and available transamidating activity of TG2 is clearly crucial (Piacentini, et al., 2011).

The first direct evidence that the cytosolic form of TG2 acts as proapoptotic factor was achieved by transfecting cells with cDNAs coding for mutant (inactive), or wild type TG2, the protein products of which were targeted to different compartments of cells. The inactivation of cross-linking activity of TG2 in the nucleus was shown to lead to a reduction in apoptosis, indicating the importance of TG2 effects on cell death (Piacentini, et al., 2011).

Further proof of the involvement of TG2 in apoptosis derives from experiments that showed that dual leucine zipper-bearing kinase (DLK) was oligomerised by TG2, when cells were treated with the apoptotic stimulus calphostin- C (Hebert, et al., 2000). Interestingly, the oligomerisation of DLK appears in early phases of apoptosis. The proapoptotic nature of TG2 was confirmed by conducting the studies on wild type DLK, where oligomerised DLK sensitizes cells to calphostin- C -induced apoptosis, but cross-linked DLK does not (Hebert, et al., 2000). A recent *in vivo* study showed that TG2 plays a role in alcohol-induced hepatocyte apoptosis by cross-linking nuclear specificity protein 1 (SP1)-transcription factor. Cross-linked SP1 inactivation results in a reduction of c-Met expression, which is necessary for hepatocyte cell

survival (Tatsukawa, et al., 2011; Kojima, Kuo and Tatsukawa, 2012). In Jurkat T cells, TG2 helps in apoptosis induction by modifying calcium homeostasis, via cross-linking RAP1, a GTP-GDP dissociation stimulator 1 (Hsieh, et al., 2013).

#### **1.8.5.2 Anti-apoptotic roles of TG2**

Recent evidence suggests that TG2 plays a dual role in transformed cells, not only acting as proapoptotic factor but also as an antiapoptotic factor. It has been suggested that TG2 works as a switch between pro- and anti-apoptotic functions, depending on the environment as well as the genetic background of the cells (Piacentini, et al., 2011). The first evidence of TG2 acting as prosurvival factor came from the study of Oliverio, et al., (1997), where it was found that the modification of Retinoblastoma (Rb) protein by TG2 is necessary for cell survival (Oliverio, et al., 1997). Pre-treatment of cells undergoing apoptosis with retinoic acid (RA) showed the blocking of degradation of Rb by the action of caspases, which are important inducers of TG2 during transcription (Oliverio, et al., 1999). It has also been shown that translocation of TG2 to the nucleus in some cases may antagonise apoptosis, though this phenomenon is at present controversial (Piacentini, et al., 2015).

In TG2-expressing cells, phosphoinositide 3-kinase (PI3K) regulates the transamidating and GTP-binding activity, by inhibiting the cross-linking activity of TG2 (Lin, et al., 2011). TG2 inhibits apoptosis in several tumour cell lines, by modifying caspase-3 and Nuclear Factor-kappa B (NF-kB) activity (Cao, et al., 2008; Jang, et al., 2010; Kumar and Mehta, 2012). TG2 protects HEK293 cells from calcium-overloaded apoptosis, by reducing Bax protein levels, (one of the Bcl2 family protein involved in apoptotic induction) (Cho, et al., 2010). It also acts as a cytoprotector to H9c2 cells, in which it was overexpressed during protein kinase A- and protein kinase C-dependent cell survival pathways (Almami, et al., 2014). This suggests that TG2's dual roles may be regulated by conformational changes in the TG2 protein structure, where GTP converts TG2 from an "open" cell death- stimulating form, to a "closed"

cell-survival form (figure 1.2B) (Gundemir and Johnson, 2009b; Piacentini, et al., 2011).

## **1.9 Transglutaminase 2 in diseases**

Transglutaminase 2 is involved in various disease pathologies, either in overexpressed or repressed states. To date, it has been reported to contribute to inflammation, autoimmune diseases, diabetes, occupational asthma, neurodegenerative diseases, and cancer.

### **1.9.1 Inflammation**

TG2 is an activator of inflammatory mediators and has been shown to enhance the activity of phospholipase A2 isoform, by forming an isopeptide bond within the enzyme; as a result the biosynthesis of pro-inflammatory eicosanoids increases (Elli, et al., 2009).

NF- $\kappa$ B, one of the central downstream mediators of inflammation in inflammatory bowel disease (IBD), is activated by TG2, which exerts inflammatory and anti-inflammatory effects in immune cells and colonic epithelial cells respectively (Elli, et al., 2009). Aggregation and sequestration of the anti-inflammatory factor peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) by TG2 has also been shown to stimulate inflammation (Elli, et al., 2009; Luciani, et al., 2010; Oh, et al., 2011).

Recently, TG2 antibodies have been found as one of the potential biomarkers in occupational asthma (OA), induced by low molecular weight agent, isocyanate (Hur and Park, 2015).

### **1.9.2 Autoimmune diseases**

Coeliac disease is one of the best examples of a disease in which TG2 pathology is strongly implicated. This is a digestive system disorder, causing destruction of the small intestine by undigested rigid peptide fragments (Siegel and Khosla, 2007). The first sign of development of coeliac disease is generation of modified gluten peptides,

through deamidation of specific glutamine residues in gliadin by TG2, resulting in T cell stimulation. Later, these cells produce antibodies against TG2, which can be used clinically as an early diagnostic marker of this disease (Fesus and Piacentini, 2002). TG2 is also associated with type 1 and type 2 diabetes (Porzio, et al., 2007; Maglio, et al., 2009; Salter, et al., 2012). Missense mutations in the TGM2 gene have been found in TG2 protein in patients with early onset type 2 diabetes, corresponding to amino acids at positions Met330Arg, Ile331Asn, and Asn333Ser. Further supportive evidence came from TG2 knock-out mice that were shown to express glucose intolerance and had impaired insulin secretion (Porzio, et al., 2007; Salter, et al., 2012).

### **1.9.3 Neurodegenerative diseases**

Huntington's disease (HD) is caused by expansion of CAG (cytosine-adenine-guanine) triplet repeats in the gene coding for huntingtin protein, which produces an expanded polyglutamine-containing abnormal huntingtin (HTT) protein (McConoughey, et al., 2010). TG2 mediates cross-linking of HTT protein, which aggregates in the nucleus of neuronal cells and results in cell death in the striatum and cortex. Several studies have shown that TG2 activity is increased in brain tissue in HD patients, and TG2 co-localization has been shown with HTT aggregates (Grosso and Mouradian, 2012).

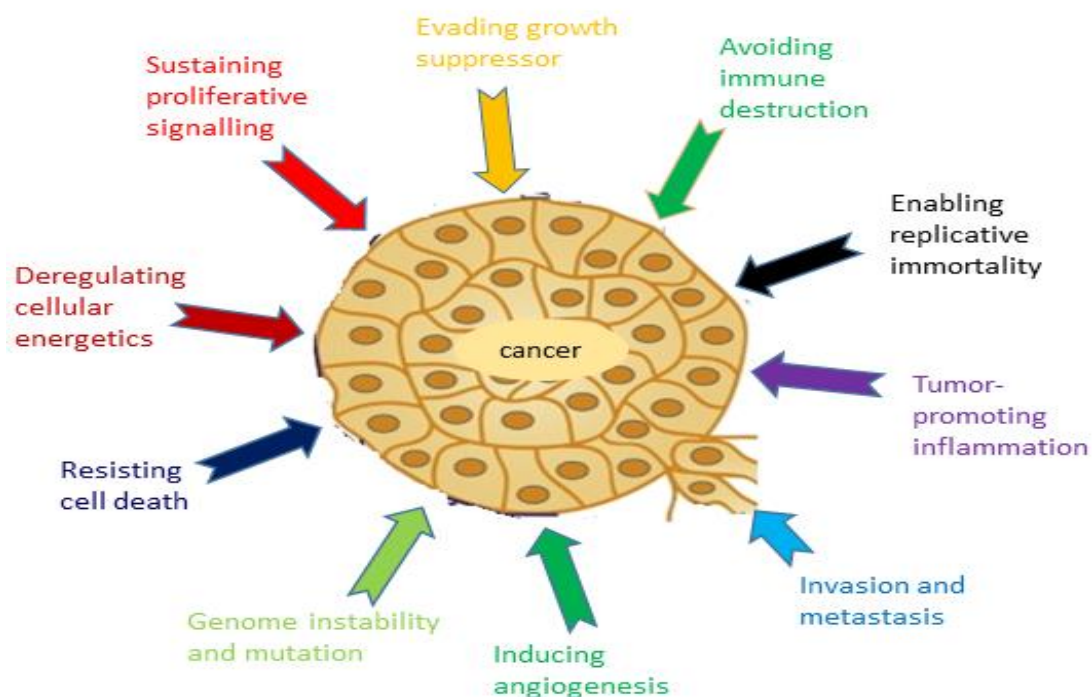
Alzheimer's disease (AD) is caused by extensive formation of aggregated amyloid beta-(A $\beta$ ) containing senile plaques and cerebral amyloid angiopathy, and aggregated tau-containing neurofibrillary tangles. TG2 has been implicated as a factor involved in the formation of A $\beta$  and tau protein aggregates, through its cross-linking activity. TG2 also polyaminates tau protein in AD (Wilhelmus, et al., 2009; Martin, De Vivo and Gentile, 2011; Grosso and Mouradian, 2012).

The pathology of Parkinson's disease and dementia also feature damage of neurological cells by formation of  $\alpha$ -synuclein aggregates in Lewy bodies, mediated

by TG2 cross-linking of  $\alpha$ -synuclein protein (Verhaar, et al., 2011; Grosso and Mouradian, 2012).

#### **1.9.4 Transglutaminase 2 in cancer**

Cancer is one of the deadly diseases of uncontrolled cell growth and proliferation, responsible for many deaths worldwide. Currently, one of the major obstacles in combating cancer is a failure of chemotherapy due to development of drug resistance by cancer cells to novel chemotherapeutic drugs, which later results in tumour recurrence and multidrug resistance. There are different risk factors for the development of cancer, including mutagenic and toxic chemicals, infections, and environmental factors, which induce genetic and epigenetic changes, resulting in continuous activation of signalling events independent of growth factors (Eckert, et al., 2014). So far, ten hallmarks of cancer have been proposed. These include sustained proliferative signalling, evasion of growth suppressors, activation of invasion and metastasis, enabling replicative immortality, induction of angiogenesis, resistance to cell death, avoidance of immune destruction, tumour-promotion of inflammation, genome instability and mutation, and deregulation of cellular energetics (Hanahan and Weinberg, 2011) (figure 1.5). TG2 has been implicated in most of these hallmarks, mainly as a mediator protein, rather than through direct involvement.



**Figure 1.5 The hallmarks of cancer**

Ten hallmarks of cancer cell are considered to be responsible to enable them to become tumorigenic. TG2 has been found to be involved in these hallmarks (self-generated diagram).

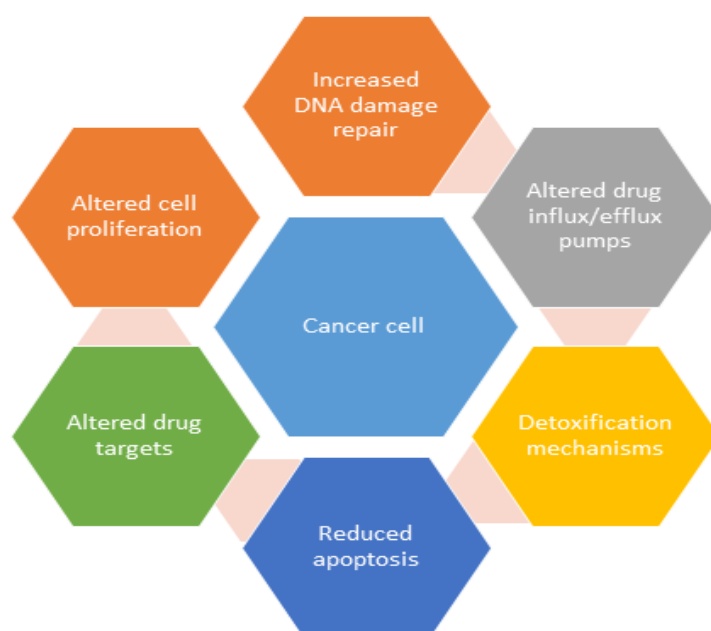
#### 1.9.4.1 TG2 in drug resistance

Over the past three decades, drug resistance has become a major challenge in cancer chemotherapy. The mechanisms that cause cancer cells to become resistant to drugs have been under investigation, and several enzymes, proteins and genes, including TG2, have been identified as having roles in such processes. High levels of TG2 expression have been found in a wide variety of cancers (Verma and Mehta, 2007). Surprisingly, TG2 silencing or TG2 enzymatic activity inhibition has reversed drug resistance in drug-resistant cancer cells to stress- or drug-induced apoptosis (Kim, et al., 2006; Kim, Park and Kim, 2009).

One of several mechanisms that contribute to drug resistance appears to result from activation of nuclear factor-kappa B (NF- $\kappa$ B), through the cross-linking and polymerisation of free I $\kappa$ B $\alpha$ , induced by TG2 (Lee, et al., 2004). Another mechanism

of drug resistance results from activation of EGFR via JNK and ERK pathway, induced by TG2 (Li, et al., 2011). The degradation of phosphatase PTEN by TG2 results in the constitutive activation of the FAK/PI3K/AKT cell survival pathway and so contributes to drug resistance (Verma, et al., 2006; 2008). A further mechanism of drug resistance occurs through depletion of nucleoplasmin by TG2 cross-linking activity (Park, et al., 2009).

Epithelial to mesenchymal transition (EMT) is the conversion of an epithelial cell phenotype to a mesenchymal cell phenotype. Recently, the presence of TG2 has also been found during the conversion of epithelial cell to mesenchymal cell, which later becomes resistant to anti-cancer drugs and metastasises to different parts of the body (Agnihotri, Kumar and Mehta, 2013).



**Figure 1.6 The hallmarks of drug-resistance**

TG2 has been found to be involved specifically in some of the known hallmarks of drug-resistance, e.g., having roles in reduced apoptosis and altered cell proliferation, possibly controlled by TG2 modifying key mechanism-related proteins (self generated diagram).

So far, the role of TG2 has been strongly associated with reduced apoptosis and the altered cell proliferation that eventually leads cells to development of drug resistance. TG2 is involved in reduced apoptosis by specifically modifying apoptosis inducing proteins, e.g., Bax, Bim, Bad, DR5, and surviving; on the other hand, it is involved in altered cell proliferation by modulating the expression of proteins that are involved in cell survival pathways, e.g., NFkB, Bcl2, BclX1, EGF, JNK, ERK, AKT and FAK, leading cells to proliferate uncontrollably.

Despite multiple lines of evidence that implicate TG2 involvement in drug resistance, the exact mechanism of TG2 induction during the drug resistance of cancer cells is not completely understood. Indeed, it seems likely that TG2 is involved in drug resistance because of its involvement in multiple pathways that regulate several hallmarks of drug-resistance (figure 1.6).

### **1.10 Transglutaminase 2 inhibitors**

Although there is a known naturally-occurring inhibitor of factor XIII, no naturally-occurring specific inhibitor of TG2 has so far been found. Some synthetic peptide inhibitors have proven effective, but these work only under specific conditions *in vitro*. Some inhibitors are available in synthetic peptide form (Siegel and Khosla, 2007). Thus, there is need to find specific TG2 inhibitor(s), which will inhibit TG activity without directly affecting key modulators of other cellular processes.

Transglutaminase 2 inhibitors can be categorised into three classes, depending on their mechanism of inhibition. These three classes of inhibitors are 1) competitive amine inhibitors, 2) reversible inhibitors, and 3) irreversible inhibitors (Siegel and Khosla, 2007; Pinkas, et al., 2007).

#### **1.10.1 Competitive amine inhibitors**

Competitive amine inhibitors are those that compete with natural amine substrates for the TG2 active site, in a transamidation reaction. The substrate and inhibitor are



unable to bind to the active site at the same time, hence cross-links are formed between the natural glutamine and the inhibitor's amine, instead of natural glutamyl TG2 substrate(s). Competitive inhibitors are often similar in structure to the natural substrates (Choi, et al., 2005; Siegel and Khosla, 2007).

Some of the most commonly used and commercially available, chemically stable, and relatively non-toxic, competitive amine inhibitors of TG2 are cystamine, monodansylcadaverine, putrescine, cadaverine, and 5-(biotinamido)-pentylamine. Cystamine is probably the most used TG2 inhibitor, because it uniquely has multiple inhibition mechanisms, affecting disulphide bonds and maintaining sulphydryl containing residues in a reduced state (Siegel and Khosla, 2007).

### **1.10.2 Reversible inhibitors**

Reversible inhibitors prevent enzyme activity by blocking substrate access to the active site without covalently modifying the enzyme; e.g., TG2 cofactors, like GTP and GDP, reversibly inhibit TG2 *in vivo*. In some cases, the ion  $\text{Zn}^{2+}$  reversibly inhibits TG2 activity by competing with  $\text{Ca}^{2+}$  for the binding site. Some GTP analogues can inhibit TG2 activity reversibly, for example, GTP $\gamma$ S and GMP-PCP (Pinkas, et al., 2007; Siegel and Khosla, 2007).

### **1.10.3 Irreversible inhibitors**

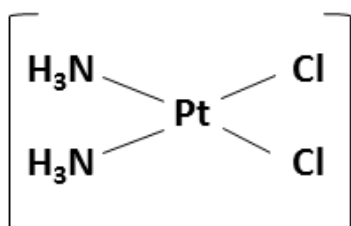
Irreversible inhibitors, also called suicide inhibitors, prevent enzyme activity by covalently modifying enzymes, thereby blocking the binding of substrate. Most of these inhibitors are designed to directly target cysteine in the catalytic site of TGs, and are often not found naturally, like other inhibitors (Siegel and Khosla, 2007).

Iodoacetamide is one of the simplest irreversible TG2 inhibitors. It has been shown that iodoacetamide reacts with nucleophilic residues other than the active site cysteine. Another irreversible inhibitor of TG2 is 3-halo-4,5-dihydroisoxazoles, which is chemically and biologically suitable for inhibition (Choi, et al., 2005; Siegel and

Khosla, 2007). However, these inhibitors are not sufficiently strong enough to completely ablate the effects of TG2 and therefore may have been neglected by researchers.

### 1.11 Use of cisplatin in Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) (liver cancer) is one of the primary liver malignancies and has been recorded as the 2<sup>rd</sup> most common reason for cancer-related deaths worldwide (Ashtari, et al., 2015). Chronic infection of the liver with Hepatitis B and C viruses (HBV & HCV) is the main cause of HCC development (Attwa and El-Etreby, 2015; Ashtari, et al., 2015), whereas alcohol consumption, obesity, aflatoxin toxicity, diabetes and fatty liver acid are the secondary causes (Attwa and El-Etreby, 2015). Recent studies have also identified the stem cells of HCC which are supposed to be another cause for the recurrence of cancer, despite long term treatment with anti-cancer drugs. Long-term survival has been found to be poor (Tomuleasa, et al., 2010).



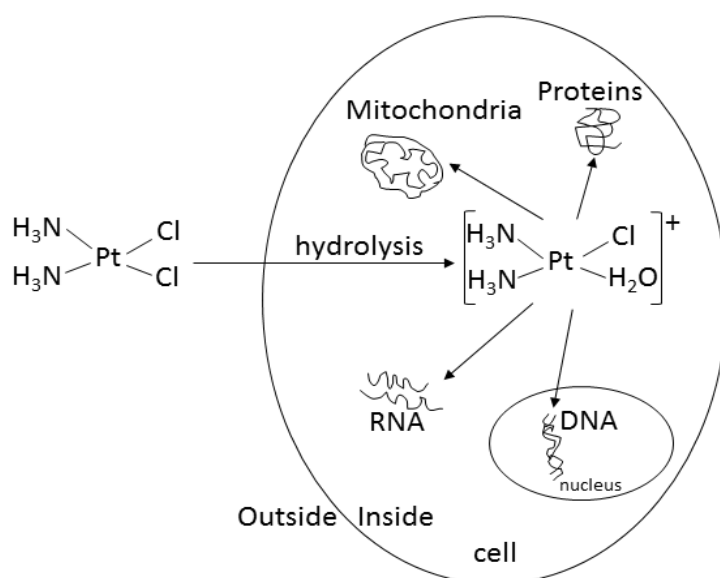
**Figure 1.7 Chemical structure of cisplatin.**

The platinum derivative cis-diaminedichloroplatinum (CDDP), best known as cisplatin (figure 1.7), is one of the most effective and currently-used anti-cancer drugs for patients affected by testicular, ovarian, head and neck, colorectal, bladder, lung, (Galluzzi, et al., 2014), and liver cancer (Song, et al., 2015). Over the last five years, the use of cisplatin in the treatment of advanced HCC has increased and shown several encouraging results. However, in many cases cisplatin response has relapsed after initial treatment and the tumour fails to respond, which results in

cisplatin resistance. To overcome this problem higher concentrations of cisplatin are recommended, which causes serious side effects, i.e. nephrotoxicity and ototoxicity (Ishida, et al., 2002). Therefore, understanding of specific mechanisms is crucial to overcome cisplatin resistance and improve the treatment.

#### **1.11.1 Mode of cisplatin action**

It is known that cisplatin cross-links DNA, and in this way inhibits DNA synthesis to induce apoptosis or to simply arrest the cell cycle (Gonzalez, et al., 2001; Qin and Ng, 2002; Zhang, Niu and Zhou, 2010). Cisplatin enters into the cellular environment by passing through the plasma membrane, to the cytoplasm, where it may come in contact with various proteins or enzymes, and then finally it enters the nucleus (figure 1.8); the way cisplatin works in the body is not fully understood. For example, it is not always the case that simple arrest of DNA synthesis is sufficient in itself to induce apoptosis, as there are many other proteins that are involved in cisplatin-induced apoptosis (Dasari and Tchounwou, 2014). Cisplatin is in fact a non-specific drug and can bind to any molecules that contain nucleophilic sites, such as DNA, RNA, and proteins. Indeed, it has been suggested that cisplatin may initiate apoptosis by damaging cytoplasmic proteins (Gonzalez, et al., 2001).



**Figure 1.8 Hydrolysis of cisplatin and its binding to different molecules** (self generated diagram).

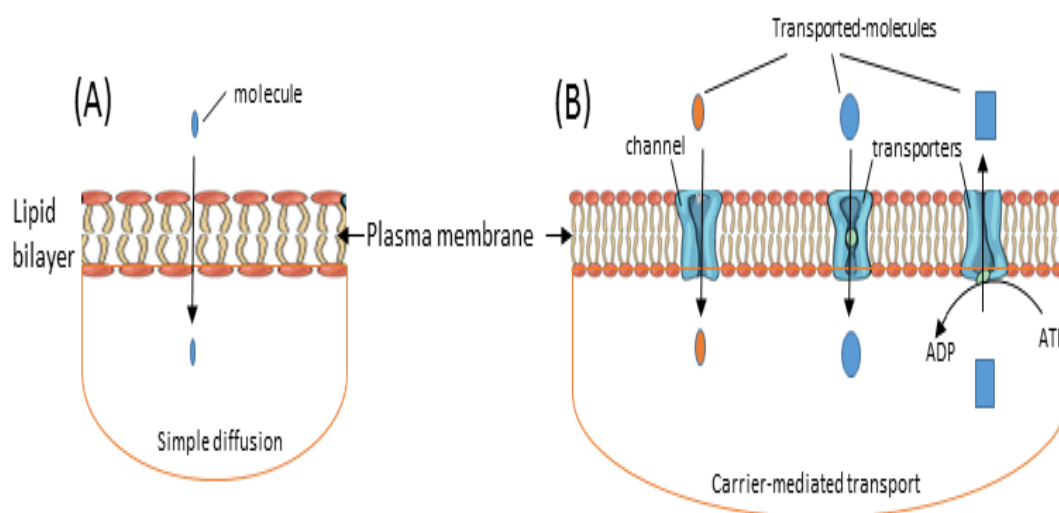
### 1.11.2 Cisplatin resistance

Cisplatin is mostly used for treatment of solid tumours, and for treating metastatic cancer. Development of cisplatin resistance in different cancers has shown that cancer cells have self-defence mechanisms pertaining to drug uptake. It appears that these self defence mechanisms arise from multiple epigenetic and genetic changes at the molecular and cellular level, including reduced accumulation of cisplatin, increased DNA damage repair, apoptosis inhibition, defective endocytosis and control of membrane transporters during cisplatin trafficking, changes in transcription factors, histone modification, and activation of EMT pathway (Shen, et al., 2010; 2012). It is also evidenced from drug-resistant cells that, during cisplatin entry, several proteins or enzymes interact with cisplatin and that this may reduce the cytotoxicity of cisplatin (Zhang, Niu and Zhou, 2010), and in this way may contribute to the development of drug resistance.

#### 1.11.2.1 General mechanisms of membrane transport for drugs

Cells live by exchanging molecules with their environment. The exchange of molecules is controlled by the plasma membrane, which acts as a barrier to transit

molecules into and out of the cell. Entry of molecules into and out of the cell occurs through various membrane transport mechanisms. There are four main mechanisms, of which two are more important in the transport of drugs: 1) diffusion across the lipid bilayer of the membrane, and 2) carrier-mediated transport (Alberts, et al., 2013). Diffusion across the lipid bilayer is concentration gradient-dependent and transports molecules from high to low concentrations. Carrier-mediated transport occurs by passive transport, through channels or transporters, and by active transport through specific transporters that require energy (Alberts, et al., 2013) (figure 1.9).



**Figure 1.9 Mechanisms of membrane drug transports.**

(A) Simple diffusion - down concentration gradient, (B) Carrier-mediated transport: Passive transport - down concentration gradient; Active transport – against concentration gradient (self generated diagram).

Emerging research indicates that TG2 is involved in drug resistance, but how it is involved and what roles it plays are still unsolved. The relationship between defective uptake of cisplatin, and overexpression/involvement of TG2 on the cell membrane or cytoplasm during cellular uptake of cisplatin and other anti-cancer drugs has been little studied so far. Therefore, this current study seeks to advance knowledge in this area, specifically focusing on the mechanisms of modulation of influx of anti-cancer drugs with relation to expression of TG2.

### **1.12 Aims of the current study**

So far, experimental evidence suggests that TG2 is an enigmatic enzyme, which appears to be ubiquitous, and is found in the extracellular matrix, cell surface, plasma membrane, cytosol, mitochondria, recycling endosomes, and nucleus, and that its subcellular localisation is an important determinant of its functions. However, the exact physiological role or roles of TG2 are still unclear. The aim of this research is therefore the following: 1) to investigate the association of TG2 with apoptosis and/or cell survival after cisplatin treatment in hepatocarcinoma HepG2 cell lines; 2) to investigate the relationship of TG2 with respect to development of cisplatin-resistance in HepG2 cell lines; 3) to investigate the role of TG2 in cellular uptake of cisplatin in cisplatin-resistant HepG2 cell lines; 4) to perform a preliminary investigation into the scale of modification and molecular characterization (at the level of determination of molecular weight) of cellular TG2 substrate protein(s) during the cellular uptake of dansylcadaverine in HepG2 cell lines.

Collectively, this research provides more detail, concerning the role of TG2 in cancer and drug resistance and so may help to develop novel strategies for the use of TG2 as a novel cancer biomarker and as a novel target for chemotherapeutic therapy.

## **CHAPTER 2:**

### **MATERIALS AND METHODS**

#### **2.1 Stock solutions' preparation**

**Cisplatin (2 mM):** Stock solutions of 2 mM cisplatin were prepared in 18 mΩ ultrapure water under subdued light and stored in the dark at room temperature before use. Freshly-prepared cisplatin solution was used for every experiment, owing to its instability in aqueous solution.

**Retinoic acid (10 mM):** Special precautions were taken during preparation of retinoic acid (RA) solutions, as it is sensitive to UV light, air and oxidizing agents. Preparation of 10 mM (3 mg/ml) RA in absolute ethanol was performed under subdued light and in a glove bag under an atmosphere of inert gas and stored at -20 °C. RA solutions were diluted with tissue culture medium prior to any treatments and used within two weeks.

**Cystamine (0.5 M):** Stock solutions of 0.5 M cystamine were prepared in ultrapure water and stored at 4 °C.

#### **2.2 Cell lines and cell culture preparation**

The Hepatocellular carcinoma (HepG2) and Renal carcinoma (CAKI2) cell lines used in this study were procured from the European Collection of Cell Cultures (ECCC) (Sigma Aldrich, UK). Cell authentication was confirmed by comparison with online cell morphology.

The HepG2 cells were cultured in RPMI 1640 culture medium (Life Technology, UK), fully supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, UK).

The CAKI2 cells were cultured in McCoy's culture medium (Life Technologies, UK), fully supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

The cell cultures were maintained in an incubator at 37 °C in the presence of a humidified 5% CO<sub>2</sub> atmosphere and were kept free of mycoplasma contamination by adding Plasmocin, a commercially available antibiotic, during initial maintenance of cell cultures. Cells were observed on a regular basis and fed with fresh culture medium after every 2-3 days. Cell cultures were transferred to a new culture flask by trypsinization (0.1% trypsin plus 0.02% EDTA) once cells reached 90% confluence. Cell viability and cell counts were performed by trypan blue assay and handheld automated cell counter (Scepter Millipore, UK), respectively. Passage numbers 6-12 were used for all experiments.

### **2.2.1 Cell viability by trypan blue assay**

Cell viability was assessed by trypan blue assay; viable cells exclude trypan blue, whereas non-viable cells retain it. After trypsinisation, cell suspensions were diluted with culture medium and mixed with trypan blue dye (0.4% in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4)) in a ratio of 1:1. The mixture was incubated for 15 minutes at room temperature on a bench-top. Then, 10 µl of trypan blue-cell suspension were transferred to a hemocytometer chamber, with a cover-slip in place, and cells were observed, using an inverted phase contrast microscope.

### **2.2.2 Cell passaging**

Cell passaging was performed once cells reached 90% confluence. The old culture medium was aspirated from the culture flask and cells were washed once with sterile PBS. Then, trypsin was added according to the size of culture flask (e.g. 1 ml/25 cm<sup>2</sup>, 3 ml/75 cm<sup>2</sup>, and 6 ml/160 cm<sup>2</sup>) and incubated for 5-10 minutes at 37 °C, until cells detached from the bottom of the flask. After incubation, the trypsinization reaction was



stopped by adding the same amount of warm fully-supplemented culture medium. Then, cells were re-cultured in the new culture flask, at a ratio of 1:3 and incubated at 37 °C in the presence of a humidified 5% CO<sub>2</sub> atmosphere.

### **2.2.3 Cells cryopreservation**

Cells were cryopreserved in a freezing medium (90% RPMI 1640 culture medium/10% dimethylsulphoxide (DMSO)). Briefly, cells growing in a culture flask at 90% confluence were collected by trypsinization and spun at 1000 rpm for 5 minutes. The cell pellet was gently broken up in freezing medium by slow pipetting up and down, and 1 ml of freezing medium, containing cells at  $1 \times 10^6$  cells/ml, were transferred to several freezing vials, and then immersed in an isopropanol solution, within a “Mr. Frosty” container (Sigma Aldrich, UK), and incubated at -80 °C. After 24 hours, all cryovials were preserved in liquid nitrogen at -196 °C in a hanging position.

## **2.3 Single dose treatment method for the development of drug resistance cells**

Cisplatin resistant HepG2/cr cells were developed by a single dose treatment method, according to the guidelines described in McDermott, et al., (2014), with minor modifications. Approximately  $1 \times 10^6$  cells were grown in a 75 cm<sup>2</sup> culture flask at 37 °C in the presence of a humidified 5% CO<sub>2</sub> atmosphere. After 24 hours, cells were treated with freshly made 8 µM cisplatin, continuously, for 4 days. After incubation, cells were washed twice with warm culture medium and incubated in cisplatin-free culture medium for 4 weeks, changing the media after every 2-3 days. The surviving colonies were recovered by passaging and then frozen down in liquid nitrogen as described in section 2.1.3; the treatment was conducted in duplicate. Control flasks were left untreated, but were passaged alongside the treatment flasks. The cell colonies collected from the cisplatin-treated flasks were named HepG2/cr cells. The morphology of both cell lines was monitored using an inverted phase contrast microscope. Then, cells were exposed to 0-40 µM cisplatin for 24 and 48 hours, and drug sensitivity was tested, and compared to non-drug resistant parental cells.

## **2.4 Cell migration assay**

Cell migration assays were performed according to the method described by Liang (Liang, Park and Guan, 2007). Approximately 400,000 cells/well were grown in 12-well culture plates for 24 hours. Then, media was removed and a horizontal scratch wound across the monolayer was created using a sterile 10  $\mu$ l pipette tip. Cells were washed twice with fresh culture media and detached cells in the media were aspirated. Then, cells were inspected using an inverted phase-contrast light microscope, prior to incubation at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for further observation.

## **2.5 Cytotoxicity assay with the cell counting kit-8**

The cell counting kit-8 (CCK-8) assay is a colorimetric procedure, based on the ability of viable cells to reduce a yellow-coloured formazan dye. The assay utilises WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] in conjugation with an electron mediator, 1-methoxy-5-methylphenazinium methylsulphate, to assess cell viability.

HepG2 and HepG2/cr cells were trypsinized and re-suspended in fresh culture medium at a concentration of  $1 \times 10^5$  cells/ml. The cells were seeded into 96-well cell culture plates, at 10,000 cells per well, in a 100  $\mu$ l volume of fully-supplemented RPMI 1640 medium. Blank control wells were filled with 100  $\mu$ l fresh culture medium, with omission of the cells. The cells were then grown for 24 hours at 37 °C, in the presence of a humidified 5% CO<sub>2</sub> atmosphere. Following incubation, the culture medium was removed and 100  $\mu$ l of fresh culture medium, containing freshly-prepared drugs, were added and samples were incubated prior to assay over defined time courses. At defined incubation times, 10  $\mu$ l of the CCK-8 reagent were added directly to the cell cultures and incubated for another 4 hours at 37 °C in the presence of a humidified 5% CO<sub>2</sub> atmosphere. The WST-8 formazan product was then measured at 450 nm using a monochromator-based multi-mode microplate reader equipped with Megalan

software (Sunrise, UK). The viable cell numbers were calculated according to the following formula:

$$\text{Viable cells (\%)} = [(OD_{450\text{nm}} \text{ of Test} - OD_{450\text{nm}} \text{ of Blank}) / (OD_{450\text{nm}} \text{ of Control} - OD_{450\text{nm}} \text{ of Blank})] \times 100$$

The IC<sub>50</sub> value is defined as the concentration of toxic agent required to reduce cell survival by 50%. All samples were assayed in triplicate, with a minimum of three independent experiments.

## **2.6 Nuclear staining with DAPI**

HepG2 cells were grown on coverslips, in 6-well culture plates, in the presence of a humidified 5% CO<sub>2</sub> atmosphere. After 24 hours, cells were treated or not-treated with a range of concentrations of cisplatin and incubated for a further 24 hours. After incubation, cells were washed briefly with 1x PBS. Then, samples of treated and non-treated (control) cells were fixed and permeabilized with 4% formaldehyde/0.2 M sucrose, for 20 minutes, at room temperature, in order to disrupt the cells' plasma membranes. Coverslips, with cells, were washed briefly with PBS and then mounted using a mounting medium, containing DAPI (4', 6-diamidino-2-phenylindole) (5 µg/ml). The slides were then inspected by DMR-X fluorescence microscopy (Leica, Germany).

## **2.7 Cell cycle and apoptosis analysis by propidium-iodide staining using flow cytometry**

Briefly, 1x10<sup>6</sup> cells were grown in culture flasks for 24 hours, and then treated with freshly prepared cisplatin (see section 2.1 for stock preparation), at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. After defined incubation times, floating cells from aspirated medium were collected by centrifugation at 1000 rpm for 5 minutes. Adherent cells were washed with PBS and harvested by trypsinization and the cell pellets were washed once with ice-cold PBS. Cell pellets were resuspended in 0.5 ml ice-cold PBS and 70% ethanol was added drop-wise to the cell suspension, up to a

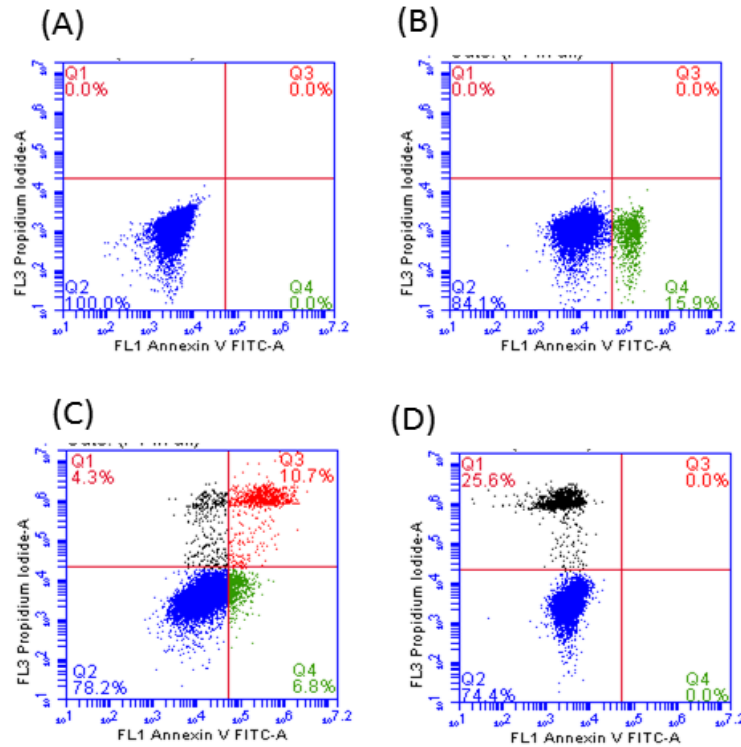
final volume of 5 ml, while vortexing. Cells in 70% ethanol were then incubated at 4°C for 2 hours, with gentle agitation every half an hour to avoid clump formation. Then, cells were spun at 2500 rpm for 10 minutes, and cell pellets were washed twice with ice-cold PBS. 400 µl of propidium iodide (PI) (50 µg/ml) solution, containing 50 µl of DNase-free RNase A (100 µg/ml) were then added to the cells and the mixtures were incubated in the dark at room temperature (RT) for 30 minutes, before assay by flow cytometry. Assessment of apoptotic cells by flow cytometric analysis was carried out on a Fluorescence-activated Cell Sorter (FACS) equipped with Cell Quest Pro Software (Becton Dickinson, UK).

Apoptotic cells were distinguished from live cells on the basis of PI binding to low molecular weight DNA (fragmented DNA), compared to non-fragmented DNA.

## **2.8 Apoptosis analysis by FITC-labelled Annexin V with flow cytometry**

After treatment of cells with drugs, floating cells from aspirated medium were collected by centrifugation and adherent cells were harvested by trypsinization. The cell pellets were washed 2 times in ice-cold PBS. Cells were then diluted with ice-cold binding buffer (0.01 M Hepes, pH 7.4, 0.14 M NaCl, 2.5 mM CaCl<sub>2</sub>) at 1x10<sup>6</sup> cells/ml. To 100 µl of cell suspension (10<sup>5</sup> cells), 5 µl of Fluorescein isothiocyanate (FITC)-labelled Annexin V (5 µg/ µl) and 10 µl of propidium-iodide (PI) (20 µg/µl) (BD Biosciences, UK) were added, and mixtures were incubated in the dark, for 15 minutes at RT. Then, 300 µl of binding buffer was added, and samples were analysed using a BD Accuri flow cytometer, equipped with C6 software (BD Biosciences, UK).

The cells were divided into 4 categories: 1) Live cells, i.e., those that excluded FITC Annexin V and PI; 2) Early apoptotic cells, i.e., those that bound only FITC Annexin V; 3) Late apoptotic or already dead cells, i.e., those cells that bound both FITC Annexin V and PI; 4) apoptotic or necrotic (death reason unknown), i.e., those cells that bound only PI (an example is shown in Figure 2.1).



**Figure 2.1 Flow cytometric analysis of FITC Annexin V staining and PI.**

(A) cells were stain-free, (B) cells were incubated with only FITC Annexin V stain, (C) cells were incubated with FITC Annexin V and PI stain, (D) cells were incubated with only PI stain. Blue colour indicates live cells; green colour indicates early apoptotic; red colour indicates late apoptotic or already dead cells; black colour indicates necrotic or apoptotic cells.

## 2.9 RNA isolation

HepG2 parental and HepG2-cisplatin resistant cells were seeded at a density of  $\geq 1 \times 10^6$  cells per 25 cm<sup>2</sup> culture flask and pre-incubated for 24 hours, to facilitate attachment to the bottom of flasks. Following subsequent cisplatin treatment, total RNA was isolated from cells, using the RNeasy kit (Quiagen, UK), according to the manufacturer's instructions. Briefly, cells were lysed in RLT buffer and supernatant was obtained by centrifuging for 3 minutes at 13,000 rpm. The supernatant was mixed with 70% ethanol at a ratio of 1:1. Then, total mixture was transferred to an RNeasy spin column and centrifuged for 15 seconds at 8,000 rpm and the flow-through was discarded. Then, 700  $\mu$ l RW1 buffer were added to the spin column and centrifuged for 15 seconds at 8,000 rpm and again the flow-through was discarded. Next, 500  $\mu$ l

of RPE buffer were added to the spin column and centrifuged for 15 seconds at 8,000 rpm and again the flow-through was discarded. Next, 500 µl RPE buffer were added to the spin column and centrifuged for 2 minutes at 10,000 rpm and the flow-through was discarded. After DNase treatment, total RNA was collected in 30 µl of RNase-free H<sub>2</sub>O and was quantified by measurement of the absorbance at 260 nm ( $A_{260}$ ) using a Nanodrop spectrophotometer (Thermo Scientific, UK). The purity of RNA was confirmed using the  $A_{260}/A_{280}$  method, where RNA has an  $A_{260}/A_{280}$  ratio of 2.0. This method provides an estimate of the purity of RNA, with respect to other contaminants, such as protein, that typically absorb at 280 nm in the UV spectrum. For pure RNA, the value of  $A_{260}/A_{280}$  ratio must be 1.9 - 2.1. The value of  $A_{260}/A_{280}$  ratio was found to be 2.0 for the RNAs isolated in this study.

### **2.10 Reverse transcription quantitative real time-PCR (qRT-PCR)**

The quantitative estimation of expression of TG2 mRNA and the “housekeeping” enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls were based on real-time monitoring of amplification and melting curves. Specific sequences of the primers for TG2 and other genes are summarized in Table 2.2. The reverse transcription was performed at 50 °C for 10 minutes, with 500 ng of total RNA, using specific reverse primers (Eurofins, UK) (Table 2.2) and iScript reverse transcriptase, from the one-step RT-PCR kit (Bio-Rad, UK), with some modifications to the manufacturer’s protocol. The reaction mixture contained: 12.5 µl 2x SYBR green PCR master mix; 1 µl (400 nM) forward primer; 1 µl (400 nM) reverse primer; 0.5 µl RT-enzyme; total RNA and RNase-free H<sub>2</sub>O; in a total volume of 20 µl. Quantification based on real-time monitoring of amplification and melting curves was carried out using a Roche RT-PCR thermal cycler equipped with light cycler 4.1 software (Roche-science, UK). The reaction mixtures were incubated in the following manner: cDNA synthesis during 1 cycle at 50 °C for 10 minutes; denaturation during 1 cycle at 95 °C for 5 minutes; 3 step amplification during 40 cycles, at 95 °C for 30 seconds, 60 °C

for 30 seconds, and 72 °C for 90 seconds; final extension during 1 cycle at 72 °C for 10 minutes; melting curve generation during 1 cycle at 95 °C for 10 seconds, 44 °C for 1 minute, and 72 °C for 1 second; and a final cooling step during 1 cycle at 37 °C. All determinations were done in triplicate, together with one control reaction, in which RT enzyme was omitted. The latter was used to test for potential DNA contamination, and to act as a negative control with respect to primer dimer -oligomerization. Absolute numbers of mRNA molecules were normalised to GAPDH, to correct for differences in RNA concentration. Sequences of the primers used for subsequent amplification reactions were designed from available data on specific genes obtained from NCBI website. Amplified products were monitored by running samples on a 1.2% agarose gel and confirmed against available data. The relative quantification was calculated using the  $\Delta\Delta C_T$  ( $\Delta\Delta C_q$ ) method.

The formulae used to calculate relative quantification are given in Table 2.1:

**Table 2.1 Equations used to calculate relative expression of genes**

| Samples<br>e.g.,<br>1.Treated<br>2. Not-<br>treated | Cq<br>value<br>of<br>GAP-<br>DH | Cq<br>value<br>of<br>TG2 | $\Delta Cq$<br>= (CqTG2-<br>CqGAPDH) | $\Delta Cq$<br>Expression<br>= $2^{-\Delta Cq}$ | Mean of<br>$\Delta Cq$<br>expression<br>(average of<br>replicates) | $\Delta\Delta Cq$<br>expression<br>(normalise<br>to control) | Std Dev<br>of $\Delta\Delta Cq$<br>expressi-<br>on |
|---|---------------------------------|--------------------------|--------------------------------------|---|--|--|--|
|---|---------------------------------|--------------------------|--------------------------------------|---|--|--|--|

Ratio expression ( $\Delta\Delta Cq$ ) = Test expression/control expression

**Table 2.2 Primers used for transcript quantification**

| Transcript Name | Sequence  |
|-----------------|---|
| GAPDH           | (F) – CACTAGGCGCTCACTGTTCTC<br>(R) – GACTCCACGACGTACTCAGC |
| TG2             | (F) – CTGGGCCACTTCATTTTGC<br>(R) – ACTCCTGCCGCTCCTCTTC    |
| TG1             | (F) – CTGCTCAATGTCTCAGGCCA<br>(R) – CTTGGGCCTCTGTAACCCAG  |

F = Forward primer, R = Reverse primer.

### **2.11 Isolation of the nuclear fraction**

Cisplatin-treated HepG2 cells, and controls, were disrupted in homogenization buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA,), containing 1% complete protease inhibitor cocktail (Aprotinin, Bestatin, Leupeptin, Pepstatin A) (SigmaAldrich, UK). The homogenates were centrifuged at 600 x g for 5 minutes at 4 °C, to yield a nuclear pellet and a non-nuclear supernatant. Nuclei were then resuspended in nuclei isolation buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol), again containing a cocktail of protease inhibitors. The mixtures were incubated at 4 °C for 1 hour with constant rotation, and then centrifuged at 17,000 xg for 20 minutes to obtain a supernatant fraction enriched with nuclear proteins. The non-nuclear supernatant was centrifuged at 17,000 xg for 20 minutes at 4 °C. The supernatant that contained the remaining organelle proteins was collected and the total protein concentrations of samples were determined using the Bradford dye-binding assay (Bradford, 1976).

### **2.12 Isolation of membrane proteins**

The membrane proteins were isolated using Mem-PER Plus Membrane protein extraction kit (Thermo Scientific, UK) according to the manufacturer's instructions. Briefly, cells were collected by centrifugation and cell pellets were washed once with



wash solution (PBS). Cell pellets were permeabilized in a state type buffer, containing a cocktail of protease inhibitors and incubated for 10 minutes at 4 °C, with constant mixing. Then, mixtures were centrifuged at 16,000 x g for 15 minutes at 4 °C and the supernatant containing cytosolic proteins was collected. The remaining pellets were re-suspended in solubilisation buffer (1-propanaminium, N,N-dimethyl-N-(3-sulfopropyl)-3-[[[(3 $\alpha$ ,5 $\beta$ ,7 $\alpha$ ,12 $\alpha$ )-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]-, inner salt) by slow pipetting up and down, until the pellets were broken. The cell suspensions were incubated for 30 minutes at 4 °C with constant mixing on a sample mixer. Then, samples were centrifuged at 16,000 x g for 15 minutes at 4° C and supernatants containing membrane proteins were collected and stored at -20 °C until required for assays.

### **2.13 Whole Cell lysate preparation**

Cisplatin-treated, and untreated (control), HepG2 and HepG2/cr cells were washed twice with ice-cold PBS for 5 minutes each. Then, cells were directly lysed for 5 minutes at 4 °C in cell lysis RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) (Sigma Aldrich, UK) containing 1% complete protease inhibitor cocktail. Cell suspensions were then vigorously vortexed and incubated on ice for 5 minutes, and then centrifuged at 17,000 x g for 20 minutes at 4 °C. The protein-enriched supernatants were collected and stored at -80 °C prior to analysis.

### **2.14 Protein quantification by Bradford assay**

The total protein concentration of cell lysates were determined using the Bradford dye-binding assay (Bradford, 1976). Briefly, 3 ml of Bradford reagent (0.05 mg/ml Coomassie Brilliant Blue G-250, 10% phosphoric acid) were added to 50  $\mu$ l of samples, before incubation of samples at RT for 45 minutes. After incubation, the optical density (absorbance) of colour formed was measured at 595 nm, using a spectrophotometer (Thermo Scientific, UK). The concentration of protein samples

was calculated by reference to a standard curve that had been constructed using known concentrations of Bovine Serum Albumin (BSA) as standards.

### **2.15 Gel electrophoresis**

One dimensional (discontinuous) sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Samples were mixed with Laemmli 2x SDS Sample buffer (1:1) (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue) and proteins were further denatured by heating at 90-95 °C for 5 minutes. Samples were then cooled and 20  $\mu$ l aliquots were loaded and separated, using 10% Bis-Tris plus gels (Invitrogen, UK) under reducing conditions, with 1x MES SDS running buffer, in a bolt mini-gel electrophoresis tank (Life Technology, UK). The gels were run at 160 V and 80 mA, until the dye-front reached the bottom of the gels. Then, gels were either stained with Coomassie blue, or transferred to a nitrocellulose membrane for Western blot analysis.

### **2.16 Western blotting**

SDS-PAGE gels (see section 2.15) were electrophoretically transferred to nitrocellulose membranes (Abcam, Cambridge, UK), using semi-dry transfer (Thermo Scientific, UK) for 1 hour, at 100 mA, 5 V. Transferred proteins were initially visualised by Ponceau S red (0.5 g in 100 mL 1% acetic acid) staining, before further processing. Blots were then blocked in a 5% non-fat dried milk powder, in Tris-buffered saline, with 0.1% Tween-20 (TBST) for 1 hour. The blots were gently washed, briefly, with TBST and incubated with anti-TG2 polyclonal antibody, at 1:3000 (v/v) dilution in TBST, for 3 hours at RT, or overnight at 4 °C, with rotation agitation. The blots were subsequently washed 3 times for 5 minutes with TBST, replacing the buffer each time. Then, HRP-conjugated secondary antibody was added at a 1:3000 (v/v) dilution and the blots were incubated for 1 hour at RT. The blots were then washed 3 times with TBST, for 5 minutes each time. The blots were subsequently developed, using an

enhanced luminol-based chemiluminescence substrate for the detection of horseradish peroxidase (HRP) (ECL-HRP Western blotting substrate) (Pierce, Thermo Scientific, UK). The antibodies were stripped off of the blot with stripping solution (0.5 M Tris-buffer, pH 6.7, 2% SDS, 0.7%  $\beta$ -mercaptoethanol) for re-incubation with a loading control, using an antibody against  $\beta$  actin (Abcam, Cambridge, UK). The stripped blots were re-incubated with rabbit anti- $\beta$  actin antibody at 1:3000 (v/v) dilution for 3 hours at RT, or overnight at 4 °C. Then, blots were washed as described above and incubated with secondary anti-rabbit antibody (1:3000 (v/v)) for 1 hour, then washed and developed as previously described.

**Table 2.3 Antibodies used for measurement of protein**

| Antibody                          | Concentration | Company              |
|-----------------------------------|---------------|----------------------|
| TG2 (rabbit) (SAB4200072)         | 1:3000 (v/v)  | Sigma Aldrich, UK    |
| TG2 (rabbit) (ab421)              | 1:3000 (v/v)  | Abcam, Cambridge, UK |
| TG2 (mouse) (ab2368)              | 1:3000 (v/v)  | Abcam, Cambridge, UK |
| $\beta$ -actin (rabbit) (ab75186) | 1:3000 (v/v)  | Abcam, Cambridge, UK |
| Goat Anti-rabbit (ab6721)         | 1:4000 (v/v)  | Abcam, Cambridge, UK |
| Goat anti-mouse (ab6789)          | 1:4000 (v/v)  | Sigma Aldrich, UK    |

## 2.17 Development of immunoblots

Blots were incubated with ECL substrate reagents (1:1 v/v ratio) for 1 minute. Then, excess reagents were drained and blots were covered with a clean plastic wrap. The protected membranes were placed in a film cassette with the protein side facing up. The protected membranes were then exposed to X-ray film in the dark, in the presence of red light, for 3 minutes. The film was subsequently developed in developing (5% hydroquinone in H<sub>2</sub>O) and fixing (25% ammonium thiosulphate in H<sub>2</sub>O) reagents and then washed with water and dried.

### **2.18 *In vitro* specific TG2 colorimetric microassay (TG2-CovTest)**

Cell lysates were prepared from HepG2 test samples and TG2 activity was measured using a commercially-available specific TG2 colorimetric microassay kit (Covalab, France), according to the manufacturer's instructions. Briefly, the wells of a microtiter plate were pre-coated with amine substrate (spermine), and washed once with 1x PBS for 15 minutes at 37 °C. The buffer was removed and 50 µl of assay mixture (containing freshly-made dithiothreitol (DTT), biotin-pepT26/CaCl<sub>2</sub>) in double distilled water, were added. Then, 60 µl of lysate was added to each well. Samples were mixed by pipetting and incubated at 37 °C for 30 minutes. Wells were washed once with PBS, then washed once with 0.1 M NaOH, then washed twice with PBS. Streptavidin-labelled peroxidase solution (SAv-HRP) was added (100 µl) and the samples were incubated for 15 minutes at 37 °C. Wells were subsequently washed thrice with PBS and 100 µl aliquots of HRP-substrate (H<sub>2</sub>O<sub>2</sub>) were dispensed into each well. The mixtures were then incubated in the dark, for 5 minutes at RT. The reaction was developed by adding tetramethyl benzidine stock solution (100 µl) and the colour that formed was measured on the basis of its optical density at 450 nm, using a microplate reader.

### **2.19 CBZ-Gln-Gly assay of Transglutaminase activity**

Guinea pig liver transglutaminase activity was measured by Benzyloxycarbonyl-Glutamine-Glycine (CBZ-Gln-Gly) assay, using the method of Folk and Cole (1966), with minor modifications. 1 ml samples contained a final concentration of 200 mM Tris-HCL (pH 7.0), 10 mM CBZ-Gln-Gly, 100 mM Hydroxylamine (NH<sub>2</sub>OH), 6 mM CaCl<sub>2</sub>, 1.6 mM DTT and 0.02 mg/ml guinea pig liver transglutaminase. Aliquots of freshly-prepared cisplatin were added to reaction mixtures over a concentration range of 0-128 µM in final volumes of 1 ml and incubated for 1 hour at 37 °C. The reactions were stopped by adding 0.3 ml of ferric chloride-trichloroacetic acid (FeCl<sub>3</sub>/TCA/HCl), and the precipitated products were centrifuged at 3000 rpm for 3 minutes. The optical

density of the supernatant was measured immediately, at 520 nm against a blank, in which TG2 was omitted. EDTA was added to negative samples.

For determination of transglutaminase activity from cell lysates by CBZ-Gln-Gly assay, the protocol was the same as described above, except 100 µl of lysate was added to mixtures, instead of commercially-available guinea pig liver transglutaminase.

## **2.20 siRNA inhibition of transglutaminase 2 (Tgase)**

For the siRNA inhibition study, the sequence of the siRNA oligomer (pre-designed and synthesized by Ambion, Life Technologies, UK) used to inhibit TG2 expression was as follows: sense:-GGCCCGUUUCCACUAAGA, antisense:-UCUUAGUGGAAAACGGGCC. Cells were transfected with oligomers, using Lipofectamine 2000 transfection reagent (Life Technologies, UK), according to the manufacturer's instructions. Briefly,  $1 \times 10^5$  cells were grown in 12-well plates or 4-well Lab Tek chamber slides prior to transfection. After 24 hours, cells were treated with a final volume of 4 µl lipofectamine and a final concentration of siRNA at 30 pmol in a total volume of 1 ml culture medium, and cells were re-incubated for 48 hours. After the indicated time, cells were prepared for further analysis. Alternatively, cells were harvested, and cell lysates were prepared to measure protein expression of TG2 (see section 6.2.4).

## **2.21 Immunocytochemistry**

HepG2 cells were grown on coverslips in 6-well culture plates at a density of  $2 \times 10^5$  cells/ml/well. Then, cells were treated or not treated with 8 µM cisplatin for 24 hours at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. After incubation, cells were washed with ice-cold PBS and fixed in 4% formaldehyde (4% paraformaldehyde/0.2 M Sucrose/1x PBS, pH 7.5), and incubated for 15 minutes at RT. Then, cells were washed 2 times with PBS prior to incubation in blocking buffer (5% donkey serum/0.3% Triton X-100/1x PBS) for 1 hour at RT. The blocking buffer was then

replaced with anti-TG2 antibody solution (TG2 antibody at 1:200 in 1x PBS/1% BSA/0.3 Triton X-100) and incubated for 2 hours at RT, or overnight at 4 °C. The cells were then washed 3 times in PBS for 5 minutes each time. Then, cells were incubated in the dark, with Alexa Fluor 488-labelled Donkey anti-rabbit secondary antibody solution for 1 hour at RT. Finally, cells were washed 3 times in PBS for 5 minutes each, and cover slips were mounted with mounting medium containing DAPI. The slides were observed using LSM510 laser scanning confocal microscope (LSCM) (Zeiss, Heidelberg, Germany) at x400 magnification, with excitation wavelengths set at 488 nm and emission filter at 530 nm, or with a DMR-X fluorescence microscope, with detector filter set at 3 (Green filter) (Leica, Germany).

## **2.22 Confocal microscopic detection of uptake of alexa fluor 546/488 labelled cisplatin**

HepG2 and HepG2/cr cells were grown in 4-well glass Lab-Tek chamber slides (Nalge Nunc International, USA). Then, cells were incubated with Alexa Fluor 546/488-labelled cisplatin (F-CDDP) (Molecular probes, Life Technologies, UK), at a final concentration of 200 U/ml (1 unit is defined as the reagent solution required to label 25 ng of DNA *in vitro*) (Yoshida, et al., 2011) for up to 2 hours. Then, cells were washed with ice-cold PBS and fixed with ice-cold 70% ethanol for 15 min at -20 °C. Subsequently, cells were washed with ice-cold PBS, mounted with fluorescence mounting medium (Sigma Aldrich, UK) and sealed with clear nail polish. The slides were then inspected using a LSM510 laser scanning microscope (Zeiss, Heidelberg, Germany), at x400 magnification, with wavelengths set at 543/530 nm for red fluorescence and 488/530 nm for green fluorescence.

## **2.23 Alexa fluor 488-labelled cisplatin uptake assay by flow cytometry**

$5 \times 10^5$  cells were grown in 6-well culture plates for 24 hours, then treated or not-treated (controls), with Alexa fluor 488-labelled cisplatin, at a final concentration of 40 U/ml for up to 2 hours. Then, cells were collected by trypsinisation, washed with ice-

cold PBS and re-suspended in 300 µl ice-cold PBS. 10,000 cells were analysed immediately by BD Accuri C6 flow cytometry (BD Biosciences, UK) with the fluorescence intensity detector set at FL-1 (FITC; excitation max 488 nm and emission max 530 nm).

## **2.24 Cellular uptake of dansylcadaverine by fluorescence microscopy**

A cellular uptake assay was performed by the method described by Hummerich and Schloss (2010), with some modifications. Briefly,  $1 \times 10^5$  cells were grown on coverslips in 6-well culture plates. After 24 hours, cells were treated with 0-20 µM of dansylcadaverine, in the presence or absence of 2 mM cystamine, for up to 4 days. Following incubation, the medium was removed and coverslips with the cells were washed 2 times with PBS, for 5 minutes each. Then, cells were then fixed in 4% paraformaldehyde at 4 °C for 20 minutes. Coverslips were mounted in fluorescent mounting medium and inspected using a DMR-X fluorescence microscope (Leica, Germany) at x200 magnification, with the detector filter set at 3 (green filter). Photographic images were then recorded, using a microscope equipped with ProgRes Capture 2.7 software.

## **2.25 Pepsin modification reaction**

The incorporation of dansylcadaverine (DNC) into pepsin was carried out in 0.5 ml of 40 mM Tris-HCl, pH 8.0, with a final concentrations as follows: 2 mg/ml pepsin; 1 mM DNC; 10 mM DTT; in the presence or absence of 10 mM  $\text{CaCl}_2$ . The commercial guinea pig transglutaminase (see section 2.19) was added in a ratio 1:10 w/w of enzyme to pepsin substrate. In negative controls, EDTA was added to give a final concentration of 30 mM. The reaction mixtures were incubated at 37 °C for up to 24 hours. After predetermined time intervals (0, 1, 2, 5, 10, 24 hours), 100 µl of the reaction mixtures were removed for quantitative analysis (section 7.2.4) and the reaction was quenched by adding 30 mM of EDTA.

## **2.26 Gel filtration chromatography**

PD-10 Sephadex G-25 desalting columns (Sigma Aldrich, UK) were used for the separation of modified pepsin. The columns were initially equilibrated with 40 mM Tris-HCl buffer, pH 8.0, before 2 ml of sample mixtures in Tris-HCl buffer were applied to the column and the proteins were recovered by collecting 25 x 1 ml aliquots of eluent in single Eppendorf tubes. The absorbance of eluent was measured at a wavelength of 330 nm, at which dansylcadaverine gives maximum fluorescence (see figure 7.2).

## **2.27 High Performance Liquid Chromatography (HPLC)**

DNC-labelled pepsin was purified, using semi-preparative HPLC (Perkin Elmer, UK) on a C18 reverse phase column (260 x 4.60 mm), attached with the ultraviolet eluent detector. For analysis, 20 µl of reaction mixtures were mixed with up to 1 ml acetonitrile and applied to a column with a linear gradient of buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile). The samples were eluted for 30 minutes, at a flow rate equal to 1 ml/minute at ambient temperature. The eluted samples were detected and measured on the basis of UV absorption at 215 nm.

## **2.28 Immunoprecipitation**

To isolate DNC-labelled proteins from cell lysates (see section 7.2.6), 200 µl of cell lysate were used. To each sample, 5 µl (5 µg) of anti-dansyl rabbit antibody (cat no. A-6398) (Invitrogen, UK) were added and samples were incubated on a rotating mixer shaker for 1 hour, or overnight, at 4 °C. Then, 30 µl of protein-A agarose slurry were added and incubated for another hour at 4 °C. The samples were then centrifuged at 1000 x g for 3 minutes, to pellet immunocomplexes from the mixture. Pellets were washed 4 times with RIPA buffer, with centrifuging each time at 600 x g for 2 minutes. Following the final wash, the buffer was completely removed and 50 µl of 2x SDS-Sample buffer was added to each pellet. Samples were then heated at 95 °C for 5



minutes, and centrifuged at 10,000 x g for 5 minutes, before supernatants containing immunoprecipitated DNC-labeled proteins were separated on 12% SDS-PAGE gels.

### **2.29 Endoproteinase digestion**

The endoproteinase Glu-C (Sigma Aldrich, UK) was used to digest the DNC-labelled pepsin. The protein digestion was carried out in 100 mM Tris-HCl, pH 7.8, for 18 hours at 37 °C, at ratios of 1:20, 1:50, 1:100, and 1:200 (w/w) of enzyme to substrate. The peptide fragments were then separated by SDS-PAGE and gels were monitored under an ultraviolet fluorescent gel reader for detection of fluorescent bands, photographed and then stained with Coomassie blue dye.

### **2.30 Statistical analysis**

All statistical analyses were performed using GraphPad Prism 6/7 software (GraphPad, USA). p values < 0.05 were considered to be statistically significant. The statistically significant differences in mRNA expression levels, enzyme activities, cell viability and cell death rates between the control groups and various treatment groups were assessed using the Student's t test, and one way ANOVA with Dunnett's multiple comparison test, wherever applicable. The p values are denoted symbolically as follows: 0.01-0.05 (\*) = significant; 0.001-0.01 (\*\*) = highly significant; 0.0001-0.001 (\*\*\*) = very highly significant; and < 0.0001 (\*\*\*\*) = extremely significant.

## **CHAPTER 3:**

# **TRANSGLUTAMINASE 2 INVOLVEMENT IN CELLULAR UPTAKE OF DANSYLCADAVERINE**

### **3.1 Introduction**

The purpose of this chapter is to preliminarily determine whether TG2 has any role in cellular uptake of amine-based compounds using dansylcadaverine as an autofluorescent reporter molecule, in parental HepG2 cell lines. This work was conducted as a pilot study before conducting further investigations on the uptake of the anti-cancer drug cisplatin both in parental and chemoresistant cells; the results of the latter work is discussed in subsequent chapters.

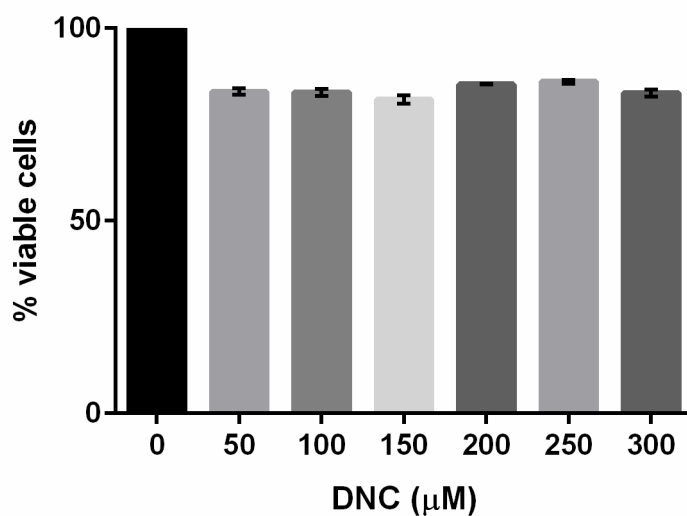
Dansylcadaverine (DNC) is a substrate and an inhibitor of transglutaminase 2 (Nunomura, et al., 2003). It is used in several studies to determine its inhibitory effects on TG2 enzymatic activity. In this chapter, the role of TG2 in cellular uptake of DNC in HepG2 and CAKI2 cell lines was investigated. Findings from this chapter suggest a role for TG2 in cellular uptake of DNC and lead on to further studies investigating the role of TG2 in cellular uptake of the anti-cancer drug cisplatin in chemoresistant cells (chapter 4-6).

### **3.2 Results and discussion**

#### **3.2.1 Determination of dansylcadaverine toxicity to HepG2 cells**

DNC was chosen for cellular uptake studies as it is a relatively non-toxic molecule (Ray and Samantha, 1996). For determination of cytotoxicity of dansylcadaverine, cells were incubated with 0-300  $\mu\text{M}$  DNC for 48 hours and toxicity was measured by CCK-8 assay. Significantly, no toxic effect was seen with DNC (figure 3.1). Although there is no cytotoxicity of DNC measured over the 0-300  $\mu\text{M}$  range, a relatively low concentration of 10  $\mu\text{M}$  was chosen for uptake studies as below 100  $\mu\text{M}$  DNC acts as

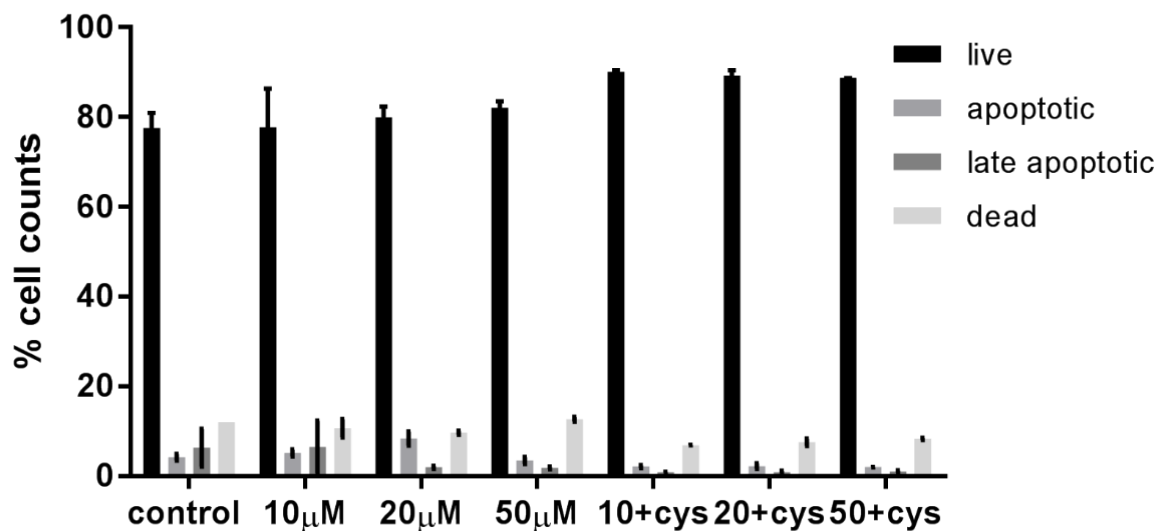
a substrate, whereas over 100  $\mu\text{M}$  DNC acts as an inhibitor of TG2 and it was felt that higher concentrations of DNC treatment might therefore add an extra level of complexity to the interpretation of results.



**Figure 3.1 Dansylcadaverine cytotoxicity to HepG2 cells.**

HepG2 cells were incubated over a range of 0-300  $\mu\text{M}$  DNC for 48 hours and toxicity was measured using the CCK-8 viability assay. Results are  $\pm$ SD of triplicate experiments.

The combined cytotoxicity of DNC and the TG2 inhibitor cystamine was measured by flow cytometric analysis, using cells that had been incubated for 4 days, either with, or without 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , 50  $\mu\text{M}$  DNC, both in the presence or absence of 2 mM cystamine.



**Figure 3.2 Apoptosis and cytotoxicity determination after DNC and cystamine treatment.**

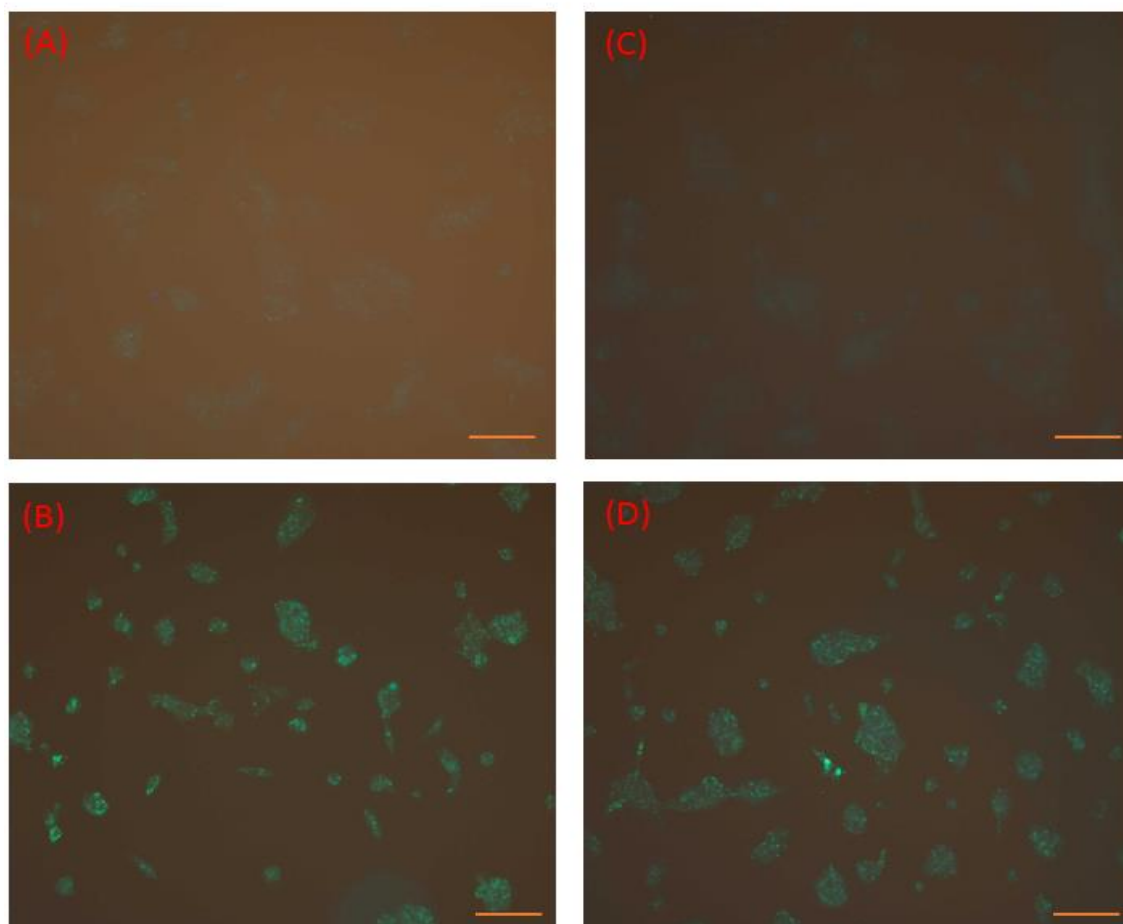
HepG2 cells were treated with 0-50  $\mu$ M DNC either in the presence or absence of 2 mM cystamine (cys). After 4 days, cell viability was observed by FITC-labelled annexin-V by flow cytometry.

Cells survived in the presence of cystamine for up to 4 days at non-lethal doses of DNC (figure 3.2).

### 3.2.2 Cystamine treatment increases internalisation of dansylcadaverine

Whereas some drugs can cross the plasma membrane by passive diffusion, it is now established that the cellular uptake of many molecules occurs through specific mechanisms of endocytosis (carrier-mediated transport) (Mellman and Yarden, 2013). In order to investigate whether transglutaminase (TGase) is involved in such mechanisms of cellular uptake, HepG2 cells were incubated with 5  $\mu$ M and 10  $\mu$ M DNC in the presence or absence of TG2 inhibitor 2 mM cystamine, and cellular uptake was examined by fluorescent microscopy. Cystamine inhibits TG2, most likely by forming a mixed disulphide bond with cysteine residue(s) of the catalytic core. Also, it has been suggested that, in addition to inhibiting TG2, cystamine may also interfere with and possibly inhibit other thiol-dependent enzymes. Although cystamine is a non-

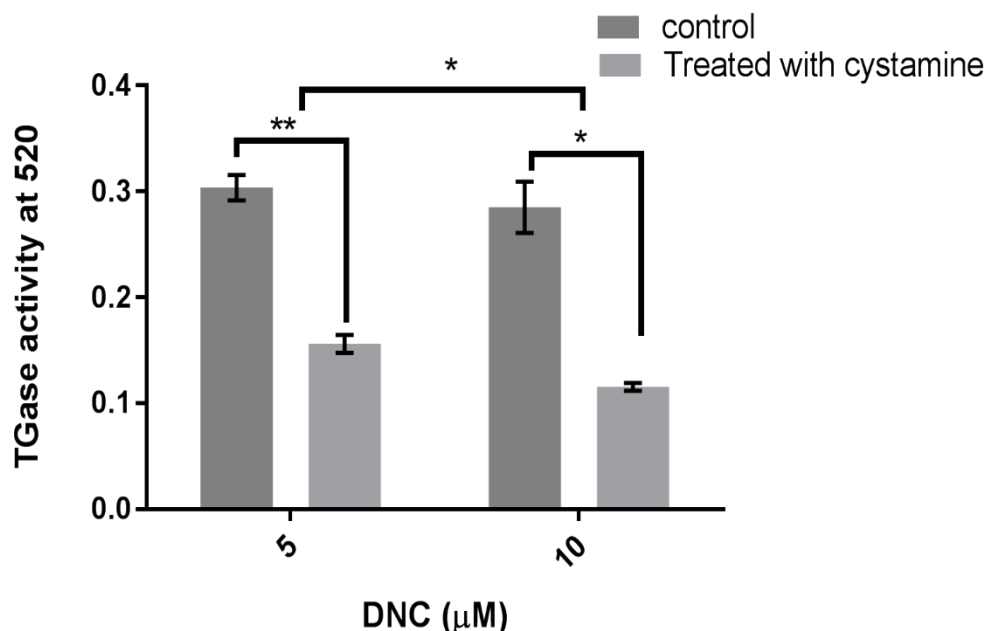
specific compound, it is a more efficient modulator of TG2 activity than many other enzymes.



**Figure 3.3 Cellular uptake of dansylcadaverine in HepG2 cells.**

HepG2 cells were grown on coverslips and incubated with DNC either in the presence or absence of 2 mM cystamine for 4 days. After 4 days, cells were washed, fixed and mounted with fluorescent mounting medium and observed using a DMR-X fluorescence microscope at x200 magnification. Scale bar = 25  $\mu$ m. a) 5  $\mu$ M DNC, b) 5  $\mu$ M DNC + cystamine, c) 10  $\mu$ M DNC, d) 10  $\mu$ M DNC + cystamine. All images were taken using Progress-Capture software with collapse time at 3215 Sec and 0 gains.

The results show that after 4 days of incubation, there was no significant uptake of DNC in control (untreated cells) (figures 3.3A and C). However, treatment of cells with TG2 inhibitor cystamine resulted in a large increase in DNC uptake by cells (figures 3.3B and D).



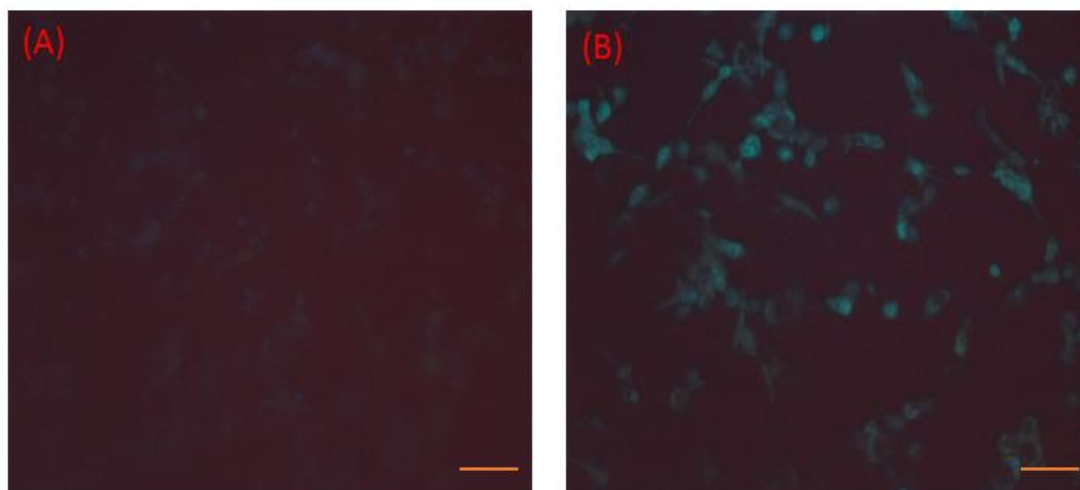
**Figure 3.4 Transaminase activity is decreased during cellular uptake of dansylcadaverine.**

HepG2 cells were treated with different concentrations of dansylcadaverine (DNC) in the presence or absence of 2 mM cystamine for 4 days. Transglutaminase activity was measured from cell lysates using CBZ-Gln-Gly assay. The activity is represented as optical density measured at 520 nm. The statistical differences between treated and untreated groups were calculated by Student's paired t-test

The total transglutaminase activity was significantly reduced in cystamine-treated cells (figure 3.4), indicating that transglutaminase may be involved in the internalisation of DNC by cells.

### 3.2.3 TG2 gene silencing increased uptake of dansylcadaverine.

To confirm that the presence of TGase in the cell was obstructing the uptake of DNC, the expression of the TG2 gene was inhibited by transfecting siRNA molecules into the cells. The HepG2 cells were treated or not-treated with 30 pmol siRNA specific to the TG2 gene in the presence of 10 μM DNC for 4 days, with the help of transfecting agent lipofectamine2000.



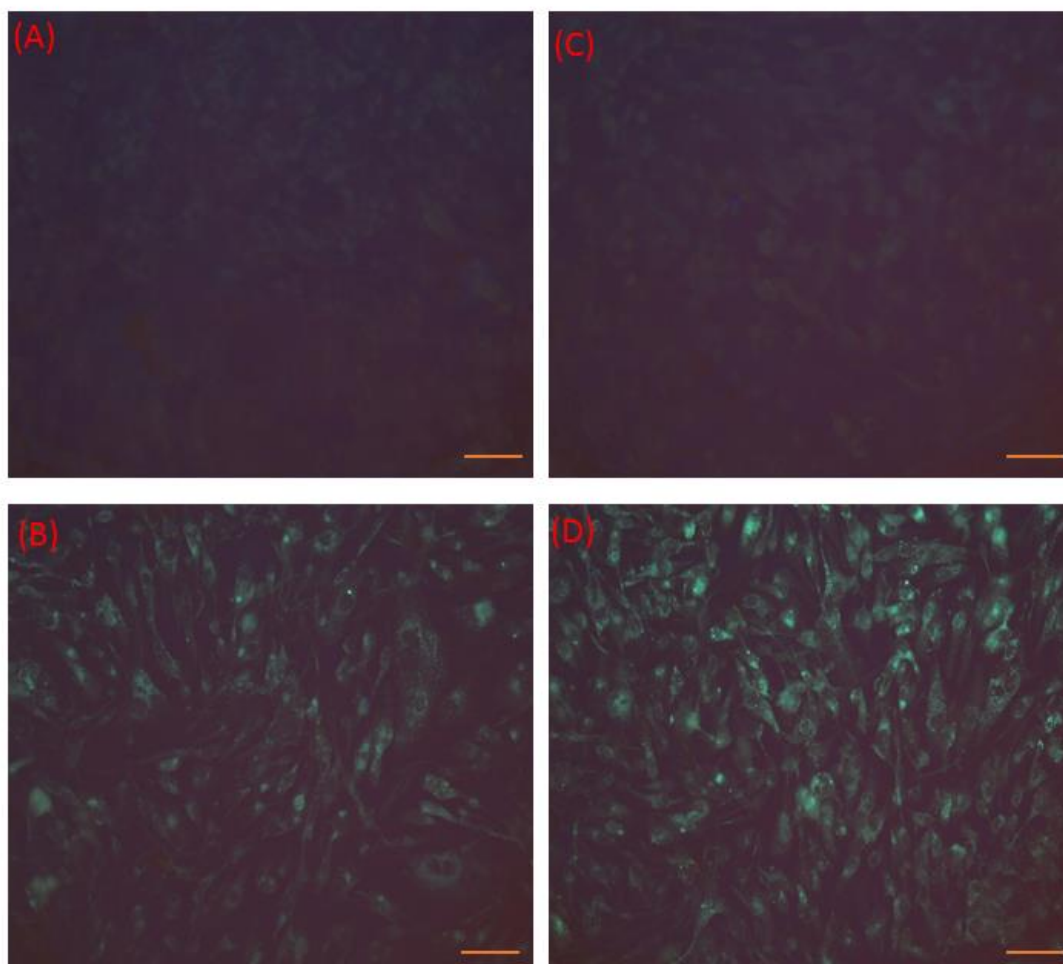
**Figure 3.5 TG2 gene silencing increased uptake of DNC.**

HepG2 cells were treated or not-treated with siRNA in the presence of 10  $\mu$ M DNC and incubated for 4 days. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde, mounted with fluorescence mounting medium and observed using a DMR-X fluorescence microscope at x100 magnification; Scale bar = 50  $\mu$ m. (A) 10  $\mu$ M DNC + lipofectamine, and (B) 10  $\mu$ M DNC + lipofectamine + siRNA.

The results indicate that the inhibition of TG2 caused accumulation of DNC into treated cells (figure 3.5B), whereas control (siRNA untreated) cells showed considerably less DNC uptake (figure 3.5A).

### **3.2.4 Cellular uptake of DNC is increased in CAKI2 cell line**

The same effects on the cellular uptake of DNC were observed in CAKI2 cells after inhibition of TG2 i.e., after 4 days of incubation, there was no significant uptake of DNC in control (cystamine untreated) cells (figures 3.6A and C), whereas there was visible uptake of DNC into cystamine treated cells (figures 3.6B and D).



**Figure 3.6 Dansylcadaverine uptake in CAKI2 cells after 4 days.**

CAKI2 cells were incubated with 5  $\mu$ M and 10  $\mu$ M DNC in presence or absence of 2 mM cystamine for 4 days. Then cells were fixed, mounted in fluorescence mounting medium and inspected using DMR-X fluorescent microscopy at x200 magnification. Scale bar = 25  $\mu$ m. (A) 5  $\mu$ M DNC, (B) 5  $\mu$ M DNC + 2 mM cystamine, (C) 10  $\mu$ M DNC, and (D) 10  $\mu$ M + 2 mM cystamine.

Results thus indicated that TG2 may have been involved in DNC transport both in liver (HepG2) and kidney (CAKI2) cells. However, the exact relationship between TG2 and transport of drugs across the cell membrane needs to be investigated.



### **3.3 Conclusion**

Collectively, these results indicate that the presence of TG2 in the cells may be responsible for reducing the cellular uptake of dansylcadaverine both in HepG2 and CAKI2 cell lines, and therefore may possibly be involved in the modulation of uptake of other compounds/drugs in these and other cell types.

## **CHAPTER 4:**

### **TRANSGLUTAMINASE 2 RESPONSE TO CISPLATIN**

### **TOXIFICATION OF HEPATOCARCINOMA CELLS**

#### **4.1 Introduction**

This chapter provides information as to how TG2 responds to cisplatin treatment before the development of resistance of HepG2 cells to cisplatin; this information is necessary in determining the role of TG2 in the mechanism(s) of the development of drug resistance.

Over the past two decades, TG2 involvement has been shown in several apoptotic as well as anti-apoptotic pathways, both *in vivo* and *in vitro*, though the precise mechanism of its action remains currently poorly understood (Fésüs and Szondy, 2005; Cao, et al., 2008; Cho, et al., 2010; Cho, et al., 2012; Yoo, et al., 2012; Rossin, et al., 2012; Hsieh, et al., 2013). Indeed, whether TG2 facilitates or ameliorates apoptosis appears to be dependent on the types of stressors and cells, and the specific isoform of TG2 that is expressed (Antonyak, et al., 2006). Some studies have shown that transamidation activity is required for cell death, whereas other studies have suggested a role for transamidation activity in cell survival (Gundemir and Johnson, 2009b; Gundemir, et al., 2013). TG2 has been found to be expressed in several cellular compartments, including the nucleus, cytoplasm, mitochondria, and extracellular matrix (ECM), depending on the cell's physiological and pathological conditions (Nurminskaya and Belkin, 2012; Eckert, et al., 2014; Piacentini, et al., 2014).

Some reports have also shown that TG2 overexpression and activation have been linked to induction of the initiation of apoptosis, after treatment with chemotherapeutic drugs (Fok and Mehta, 2007; Robitaille, et al., 2008; Park, Choi and Ha, 2010; Yoo, et al., 2012; Hsieh, et al., 2013). Other studies show that down-regulation of TG2

appears to sensitize cancer cells to apoptosis (Nadalutti, et al., 2011). Consequently, different observations related to TG2 expression might reflect altered roles for TG2 in different cell lines and may be dependent on the specific conditions used for investigation (Nadalutti, et al., 2011).

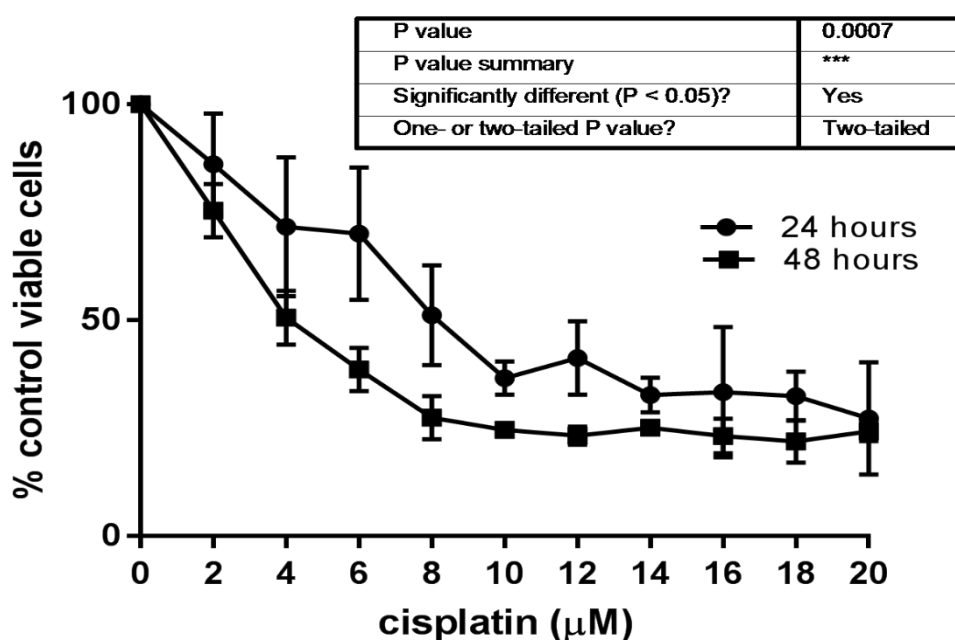
Cisplatin is one of the most effective drugs in the treatment of several types of cancer. However, owing to the limitation of its cytotoxic side effects on healthy cells and consequent damage to vital organs, it cannot be used at high doses i.e., >100 mg/m<sup>2</sup>. This problem is increased, as cancer cells that are able to evade the cytotoxic effects of low dose cisplatin treatment become resistant to normal concentrations used for treatment (Shen, et al., 2012). Mechanistically, cisplatin appears to cause cytotoxicity in cancer cells via nucleophilic attack, inducing cross-linking of DNA, defective repair of DNA and through depletion in the cellular levels of glutathione and metallothionein that are normally required to inactivate cisplatin (Gonzalez, et al., 2001; Dasari and Tchounwou, 2014; Galluzzi, et al., 2014). Cisplatin also can bind to RNA and other enzymes (Gonzalez, et al., 2001); the ways that cisplatin exerts its effects are still not fully understood.

TG2 has been found to be induced and activated upon treatment with various anti-apoptotic agents in several models of apoptosis (Uray, Davies and Fesus, 2001). However, the mechanism controlling TG2 expression after drug treatment is unknown. In order to investigate the molecular mechanism of cisplatin in relation to apoptosis, and how TG2 contributes to apoptotic pathways, the effects of the cytotoxic chemotherapeutic drug cisplatin on TG2 expression profile of mRNA, protein and enzymatic activity in the presence and absence of retinoic acid, and TG2 inhibitor cystamine were investigated in the human liver cancer HepG2 cell line.

## 4.2 Results

### 4.2.1 Determination of cisplatin inhibitory concentration (IC<sub>50</sub>)

HepG2 cells were incubated with increasing concentrations of cisplatin (0-20  $\mu$ M) for 24 and 48 hours and cytotoxicity was measured by CCK-8 assay (see section 2.4). It was confirmed from the cell survival curve that cisplatin had a concentration-dependent cytotoxic effect on HepG2 cells, and as incubation time was increased, more cells died by apoptosis. The IC<sub>50</sub> values for 24 and 48 hours were 8  $\mu$ M and 4  $\mu$ M, respectively (figure 4.1).



**Figure 4.1 Cytotoxicity of cisplatin to HepG2 cells.**

HepG2 cells were incubated with increasing concentrations of cisplatin over 24 and 48 hours and cell viability was measured by CCK-8 assay. The IC<sub>50</sub> value from 24 hours' incubation was statistically different from the IC<sub>50</sub> of 48 hours incubation. The \*\*\*p value = 0.0007 by two-tailed Student's paired t-test. Results are means  $\pm$  SD of triplicate assays from five independent experiments.

The IC<sub>50</sub> values were confirmed by flow cytometric analysis of cell death using Annexin V assay (see figure 5.1 in chapter 5). The IC<sub>50</sub> values obtained were different from the IC<sub>50</sub> previously reported from various studies (Table 4.1). However, the cells'

response to drug may be depend on the cells' condition/passage number and methods used for the cytotoxicity assay.

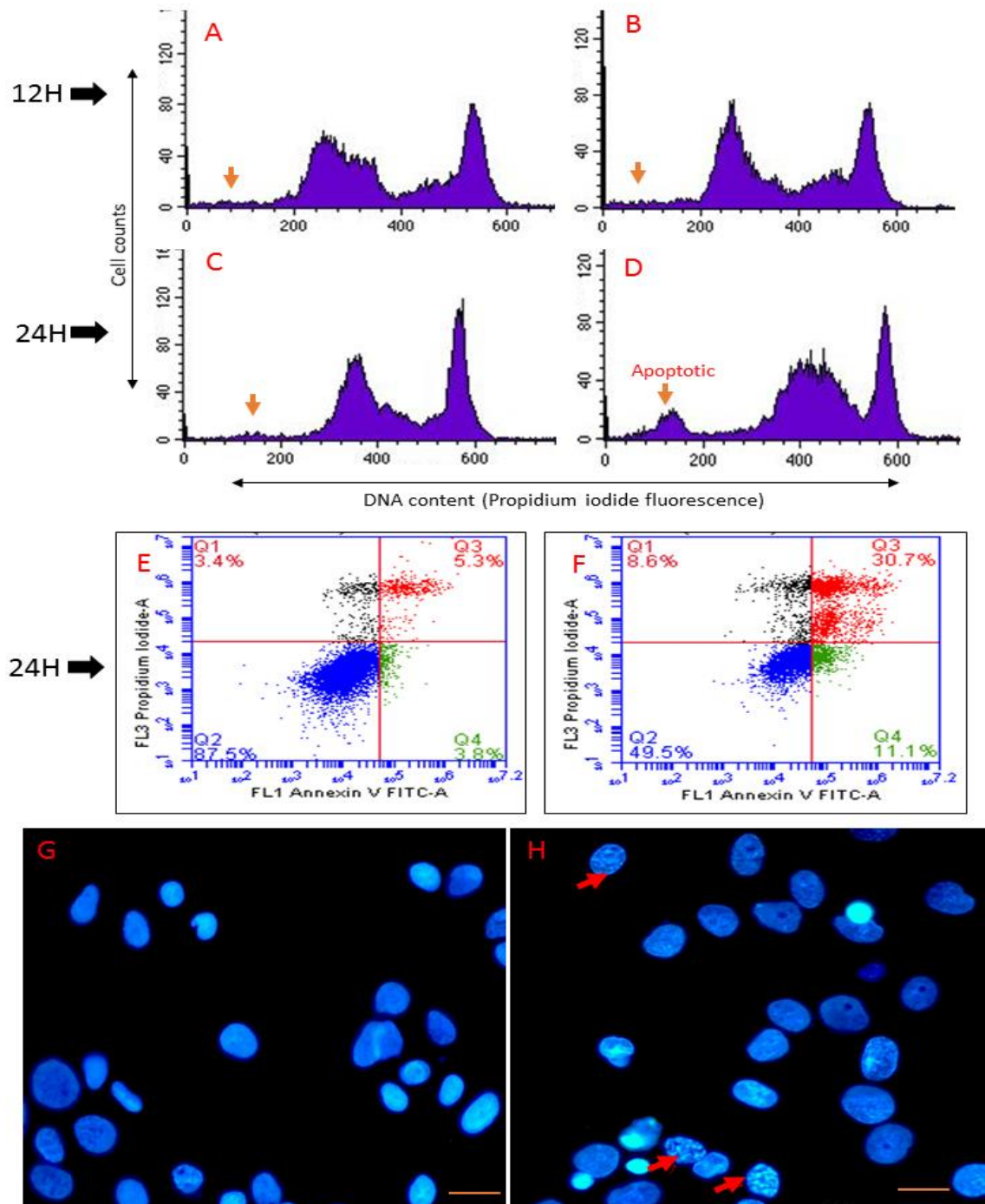
**Table 4.1 IC<sub>50</sub> values in HepG2 cell line obtained from different studies after 24 hours' incubation**

| IC <sub>50</sub> | Methods | References                     |
|------------------|---------|--------------------------------|
| 8.3 $\mu$ M      | MMT     | (Luo, Jin and Huang, 2012)     |
| 33.3 $\mu$ M     | MMT     | (Uray, Davies and Fesus, 2001) |
| 106.3 $\mu$ M    | MMT     | (Qin and Ng, 2002)             |
| 4 $\mu$ M        | CCK-8   | (Odii and Coussons, 2012)      |
| 40 $\mu$ M       | MMT     | (Zhang, Niu and Zhou, 2010)    |

#### **4.2.2 Apoptosis and cell cycle determination after cisplatin treatment**

Following treatment of HepG2 cells with 8  $\mu$ M cisplatin for 12 and 24 hours, induction of apoptosis was measured by harvesting cells and estimating the DNA-based propidium iodide levels. Although cisplatin is known to induce apoptosis as early as at 12 hours after treatment at high dose (50  $\mu$ M) (Qin and Ng, 2002), results indicated that there were no signs of apoptosis at this point with 8  $\mu$ M cisplatin (figures 4.2A and B). However, following incubation for 24 hours, cisplatin-treated cells generated an extra apoptotic peak before G<sub>0</sub>/G<sub>1</sub> phase (indicated by the arrow in figure 4.2D), that was not apparent in untreated control cells (figure 4.2C). When incubation time was increased to 48 hours, more cells became apoptotic (see figure I.3 in appendix I). The morphological nuclear changes and the formation of apoptotic cell bodies were confirmed by DAPI staining observed by fluorescent microscopy. While untreated cells had a regular nuclear shape and showed no DNA condensation and/or fragmentation (figure 4.2G), cisplatin-treated cells revealed larger and irregularly-shaped nuclei, and showed signs of both DNA condensation and fragmentation (figure 4.2H). Further evidence that apoptosis had been induced by cisplatin

treatment was provided by FITC-labelled Annexin-V flow cytometric analysis (figures 4.2E and F), that indicated the presence of phosphatidylserine on the surface of cisplatin-treated cells, following incubation for 24 hours.

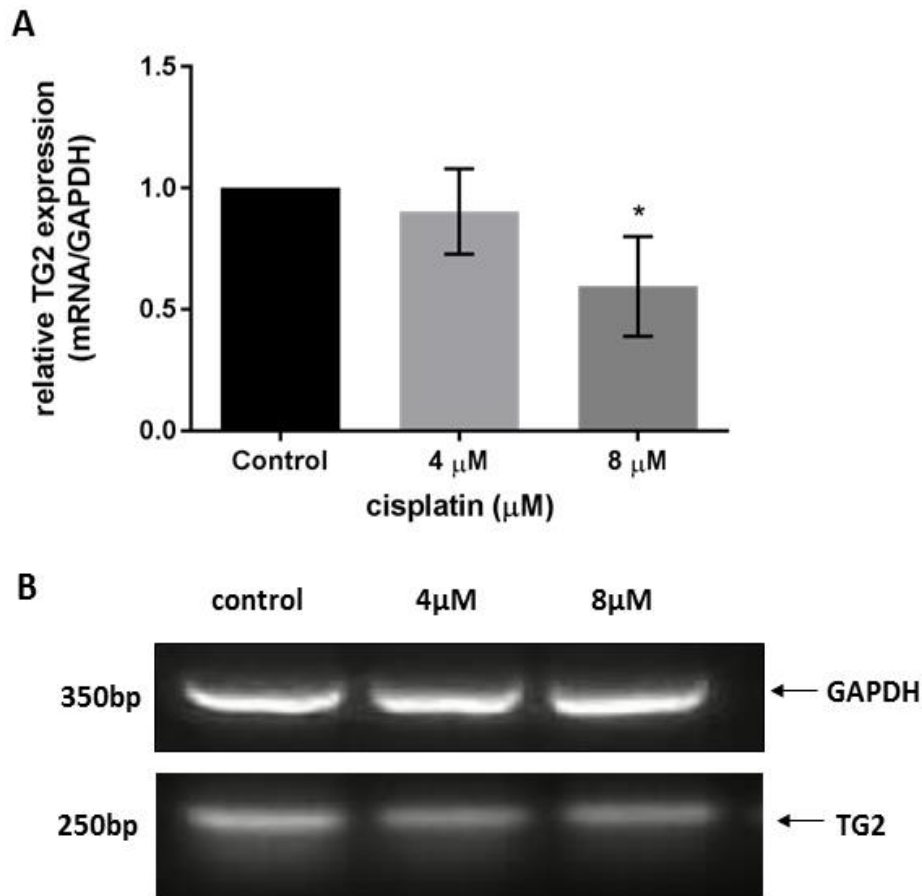


**Figure 4.2 Apoptosis induction following cisplatin treatment.**

Apoptosis in HepG2 cells was measured by flow cytometry using propidium iodide-staining. Cells were treated with 8  $\mu$ M cisplatin for 12 hours (A - control; B - cisplatin treated), or 24 hours (C - control; D - cisplatin treated). FITC-labelled Annexin V flow cytometric analysis of apoptosis was conducted after incubation for 24 hours (E - control; F - cisplatin treated). In figures E and F, Q1 indicates dead cells; Q2 - live; Q3 - late apoptotic; Q4 - early apoptotic. DNA condensation and fragmentation analysis by fluorescent microscopy with DAPI staining is shown after treatment for 24 hours with or without 8  $\mu$ M cisplatin (G - control; H - cisplatin treated), where the red arrows indicate cells undergoing apoptosis (x200 magnification); Scale bar = 25  $\mu$ m. Results are representatives of triplicate experiments. Untreated cells were used as controls.

#### 4.2.3 TG2 transcriptional expression after treatment with cisplatin

To investigate the molecular mechanism of cisplatin in relation to apoptosis, and the role of TG2 in apoptotic pathways, the effect of cisplatin treatment on TG2 gene expression in HepG2 cells was studied. Cells were treated with 0, 4 and 8  $\mu$ M cisplatin for 6, 12 and 24 hours, respectively.



**Figure 4.3 Effects of cisplatin on TG2 at the transcriptional level.**

(A) TG2 mRNA expression measured by quantitative RT-PCR. HepG2 cells were incubated with cisplatin for 24 hours. Results are means  $\pm$ SD of triplicates of three independent experiments. The \* $p = 0.01$  by one way ANOVA with Dunett's multiple comparison test relative to the control. (B) PCR products analysed by 1.2% agarose gel electrophoresis (lanes: 1 – control; 2 - 4  $\mu$ M cisplatin; 3 - 8  $\mu$ M cisplatin). PCR products were confirmed against available online data from which primers were designed (NCBI Nucleotide database).

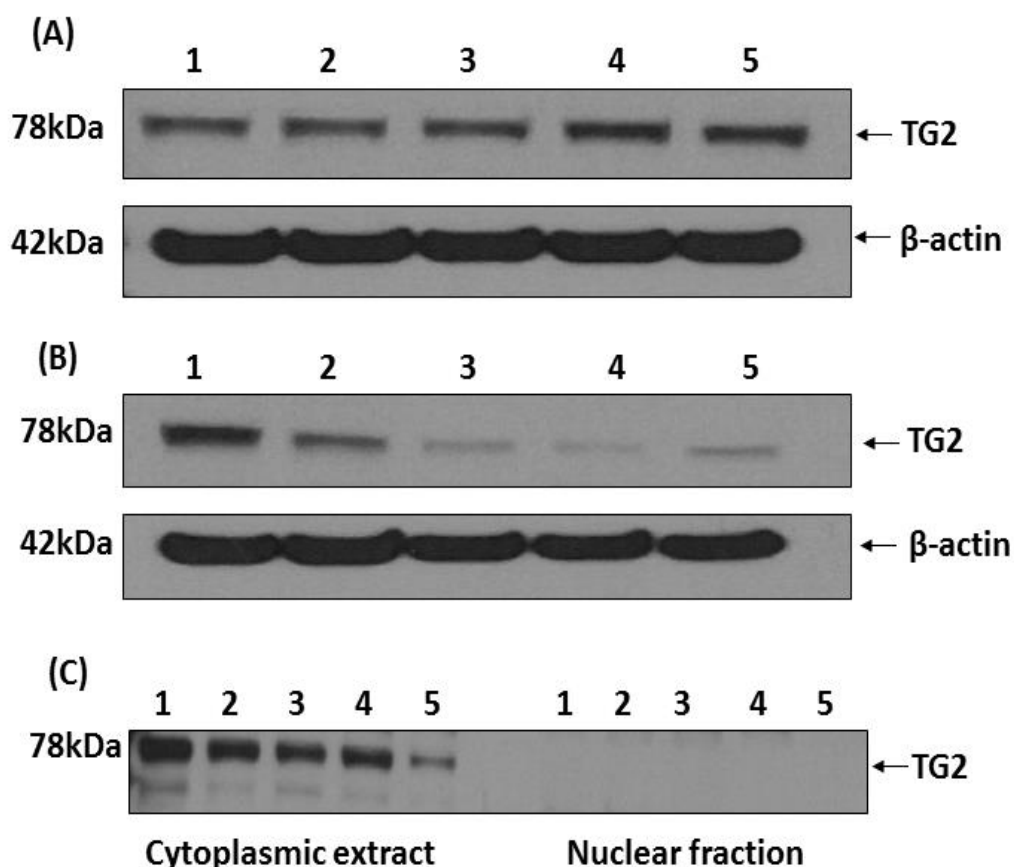
Gene expression at the mRNA level was measured by RT-PCR using specific primers designed for TG2 mRNA. HepG2 cells treated for 6 and 12 hours expressed the TG2



gene at a low level; no significant difference was observed when controls compared to treatments groups (not shown). This trend continued and after 24 hours, TG2 mRNA expression decreased in cisplatin-treated cells compared to control groups (figure 4.3A). This result was independently confirmed by isolating PCR products by agarose gel electrophoresis (figure 4.3B). The housekeeping gene GAPDH was used for normalisation of any general changes due to cisplatin.

#### **4.2.4 TG2 protein translational expression after cisplatin treatment**

To determine the relationship between cisplatin and TG2 protein expression during apoptosis, cells were treated with 0-16  $\mu$ M cisplatin for 12 and 24 hours. After incubation of cells for 12 hours, no change was detected in TG2 protein expression (figure 4.4A). However, after incubation for 24 hours, like its mRNA, TG2 protein expression was reduced in cisplatin-treated cells compared to untreated cells (figure 4.4B), and apoptosis was induced (figure 4.2D). This suggests that cisplatin may induce apoptosis initiation by inhibiting mRNA and thus TG2 protein production. To determine the subcellular distribution of TG2 during cisplatin-induced apoptosis, cytoplasmic and nuclear fractions were isolated. TG2 was only seen in samples from cytoplasmic extracts and none was detected in the nucleus (figure 4.4C).

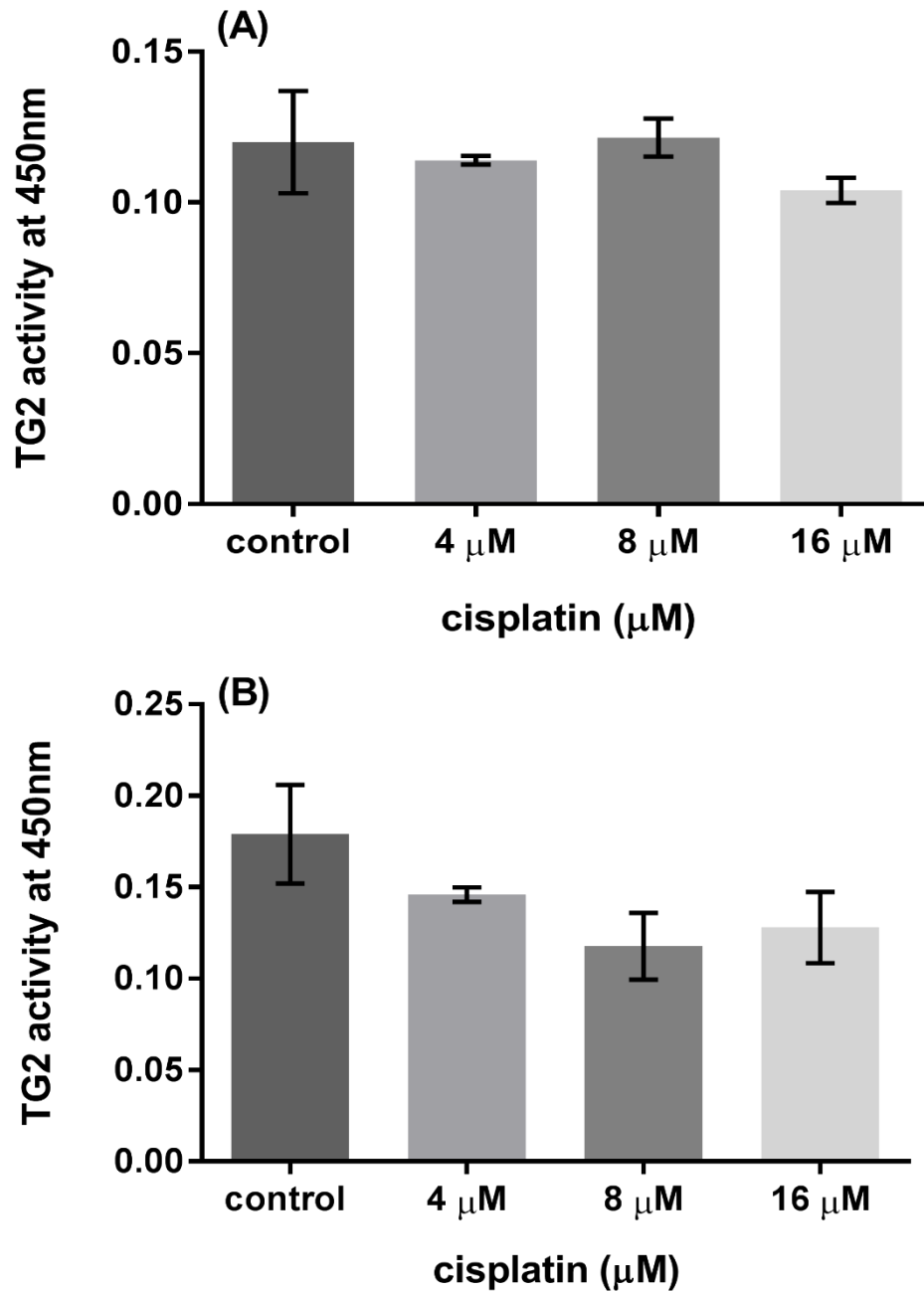


**Figure 4.4 Effects of cisplatin on TG2 protein expression.**

HepG2 cells were treated with cisplatin (lane 1 - control; lane 2 - 4 μM; lane 3 - 8 μM; lane 4 - 12 μM; lane 5 - 16 μM cisplatin) and expression was measured by Western blot analysis following treatment for (A) 12 hours, and (B) 24 hours. (C) The nuclear and cytoplasmic extracts were fractionated following incubation for 24 hours, either with or without cisplatin. The sample labelling of figure C, is same as of figure (B) for the lanes 1-5. The anti-TG2 antibody used was raised in the rabbit. Results are typical representatives of triplicate experiments.

#### 4.2.5 Effect of cisplatin treatment on TG2 transamidation activity

To investigate whether transamidation activity has any role during cisplatin-induced apoptosis, cell lysates were prepared following 12 and 24 hours' of treatment with 0-16 μM cisplatin. TG2 activity was measured in cisplatin-treated cell lysates, using a specific *in vitro* TG2 CovTest kit. The TG2 present in 60 μl of cell lysate was added to the reaction mixture and the remaining assay was performed according to manufacturer's instructions. The optical density was measured at 450 nm.



**Figure 4.5 Effects of cisplatin on TG2 transamination activity.**

Transglutaminase 2 activity was measured with the specific TG2-CovTest kit. Cells were treated with 0-16 μM cisplatin for (A) 12 and (B) 24 hours. Samples were derived from 60 μl of cell lysate. Results are means ±SD of triplicates of three independent experiments. The  $p = 0.05$  was not statistically significant between test and control groups calculated by one way ANOVA with Dunett's multiple comparison test.

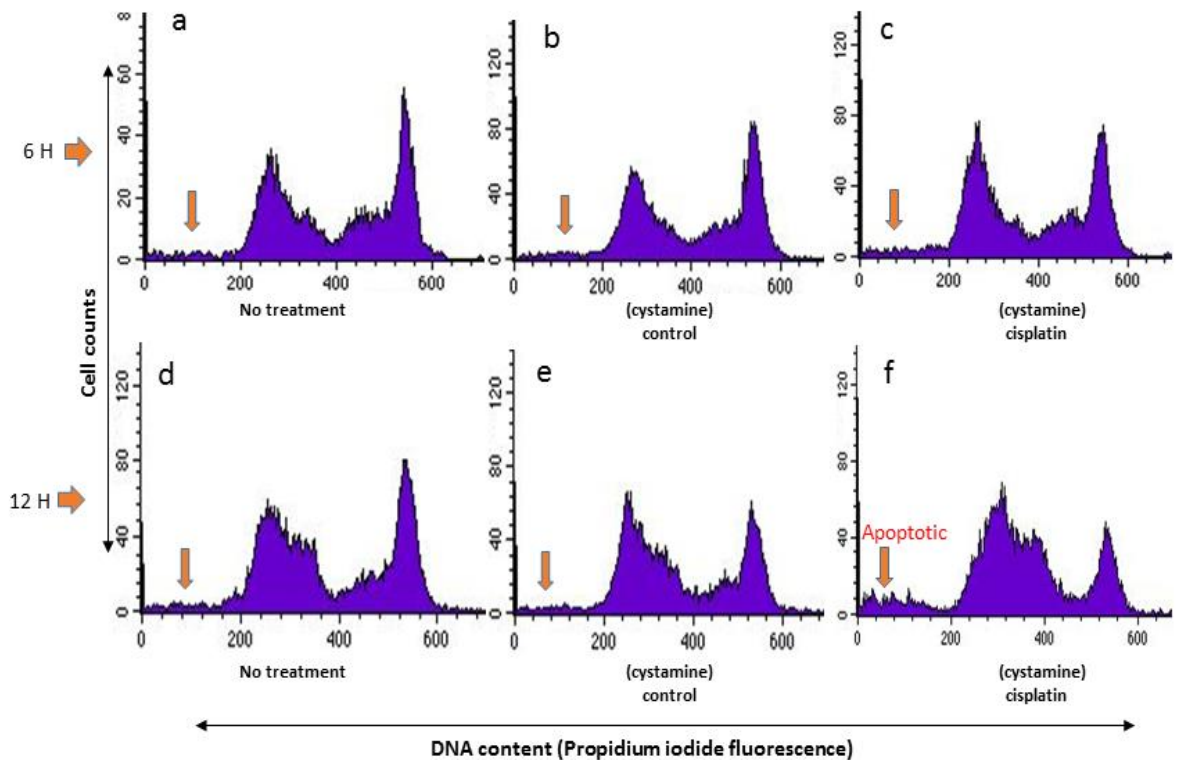
The pattern of TG2 activity broadly reflected that of TG2 protein expression. After incubation for 12 hours, TG2 transamination activity was little changed in cisplatin-

treated cells compared to untreated cells (figure 4.5A). After 24 hours, as cisplatin concentration was increased, TG2 transamidation activity decreased in a concentration-dependent manner, by approximately 50% compared to untreated cells (figure 4.5B), (although the p value was not statistically significant when compared between groups.  $p = 0.5$ ).

Although the reduction in TG2 activity appears to reflect the reduction in protein expression and the level of mRNA – there is a possibility that cisplatin could inhibit TG2 activity by competing with intracellular TG2 substrate proteins, possibly via amine groups present in cisplatin. Thus, this effect was tested directly *in vitro* on commercially-available guinea pig liver transglutaminase 2 activity (see section 4.2.10).

#### **4.2.6 Inhibition of TG2 activity by cystamine induced apoptosis from 12 hours post-treatment**

Cisplatin is known to induce apoptosis as early as at 12 hours post-treatment in the HepG2 cell line at a higher dose (50  $\mu\text{M}$ ) (Qin and Ng, 2002). To determine whether TG2 transamidating activity was involved in apoptosis induction, HepG2 cells were pre-incubated with or without a non-cytotoxic dose of 1 mM cystamine for 48 hours. Cells were subsequently left without further treatment (control), or, were treated with 8  $\mu\text{M}$  cisplatin and incubated for a further 6 to 12 hours.



**Figure 4.6 TG2 inhibition by cystamine induced apoptosis**

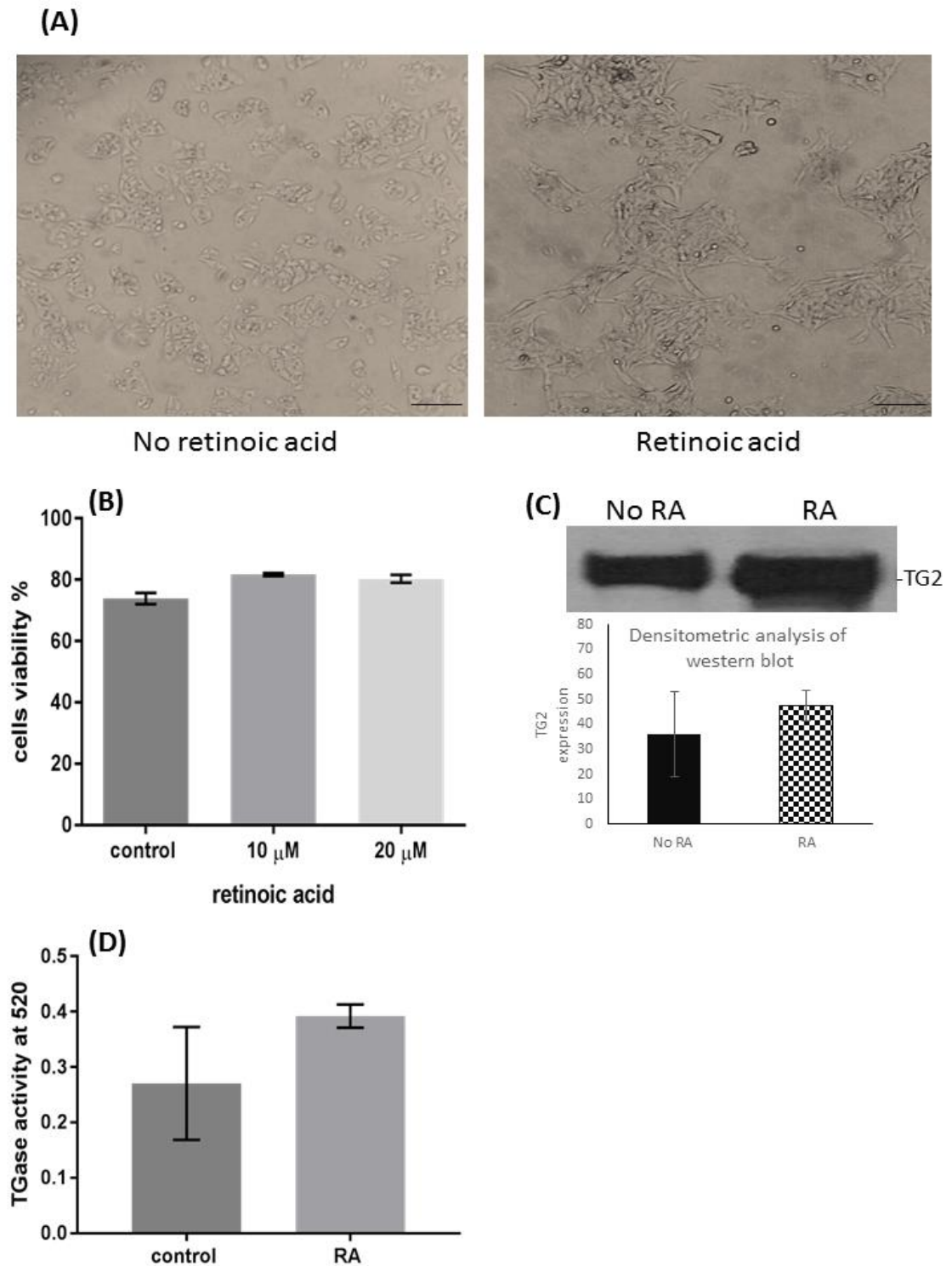
HepG2 cells were pre-incubated with or without a non-lethal dose of 1 mM cystamine for 48 hours. Then, cystamine-treated cells were left untreated or were treated with 8  $\mu$ M cisplatin for 6 and 12 hours, and cells were analysed by flow cytometry using propidium iodide staining.

Results indicate that initiation of apoptosis was induced as early as 12 hours post cisplatin-treatment in cystamine pre-treated cells (figure 4.6), whereas apoptosis was not induced in cisplatin-treated cells that had not been exposed to cystamine (see figure 4.2B).

#### 4.2.7 Retinoic acid increased TG2 expression

Retinoic acid (RA) is known to be one of the activators of TG2 at the transcriptional level, operating via nuclear receptors, in several cell types. It plays a role in cell differentiation. It has been used as a maintenance therapy for some cancers prior to testing with anti-cancer drugs. RA-activated TG2 has thus been implicated in cell survival (Singh, Li and Cerione, 1998; Ou, et al., 2000; Antonyak, et al., 2001; Singh, et al., 2001; Kweon, et al., 2004). In order to determine the level of up-regulation of

TG2 by RA, HepG2 cells were treated with 10  $\mu$ M RA continuously for 72 hours. As a preliminary step, the cytotoxicity of RA was measured by flow cytometric analysis. The results show that retinoic acid has no toxic effects on HepG2 cells at the concentration at which cells were treated; in fact RA-treated cells showed a higher percentage of live cells compared to non-treated cells (figure 4.7B). The morphology of cells was observed by inverted phase contrast microscopy, and showed that the RA-treated cells' shape changed, to resemble neuron-like structures (figure 4.7A). Both TG2 protein expression and enzymatic activity were increased in RA-treated cells compared to non-treated cells (figure 4.7C and D). TG2 was maximally expressed after 72 hours' of incubation.

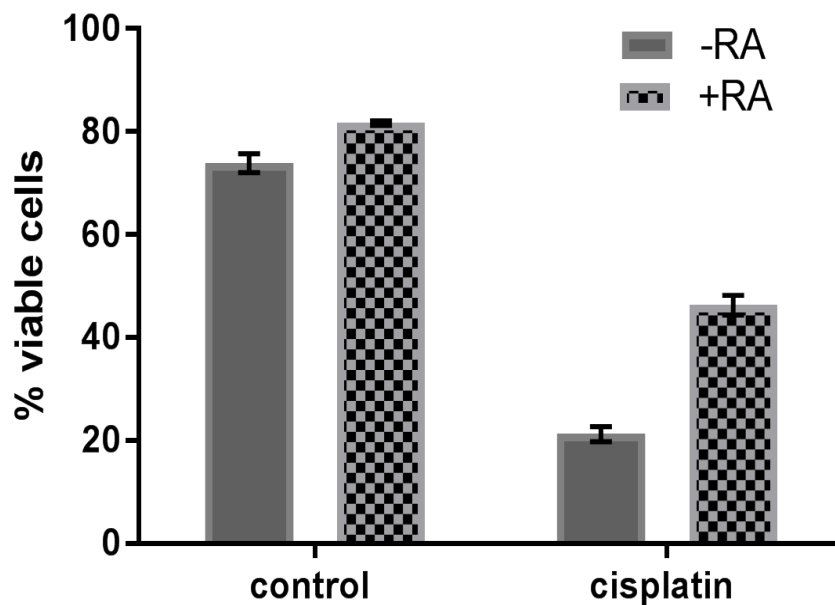


**Figure 4.7 Effect of retinoic acid treatment**

HepG2 cells ( $2 \times 10^5$  cells/25 cm<sup>2</sup> flask) were treated with 10  $\mu$ M retinoic acid (RA) continuously for 72 hours. After incubation, (A) changes in cell morphology were observed by inverted phase contrast microscopy, x200 magnification; images were taken using iphone 5 camera. Scale bar = 6.5  $\mu$ m. (B) toxicity of 0-20  $\mu$ M retinoic acid to cells was measured by flow cytometry, (C) TG2 protein expression was measured by Western blotting and densitometry analysis, (D) Transaminase activity was measured by CBZ-Gln-Gly assay. Plates A, C and D show effects of 10  $\mu$ M retinoic acid; TG2 was maximally expressed after 72 hours' of incubation.

#### 4.2.8 Protective role of RA treatment on cell death induced by cisplatin

Retinoic acid is a receptor activator for several enzymes at the transcriptional level including TG2, during the cell cycle and in cell differentiation. In order to test the hypothesis that overexpression of TG2 inhibits cisplatin-induced cell death, HepG2 cells were treated with 10  $\mu$ M RA for 72 hours. The cells were then treated with 8  $\mu$ M cisplatin for 48 hours and cell death was analysed by FITC-labelled Annexin V flow cytometric analysis, using untreated cells as controls.



**Figure 4.8 Effect of retinoic acid pre-treatment of HepG2 cells on cisplatin toxicity**

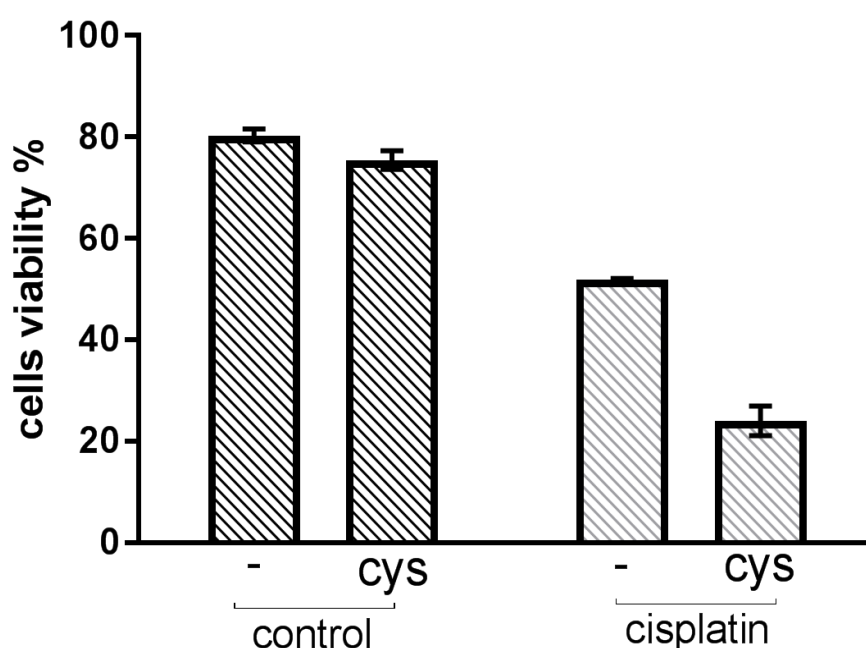
HepG2 cells ( $2 \times 10^5$  cells) were treated with 10  $\mu$ M RA and incubated for 72 hours. After incubation, cells were either not treated or were treated with 8  $\mu$ M cisplatin for a further 48 hours. Then samples were analysed by FITC-labelled Annexin V flow cytometry. Untreated cells were used as controls. The results are means  $\pm$ SD of triplicate experiments.

The results shown in figure 4.8, indicate that the RA-treated cells had a greater proportion of viable cells (50%), compared to RA-non-treated cells (20% viable cells) in cisplatin treated groups.



#### 4.2.9 Inhibition of TG2 decreased cell viability

To determine whether TG2 is involved in cellular protection against cisplatin treatment, HepG2 cells were incubated with 10  $\mu$ M RA for 72 hours. The RA-treated cells were pre-incubated with 1 mM cystamine for 48 hours and then treated (or left untreated in the case of controls) with 8  $\mu$ M cisplatin for 48 hours; cell viability was measured by flow cytometry.



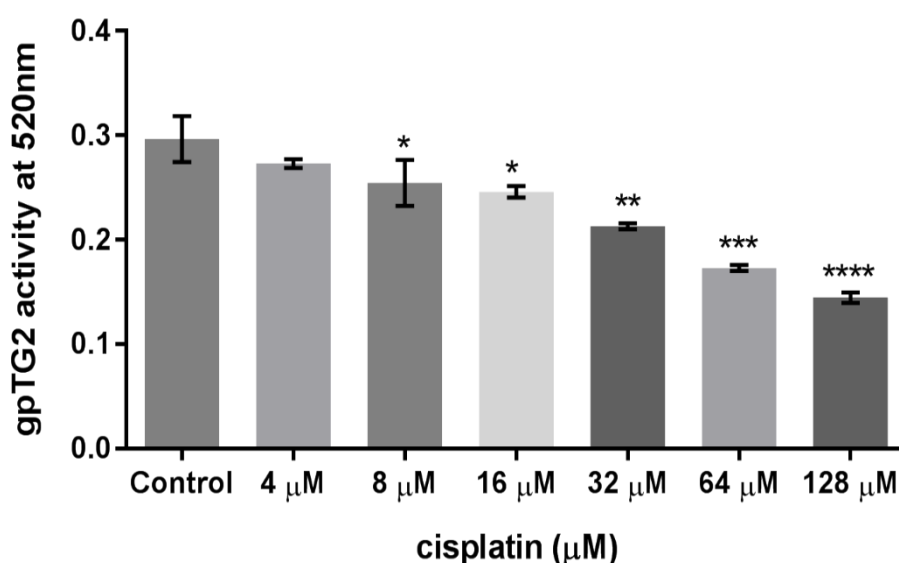
**Figure 4.9 Effects of pre-treatment of HepG2 cells with retinoic acid on cystamine potentiation of cisplatin toxicity.**

HepG2 cells ( $2 \times 10^5$  cells) were pre-treated with 10  $\mu$ M retinoic acid for 72 hours. Then, cells were treated or were not treated with 1 mM cystamine (cys) for another 48 hours. After incubation with cystamine, cells were treated or not treated with 8  $\mu$ M cisplatin for a further 48 hours, and cell death was analysed by FITC-labelled Annexin V by flow cytometry. Results are means  $\pm$ SD of triplicate experiments.

Results indicate that treatment with cystamine appeared to increase cell death (figure 4.9). These observations indicate that higher levels of TG2 activity were correlated with reduced cell death; an example of TG2 activity inhibition with cystamine alone is shown in figures 3.4 and 6.4A, in chapters 3 and 6, respectively.

#### 4.2.10 Direct effects of cisplatin on commercially-available exogenous guinea pig liver Transglutaminase

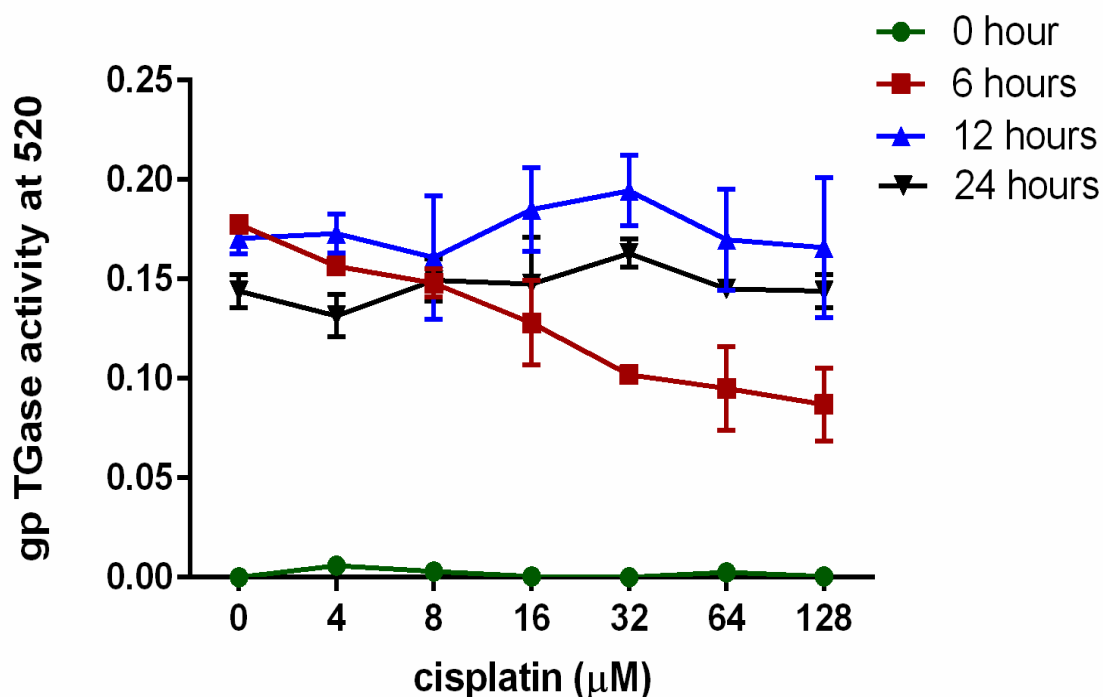
To confirm whether cisplatin can cause direct inhibition of TG2, the effect of cisplatin was tested on purified guinea pig liver transglutaminase enzyme, using the CBZ-Gln-Gly activity assay (see section 2.19). The inclusion of cisplatin into CBZ-Gln-Gly assay mixtures containing purified guinea pig liver transglutaminase caused a direct concentration-dependent inhibition of CBZ-Gln-Gly transamination over the whole 0-128  $\mu\text{M}$  range assayed.



**Figure 4.10 Effect of cisplatin on guinea pig liver transglutaminase activity *in vitro*.**

Transglutaminase activity was measured by CBZ-Gln-Gly assay. Cisplatin was used over the range of 0-128  $\mu\text{M}$  on a 0.02 mg/ml guinea pig liver transglutaminase solution for 1 hour. Results are means  $\pm$ SD of duplicates of three independent experiments. All cisplatin-treated groups were compared with control groups using one way ANOVA with Dunnett's multiple comparison test.

The results obtained were consistent with, though less pronounced than, the decrease in cellular transamination activity measured, following treatment of HepG2 cells with cisplatin, requiring 128  $\mu\text{M}$  of cisplatin to reduce the activity by 50% *in vitro* (figure 4.10).



**Figure 4.11 Time-dependent effect of cisplatin on TGase activity**

Transglutaminase activity was measured by CBZ-Gln-Gly assay. Cisplatin was used over the range of 0-128  $\mu\text{M}$  on a 0.01 mg/ml guinea pig liver transglutaminase solution for 0-24 hours. Results are means  $\pm$ SD of duplicates of three independent experiments.

Interestingly, the incubation of cisplatin together with TG2 over 6 hours resulted in a loss in apparent cisplatin activity; this may reflect deactivation of cisplatin by aqueous hydrolysis or TG2 (figure 4.11). In addition nucleophiles like amide- $\text{NH}_2$  of Gln-tripeptide in the TG2 activity assay, -SH and amino groups of proteins, and the imidazole of histidine can reduce the concentration of active cisplatin in test assays, especially over long incubation times. This observation may explain why 100  $\mu\text{M}$  cisplatin was required to induce its cytotoxic effect after 24 hours, as observed in other studies (Table 4.1).

### 4.3 Discussion

TG2 is a multifunctional protein that has been implicated in several cellular processes, including apoptosis and cell survival (Fésüs and Szondy, 2005; Nurminskaya and Belkin, 2012). Increased TG2 expression has been linked with cell survival in several cancer cells (Cho, et al., 2010; 2012). Over the past five decades, several cases of

resistance to cisplatin-induced apoptosis have evolved, and in some cases TG2 has been identified as one of the regulators of such processes (Jang, et al., 2010).

In this study, the results show that in the human hepatocarcinoma HepG2 cell line, cisplatin modulates TG2 by down-regulating its expression (figures 4.3 and 4.4) and that it is also capable of directly inhibiting TG2's enzymatic, transaminating activity during the induction of apoptosis (figure 4.5). Also, some studies have shown that upon initiation of the death response, TG2 translocates to the nucleus (Gundemir and Johnson, 2009; Kojima, Kuo and Tatsukawa, 2012). This study observed no such translocation (figure 4.4C); this effect may be dependent on differences in cellular conditions. Interestingly, Fesus & Zondey (2005) have suggested that loss of TG2 activity in mice could cause compensatory induction of other transglutaminases (TG1, 3, 5 and 7); this appears to be the case during cisplatin treatment of hepatocarcinoma, as this study observed the activation of TG1 at mRNA level following the treatment of HepG2 cells with cisplatin (see figure I.1 in appendix I). This effect of cisplatin on TG1 expression was measured along with TG2 mRNA expression.

TG2 upregulatory studies using retinoic acid confirmed that TG2 may be involved in the delay/inhibition of apoptosis induced by cisplatin, and that its inhibition by cystamine was required to reverse this effect (figures 4.6 and 4.9). This effect could be explained if the presence of TG2 in liver works as a detoxifier of cisplatin; this notion is supported by the observation that incubation of cisplatin with TG2 for 12 hours reduced the inhibitory activity of cisplatin on TG2 activity (figure 4.10. and 4.11), possibly by binding at a catalytic site.

Taken together, the findings indicate that cisplatin may required, first to inhibit TG2 prior to induction of apoptosis; which explains how the high abundance of TG2 in liver may be involved in prevention of apoptosis induction by chemotherapy drugs and could contribute significantly to the development of cisplatin-resistant in HCC

patients. On the basis of this hypothesis the TG2 expression profile is investigated in cisplatin-treated drug-resistant HepG2 cells in next chapter.

## **CHAPTER 5:**

### **EXPRESSION OF TRANSGLUTAMINASE 2 IN CISPLATIN- RESISTANT HEPATOCARCINOMA HEPG2 CELL LINE**

#### **5.1 Introduction**

As observed in chapter 4, increased TG2 expression reduces the cisplatin cytotoxicity. Thus, this chapter is focused on developing a drug resistance HepG2 cell line and comparing the TG2 expression profile to parental cells before conducting the costly cellular uptake studies using fluorescent-labelled cisplatin in the following chapter.

Drug resistance is becoming the major obstruction to successful cisplatin-based chemotherapy. Cells that become resistant to cisplatin involve both intrinsic and acquired changes at the genetic and/or epigenetic level, initiating cellular self-defence mechanisms by activating or silencing several cellular proteins and enzymes. These changes may occur during single-dose or multiple-dose treatment with anti-cancer drugs (Shen, et al., 2012; Holohan, et al., 2013).

Cisplatin is one of the most effective and extensively-used drugs to treat several cancers, but because of its high cytotoxicity to non-cancerous cells its use had been limited, and later, cancer cells have shown more resistance to cisplatin (Gumulec, et al., 2014; Galluzzi, et al., 2014). In recent years, the use of cisplatin in hepatocarcinoma treatment has been improved and increased, but chemoresistance remains a major problem. Different drug-resistant cell lines to cisplatin have been developed to elucidate the mechanisms and important key proteins and enzymes underlying this process. Thus, studies are needed to identify such proteins or enzymes that contribute to the drug resistance mechanisms, in order to target them to overcome drug resistance.

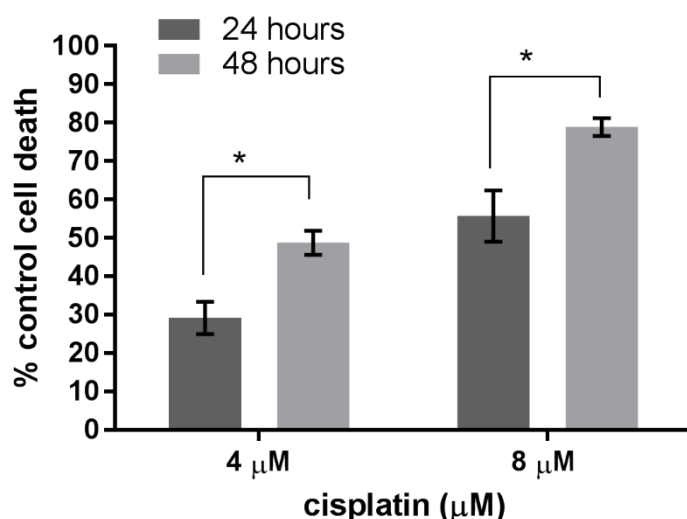
Recent studies have shown that alterations in TG2 expression occur during the development of drug resistance in several cancers, including overexpression of TG2 in drug-resistant breast, ovarian, glioblastoma, head and neck, colon, melanoma, lung, pancreas and renal cancer cells (Mehta, et al., 2004; Verma, et al., 2006). Interestingly, TG2 inhibition can sensitise drug-resistant cells to anti-cancer drugs (Kim, et al., 2006; Kim, Park and Kim, 2009), though the precise mechanism of association between TG2 and drug resistant is not clearly understood.

The aim of this chapter is therefore to investigate whether TG2 expression directly correlates with drug-resistant mechanism(s), in cisplatin-resistant HepG2 cell lines developed by a single-dose selection process.

## **5.2 Results**

### **5.2.1 Drug selection and determination of inhibitory concentration**

HepG2 cells were selected because of their ease of maintenance in cell culture, high sensitivity to cisplatin, compared to other cell lines tested in our laboratory, and their high content of TG2 protein. To determine the inhibitory concentration of cisplatin at which 50% and 80% of cells stop growth for a selected time period, cells were exposed to 0-20  $\mu$ M cisplatin for 24 and 48 hours. The cell growth was measured, using the cell counting kit-8 (CCK-8) viability assay and  $IC_{50}$  values of cisplatin for 24 and 48 hours were calculated at 8 and 4  $\mu$ M, respectively. The  $IC_{80}$  values of cisplatin for 24 and 48 hours were 20 and 8  $\mu$ M, respectively (see figure 4.1 in chapter 4). The  $IC_{80}$  was confirmed by flow cytometric analysis (figure 5.1).



**Figure 5.1 Confirmation of inhibitory concentration of cisplatin by flow cytometry.**

HepG2 cells were treated with 4 μM and 8 μM cisplatin for 24 and 48 hours. Then cells were analysed using the FITC-labelled Annexin V apoptosis detection kit by flow cytometry. The p value was calculated by Student's paired t- test.

Loss of cell proliferation was continued for 4 days after removing cisplatin-containing media from the culture flasks that were treated with 8 μM cisplatin for 48 hours. The results indicate that a single higher dose of cisplatin may have long term cytotoxicity and side effects on healthy cells. This effect was evaluated by observing cells under the inverted light microscope after regular 12 hours intervals for 4 days.

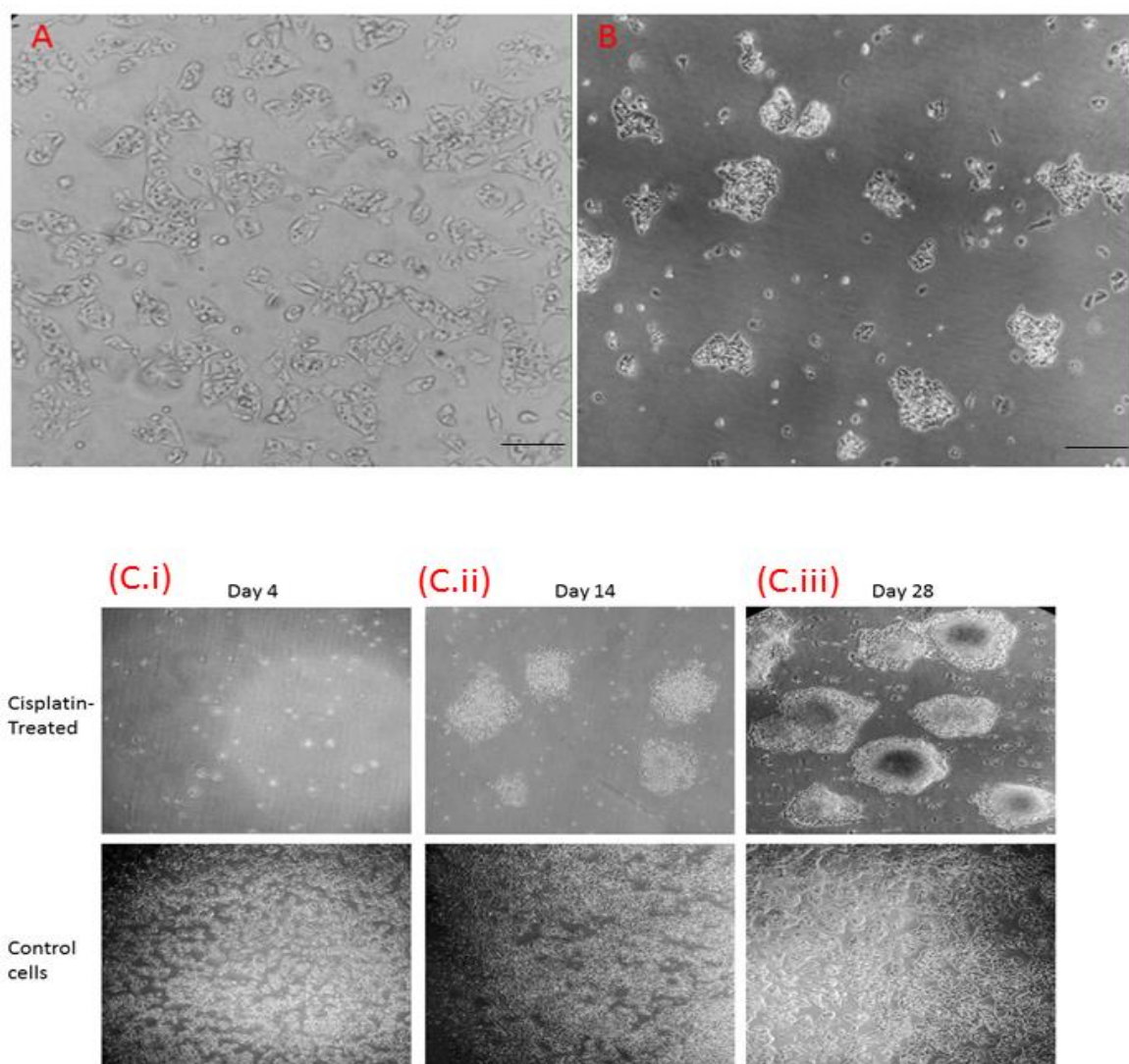
## **5.2.2 Characterisation of drug-resistant cells and determination of fold resistance**

### **5.2.2.1 Morphological characteristics of HepG2 cells and HepG2/cr cells**

Using methods of a single dose exposure to 8 μM cisplatin (IC<sub>80</sub> value at 48 hours) over a period of 0 to 4 days, cisplatin-resistant sublines of sensitive HepG2 cell lines were successfully selected. Then, cells were freed of cisplatin-containing medium and incubated for a further 4 weeks to recover from drug shock, replacing cisplatin-free fresh media every two days. Subsequently, cell colonies were isolated and frozen-down in liquid nitrogen. One flask was left untreated as a control, but was passaged



as long as treatment lasted. It is very important to passage the control cells alongside the drug treated cells, because continuously-cultured cells can alter their characteristics, including drug resistance (McDermott, et al., 2014).



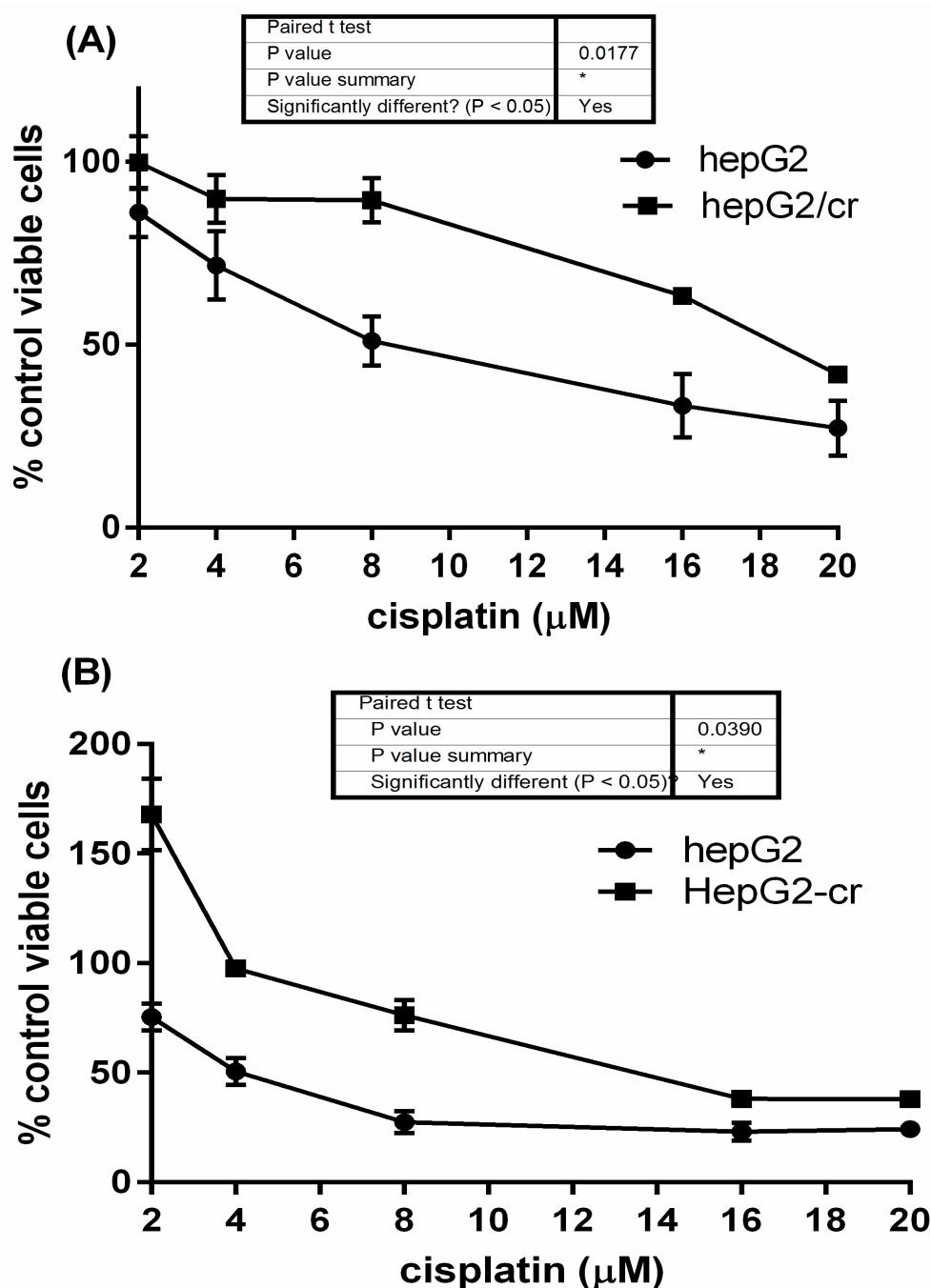
**Figure 5.2 Morphology of parental and drug-resistant cells.**

HepG2 cells were incubated with single dose of 8  $\mu$ M cisplatin continuously for 4 days. After incubation, cells were incubated with cisplatin-free medium for another 4 weeks and during that time cells' morphologies were observed using inverted phase contrast microscopy. (A) Parental HepG2 cells before treatment, (B) Cisplatin-resistant cells (HepG2/cr) recovered from drug resistant colonies, (C) Time-dependent observation of parental cells during development of resistance to cisplatin. Day 4, Day 14 and Day 28 indicate cells growing in culture media (cisplatin-free) after end of cisplatin treatment. The magnification of figure A & B is x100 whereas x40 for remaining. Images were taken using iPhone 5 camera. Scale bar = 12.45  $\mu$ m.

Viable cell colonies surviving from cisplatin treatment were established and named as the HepG2/cr cell line. Drug resistant cells (HepG2/cr) changed their morphology and growth features compared to parental cells, growing initially in spheroid colony form and then layering on each other, as observed by inverted phase contrast microscope (figure 5.2); the growth rate of resistant cells was slower than that of parental cells. Changes in resistance phenotype may occur due to daughter colony formation. After comparing results with the available literature (Lopez-Ayllon, et al., 2014), it was found that drug-resistant cells appeared to grow morphologically like tumour stem cells, however, specific biomarker analysis for stem cells would be required to confirm this stem cell-like phenotype. Although the shape of drug resistant cells reverted back to that of parental cells after a number of passages (5 passages); the initial growth rate of these cells was still slow when cells were resuscitated from liquid nitrogen, compared to parental cells.

#### **5.2.2.2 Cisplatin sensitivity decreased in single dose-treated cisplatin-resistant cells**

To determine the cisplatin sensitivity of HepG2/cr, cells were grown in cisplatin-free medium and then exposed to 0-20  $\mu\text{M}$  cisplatin for 24 and 48 hours. The results indicated that the cells that survived a single dose of cisplatin were less sensitive to subsequent cisplatin treatment. The drug sensitivity of cisplatin in HepG2/cr cells was decreased from 8  $\mu\text{M}$  to 18  $\mu\text{M}$  ( $\text{IC}_{50}$  values) after 24 hours and from 4  $\mu\text{M}$  to 12  $\mu\text{M}$  ( $\text{IC}_{50}$  values) after 48 hours compared to parental cells (figure 5.3A and B).

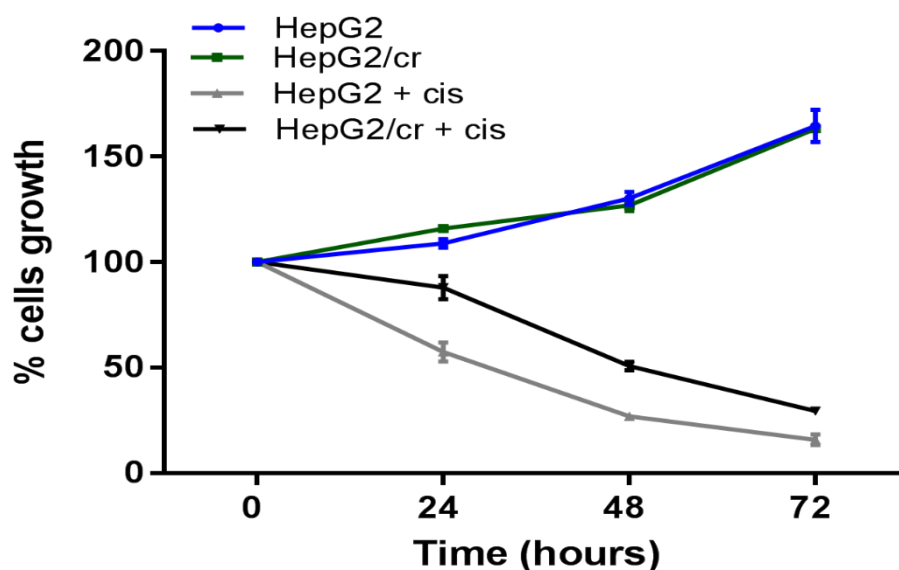


**Figure 5.3 Determination of cisplatin sensitivity in HepG2/cr cells.**

Drug resistant phenotypes were measured by incubating HepG2 and HepG2/cr cells with final concentrations of 0-20  $\mu\text{M}$  cisplatin for 24 hours (A) and 48 hours (B) and cell viability was measured and calculated by CCK-8 assay. Results are mean  $\pm$ SD of triplicate of three independent experiments. The p value was calculated by Student's paired t- test.

HepG2/cr cells were also exposed to 8  $\mu\text{M}$  cisplatin for 24, 48 and 72 hours and the cells' response to cisplatin was determined by CCK-8 cell viability assay. Interestingly, in the presence of cisplatin, growth rate of cisplatin-resistant HepG2/cr cells was

higher (90% viable cells) compared to parental cells (50% viable cells), while the proliferation rate was similar in both cell types (figure 5.4).

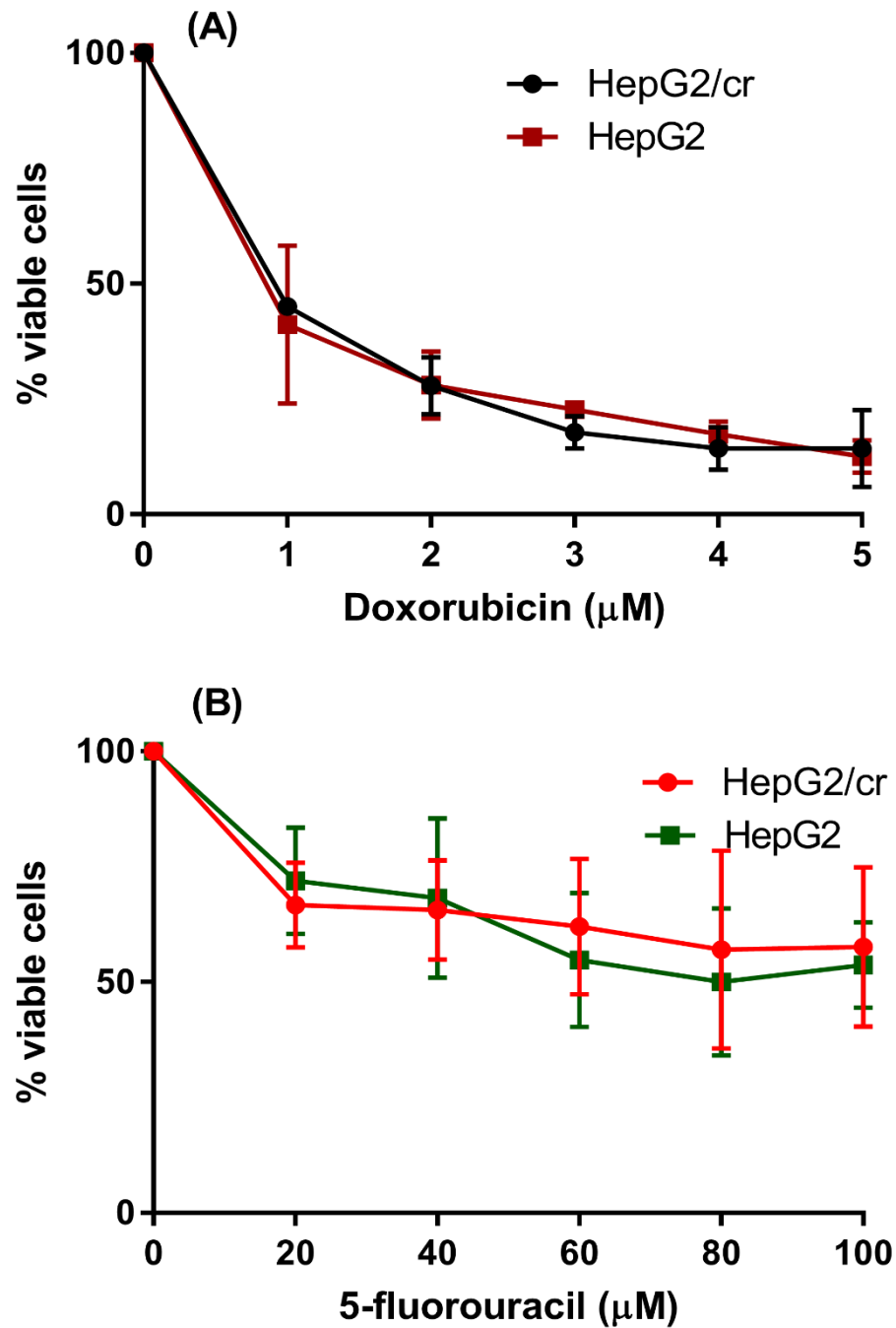


**Figure 5.4 Time course analysis of HepG2 and HepG2/cr cells in presence of cisplatin.**

HepG2 and HepG2/cr cells were grown in the presence or absence of 8  $\mu$ M cisplatin for various time points and cell growth was measured and calculated using the CCK-8 viability assay.

### 5.2.3 Cisplatin-resistant cells showed no cross-resistance to other anti-cancer drugs

Most of the cell lines used for cisplatin resistance studies have induced cross-resistance to different anti-cancer drugs, when used in combination with cisplatin (Zhou, et al., 2010). To find out whether single-step selected drug-resistant HepG2/cr cells have a cross-resistant phenotype, Doxorubicin and 5-Fluorouracil were used to test for cross-drug-resistance. The HepG2/cr and HepG2 cells were incubated with 0-100  $\mu$ M 5-Fluorouracil and 0-5  $\mu$ M Doxorubicin for 48 hours. Notably, no significant cross-resistance was observed with Doxorubicin and 5-Fluorouracil (figure 5.5).

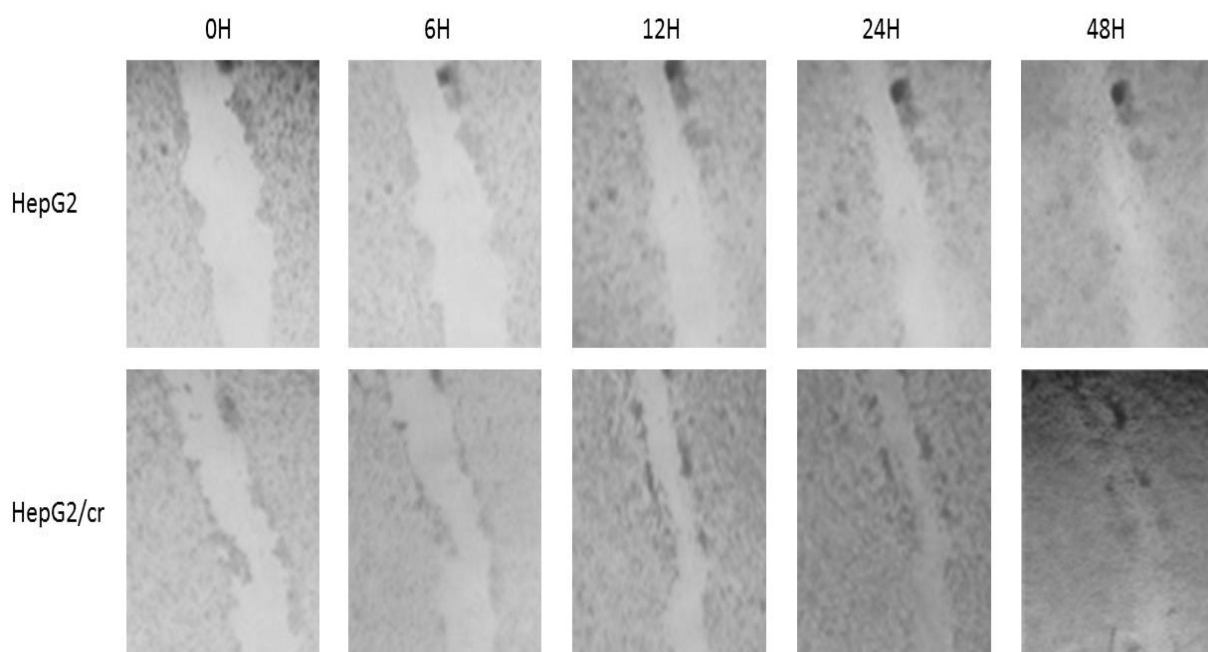


**Figure 5.5 Determination of cross-resistance to other anti-cancer chemotherapeutic drugs.**

Potential cross-resistance to doxorubicin and 5-fluorouracil was observed by CCK-8 viability assay. HepG2 parental and HepG2/cr cells were incubated with final concentrations of 0-5  $\mu\text{M}$  doxorubicin (A) and 0-100  $\mu\text{M}$  5-fluorouracil (B) for 48 hours. The results are means  $\pm$ SD of triplicate of three independent experiments.

### 5.2.4 Cell migration of drug resistant cells is increased compared to parental cells

Cell migration (metastasis) is one of the hallmarks of cancer cells. When cancer cells become drug resistant, they also become more prone to metastasis (Hanahan and Weinberg, 2011). To investigate the characteristics of drug-resistant cells, wound scratch assays were performed. Figure 5.6 indicates that after 12 hours, drug resistant cells had started migrating and had filled the wound gap after 24 hours, whereas parental cells took 48 hours to migrate to fill the wound area completely.

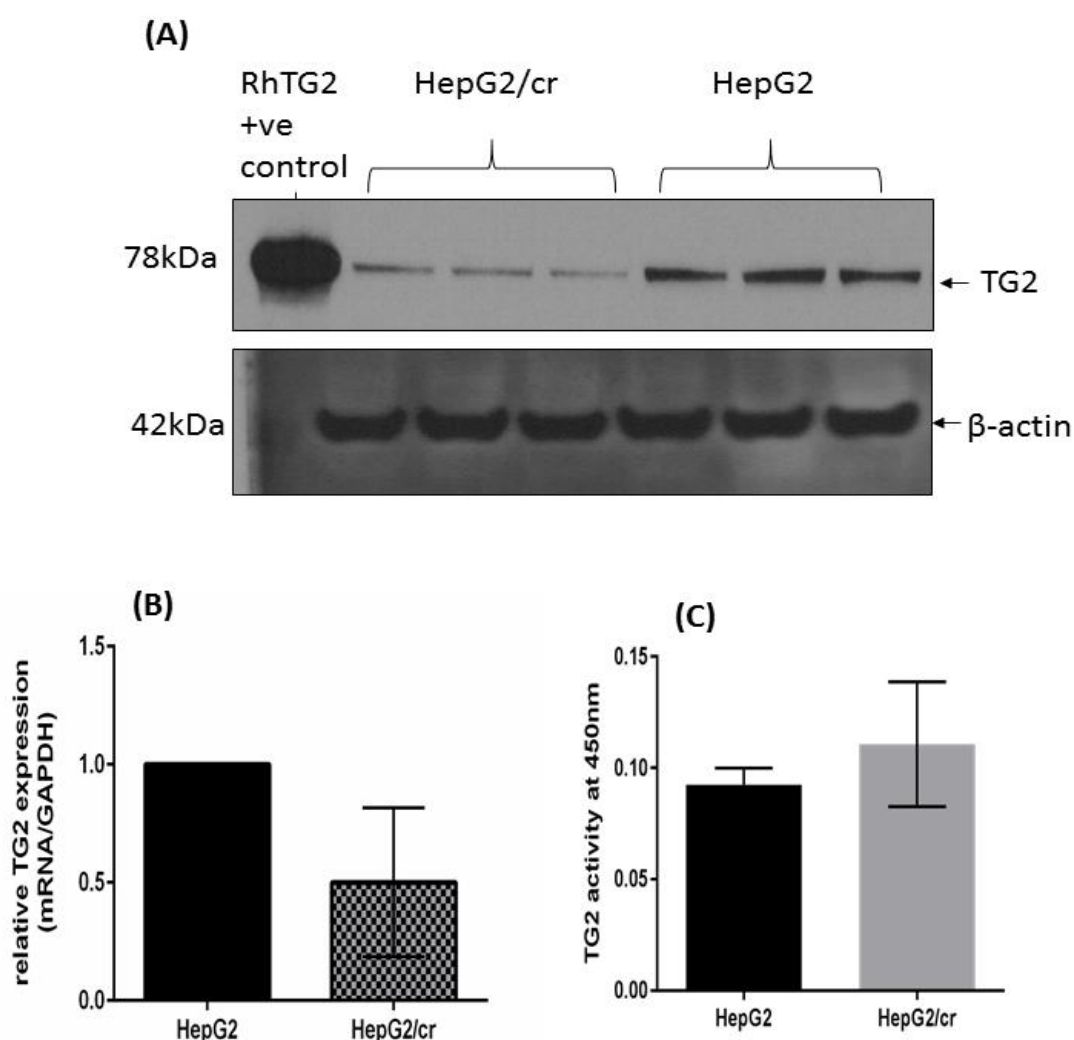


**Figure 5.6 Cell migration analysis of parental and resistant cells**

Cells ( $4 \times 10^5$  cells/well) were grown in 12-well culture plates. After 24 hours, a wound boundary was created using 10  $\mu$ l pipette tip. Then cells were washed twice with fresh culture media and incubated at 37 °C for further analysis. Images were taken using an inverted phase contrast microscope, at x100 magnification.

#### **5.2.5 TG2 mRNA and protein expression is reduced, but TG2 activity is increased, in cisplatin-resistant cells**

The overexpression of TG2 has been observed during development of drug-resistance in cancer cells in many studies of multi-dose treated cells (Verma, et al., 2006; 2008). To determine whether TG2 expression directly correlates with drug-resistance, TG2 mRNA expression, protein expression and transamidation activity were measured in HepG2/cr cells and compared to these measurements in HepG2 parental cells (see figure 5.7).



**Figure 5.7 Comparison of TG2 expression in parental and cisplatin-resistant HepG2 cells.**

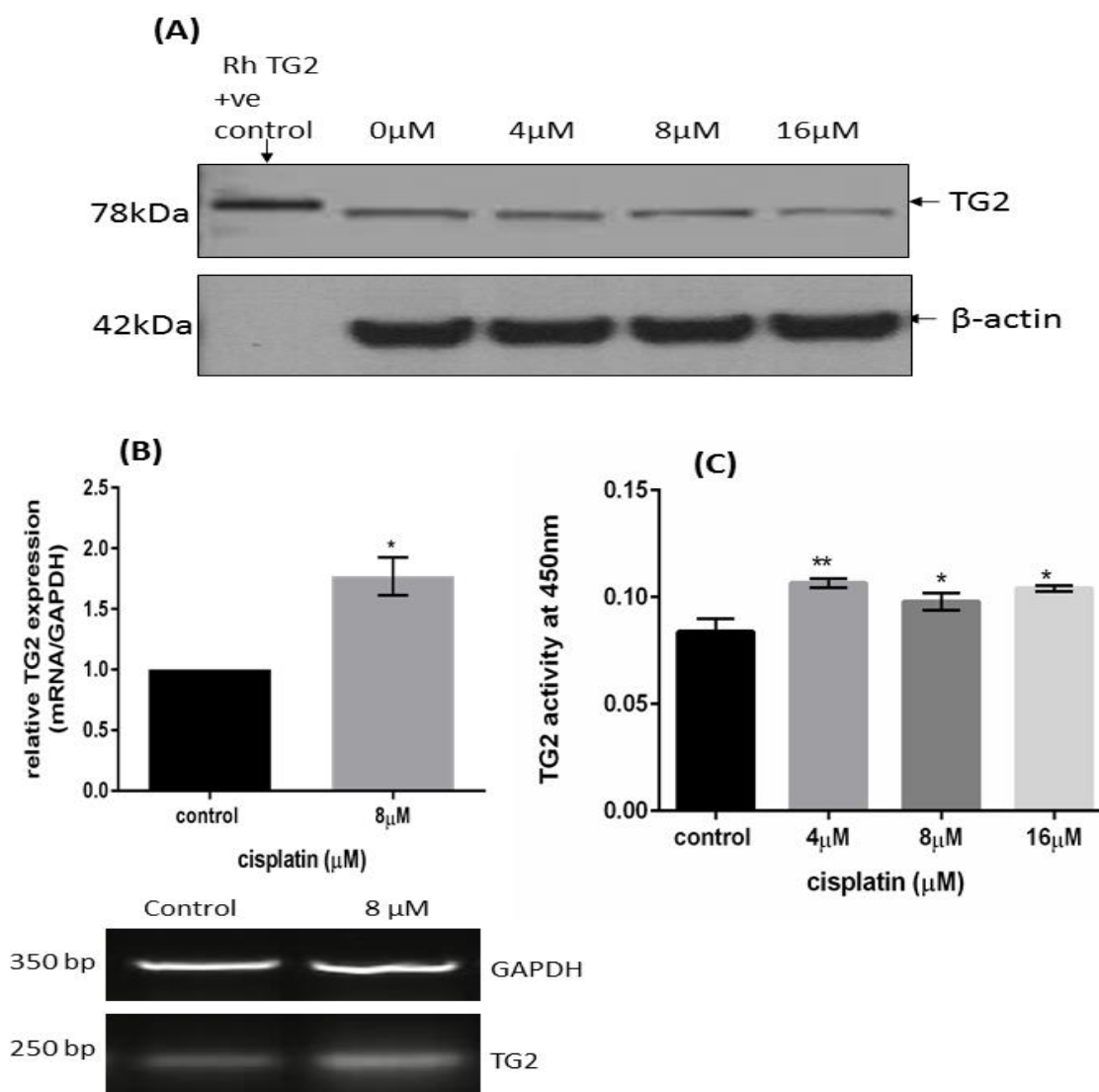
(A) Western blot analysis of TG2 protein expression. Equal numbers ( $1 \times 10^6$  cells) of HepG2 and HepG2/cr cells were lysed in cell lysis RIPA-buffer and Western blotting was performed using an anti-TG2 polyclonal antibody raised in the rabbit. (B) TG2 gene expression at the mRNA level was measured by RT-PCR, (C) TG2 transaminase activity was measured by specific TG2 colorimetric assay kit. All the results are means  $\pm$ SD of triplicate experiments. The  $p > 0.05$ . Rh TG2 denotes recombinant human TG2 protein.

The HepG2/cr cells showed very low TG2 mRNA gene expression and this reflected the TG2 protein level, which was also lowered compared to parental cells (figures 5.7A and B). However, and in direct contrast to its level of expression, TG2 transamination activity was increased in drug-resistant HepG2/cr cells compared to parental cells (figure 5.7C).



### 5.2.6 Cisplatin treatment increased TG2 gene expression and transaminase activity in cisplatin-resistant HepG2/cr cells

As observed previously in chapter 4, cisplatin modulates TG2 expression in HepG2 cells. To find out whether TG2 is expressed in a similar pattern in drug-resistant cells, HepG2/cr cells were treated or not-treated with 0-16  $\mu$ M cisplatin for 24 hours.



**Figure 5.8 TG2 expression in HepG2/cr cells after cisplatin treatment**

HepG2/cr cells were treated with 0-16  $\mu$ M cisplatin for 24 hours. Then: (A) TG2 protein expression was measured by Western blotting; (B) TG2 gene expression at the mRNA level was measured by RT-PCR and PCR products were observed by 1.2% agarose gel electrophoresis; (C) TG2 transaminase activity was measured using specific TG2 colorimetric assay kit. The p value was calculated using Student's paired t-test (B) and One Way ANOVA with Dunette's multiple comparison test (C). Rh TG2 denotes recombinant human TG2 protein.

When cells were treated with 8  $\mu$ M cisplatin, TG2 mRNA expression was increased in HepG2/cr cells compared to parental HepG2 cells (figure 5.8B). However, Western blot analysis of 0-16  $\mu$ M cisplatin-treated cells showed no difference in TG2 protein expression between cisplatin-treated and untreated groups (figure 5.8A). TG2 enzymatic activity however was increased upon cisplatin treatment over a period of 24 hours (figure 5.8C). This increase in TG2 enzymatic activity may reflect a role for TG2 activity during cellular trafficking of anti-cancer drugs in chemoresistant cells, where it may reduce or block the flow of cisplatin across the plasma membrane (see figure 6.3 in chapter 6).

### **5.3 Discussion**

Different cisplatin-resistant cell lines have been developed (McLaughlin, et al., 1991; Zhou, et al., 2010; Gumulec, et al., 2014) to elucidate the mechanisms and important key proteins and enzymes underlying its effects. Cisplatin-resistant sub-lines of the sensitive HepG2 cell line were developed by a single-dose treatment with cisplatin similar to that previously developed in a human ovarian cancer cell line (McLaughlin, et al., 1991), and a lung cancer cell line (Lopez-Ayllon, et al., 2014). Results from this study indicate that the cisplatin-resistant cells changed their shape and were less sensitive to cisplatin, as analysed by time-course incubation studies (figures 5.3 and 5.4). The drug resistant phenotype was increased by 2.25 fold at IC<sub>50</sub> level in cisplatin-resistant cells. Apart from the molecular mechanism, presence of stem cells is considered to be responsible for chemoresistance (Tomuleasa, et al., 2010). This study observed morphological characteristics of stem cells in these drug-resistant cells (figure 5.2). However, no specific biomarker (e.g., CD133 (prominin-1), an apical plasma membrane protein (Romano, et al., 2015)) study of cancer stem cells was performed because of the cost of the reagents. The drug-resistant cells were not cross-resistant to doxorubicin and 5-fluorouracil (figure 5.5), suggesting an

independent mechanism for cisplatin resistance. Cell migration rate was increased in cisplatin resistant cells (figure 5.6).

Cancer cells tend to have higher TG2 expression and activity than normal cells and this may contribute to their chemo-resistance. Following the characterisation of cisplatin-resistant cells, the TG2 expression profile was investigated in these cells. The findings from this study have created new insights into the TG2 expression pattern in drug-resistant cells, in which TG2 expression decreased but activity increased in cisplatin resistant cells (figure 5.7). Interestingly, TG2 mRNA expression increased upon subsequent cisplatin treatment in these resistant cells (figure 5.8B), which indicates the spatial pattern of TG2 expression and accumulation, when cells come in contact with anti-cancer drug upon multiple dose treatment, which may help cancer cells to become highly drug-resistant and reduce cisplatin sensitivity.

Overexpression of glutathione-s-transferase (GST) is one of the conditions of chemoresistant cells that is found to involve in reducing activity of anti-neoplastic drugs (Townsend and Tew, 2003; Balendiran, Dabur, and Fraser, 2004). As a control experiment, the expression of GST was measured in both cell lines. Results consistent with previous findings showed that the GST expression was increased in cisplatin resistant cells compared to parental cells, though there was no significant difference in GST level when compared between both cell lines (see figure I.6 in appendix I).

Collectively, these results suggest that shortly after initial treatment of HCC cells with cisplatin, increased TG2 transamidating activity contributes to the cells development of chemoresistance. From this study, it is also predicted that the detection of the level of TG2 activity at biopsy, shortly after initial treatment with cisplatin, could be used to predict a patient's likelihood of subsequently developing drug resistance.

## **CHAPTER 6:**

### **TRANSGLUTAMINASE 2 IN CELLULAR UPTAKE OF THE ANTI-CANCER DRUG CISPLATIN IN HEPATOCARCINOMA CELLS**

#### **6.1 Introduction**

As observed in chapter 5, TG2 transamination activity is increased in drug resistant HepG2 cells. As a result of such observations, by modulating its expression, this chapter investigates the role of TG2 in the cellular uptake of fluorescent-labelled cisplatin.

Cells live and grow by exchanging molecules within their local environment. The exchange of molecules is controlled at the plasma membrane, which acts as a barrier to transit molecules into and out of the cell. These processes are carefully controlled in many ways, through a variety of membrane transport mechanisms (Alberts, et al., 2013).

Studies have shown that many drug molecules, including cisplatin, are passively transported across plasma membrane against their lipophilicity (Dobson and Kell, 2008). Several lines of evidence on drug resistant cell lines have shown that the anticancer drugs that enter cancer cells depend upon specific mechanisms of carrier-mediated transport and that this process may be modified in drug-resistant cancer cells, though the cellular pathway of drug uptake, including for cisplatin, is not yet clearly defined (Mellman and Yarden, 2013).

The blockade or modification of cellular uptake mechanisms at the plasma membrane can cause failure of cellular uptake of anti-cancer drugs by cancer cells, which is one of the major challenges in the successful treatment of cancer by chemotherapy. Therefore, an understanding of the mechanisms that control cellular uptake of ligands is crucial in the development of effectiveness of drugs, specifically against drug-

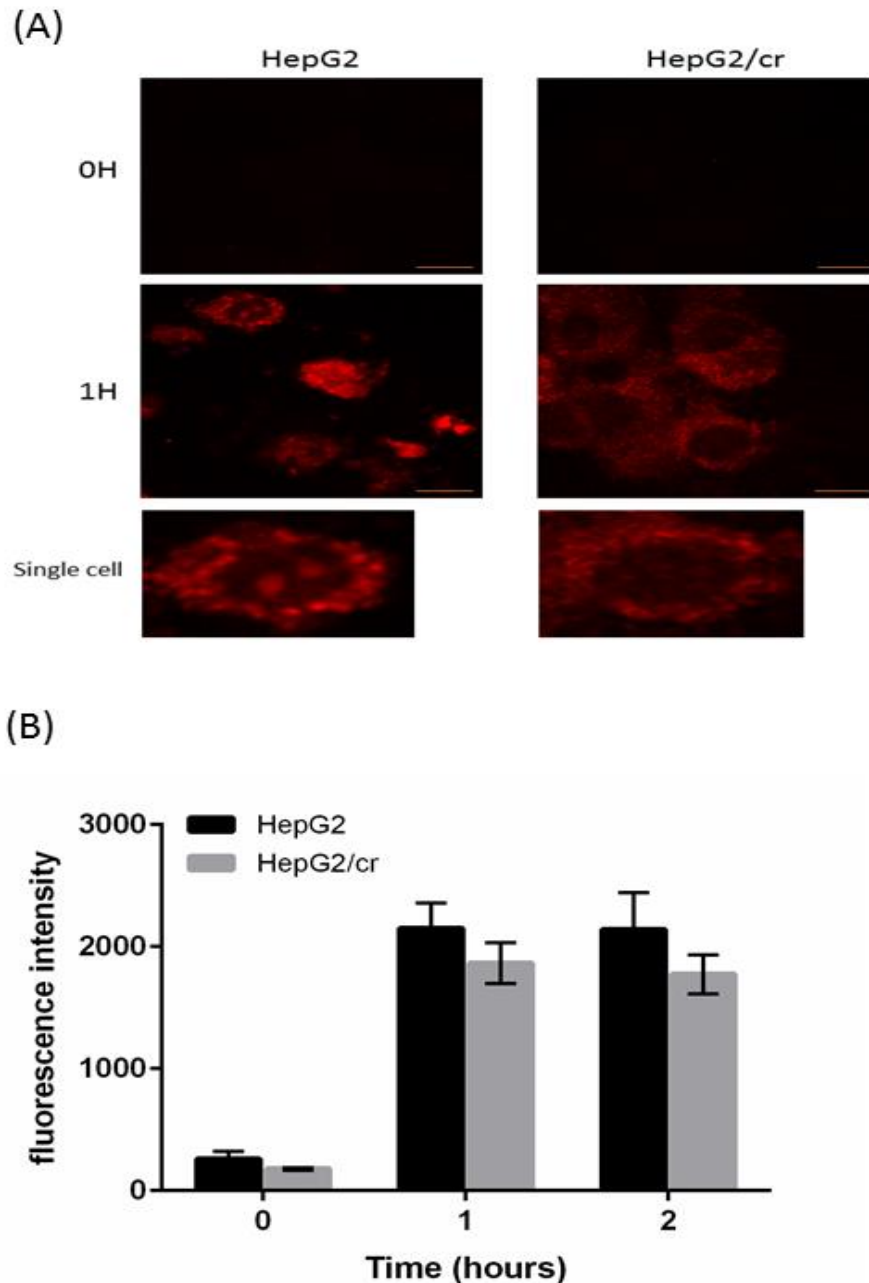
resistant cancer cells. To date, several lines of evidence support a role for TG2 involvement in receptor mediated endocytosis (Davies, et al., 1980; Schrier and Junga, 1981; Ray and Samantha, 1996; Abe, et al., 2000), though until now, no reports have specifically investigated TG2 involvement in endocytosis of anti-cancer drugs such as cisplatin in hepatocarcinoma cells.

The aim of this chapter is therefore to investigate whether the presence of TG2 is involved in cellular uptake of cisplatin, both in parental and drug-resistant cells. First, the pilot study was conducted on the uptake of a non-toxic fluorescence probe, dansylcadaverine, in parental cells (see section 3.3.2). Then, fluorescently-labelled cisplatin was used to investigate the role of TG2 in cellular trafficking of the anti-cancer drug cisplatin in HepG2 cells and its involvement in drug-resistance in a model of HCC.

## **6.2 Results and discussion**

### **6.2.1 Alexa fluor 546 labelled-cisplatin uptake is reduced in cisplatin-resistant cells**

Single dose cisplatin-resistant HepG2 cells were developed (section 5.2.2), and the accumulation or uptake of Alexa fluor 546-labelled cisplatin in both parental HepG2 and cisplatin-resistant HepG2/cr cells was analysed by confocal microscopy. Both cell lines were grown in 4-well glass Lab-Tek chamber slides and incubated with Alexa fluor 546-labelled cisplatin, at a final concentration of 200 U/ml. Following 1 hour of incubation, Alexa fluor cisplatin was visible in the nucleus of cells, with a finely granular pattern, and was seen to be distributed throughout the cytoplasm and nucleus in the majority of parental HepG2 cells, whereas in cisplatin-resistant HepG2/cr cells, the Alexa fluor cisplatin was seen to be present only on the plasma membrane (figure 6.1A).



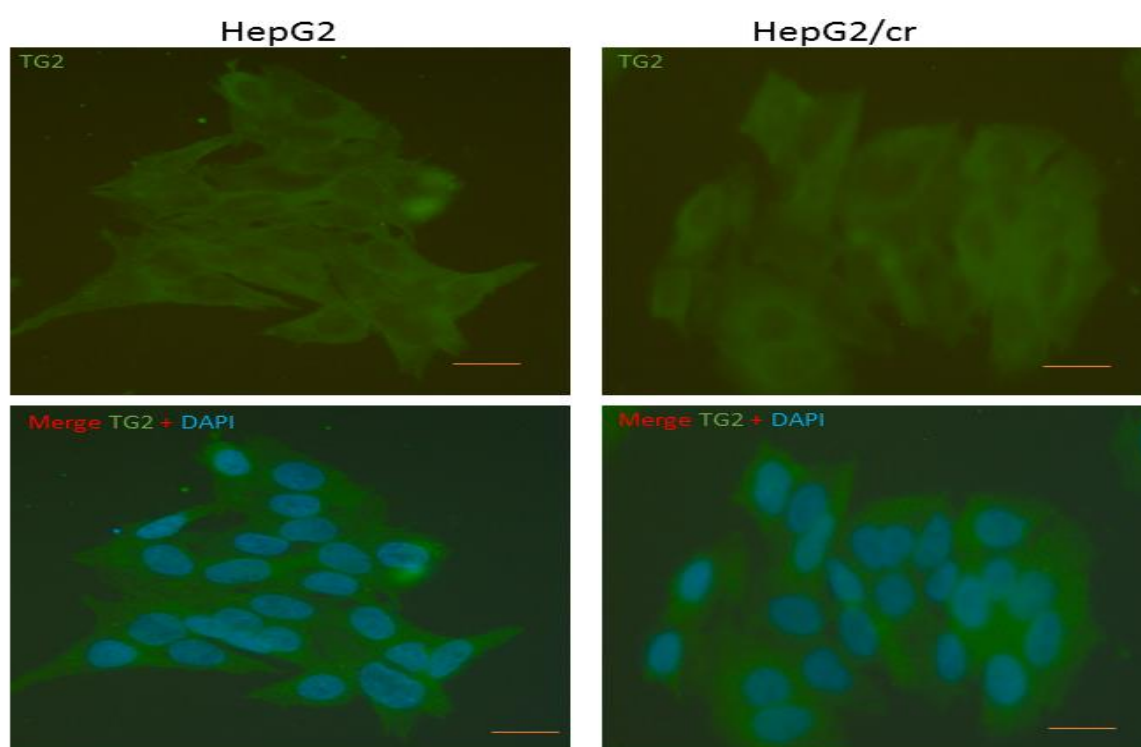
**Figure 6.1 Cellular fluorescence distribution following uptake of Alexa fluor 546/488-labelled cisplatin.**

(A) HepG2 and single-step selected cisplatin-resistant HepG2/cr cells were incubated with Alexa fluor 546-labelled cisplatin at a final concentration of 200 U/ml for different time periods. Then cells were washed with PBS, fixed with 70% ethanol, mounted in fluorescent mounting medium and observed under a laser scanning confocal microscope at x400 magnification with excitation/emission at 543/530nm. Scale bar = 0.44  $\mu$ m. Results are representative of triplicate experiments. (B) Cellular uptake of alexa fluor 488-labelled cisplatin measured by flow cytometry. Approximately  $5 \times 10^5$  cells were incubated with Alexa fluor 488-labelled cisplatin at a final concentration of 40 U/ml in 6-well culture plate for different time points. Then cells were collected, washed with PBS and re-suspended in ice-cold PBS. Then 10,000 cells were analysed immediately by flow cytometry with fluorescence intensity detector filter set at FL-1 (FITC). Results are  $\pm$ SD of triplicate experiments.

The defective cellular uptake of Alexa fluor cisplatin in cisplatin-resistant cells was also measured by flow cytometry. Approximately 500,000 cells were incubated with Alexa fluor 488-labelled cisplatin at a final concentration of 40 U/ml for up to 2 hours. The results were consistent with the confocal microscopic analysis; since as incubation time increased, the fluorescence intensity was lower in cisplatin-resistant cells compared to parental cells (figure 6.1B).

### 6.2.2 Localisation of TG2 during cellular uptake of fluorescent labelled cisplatin

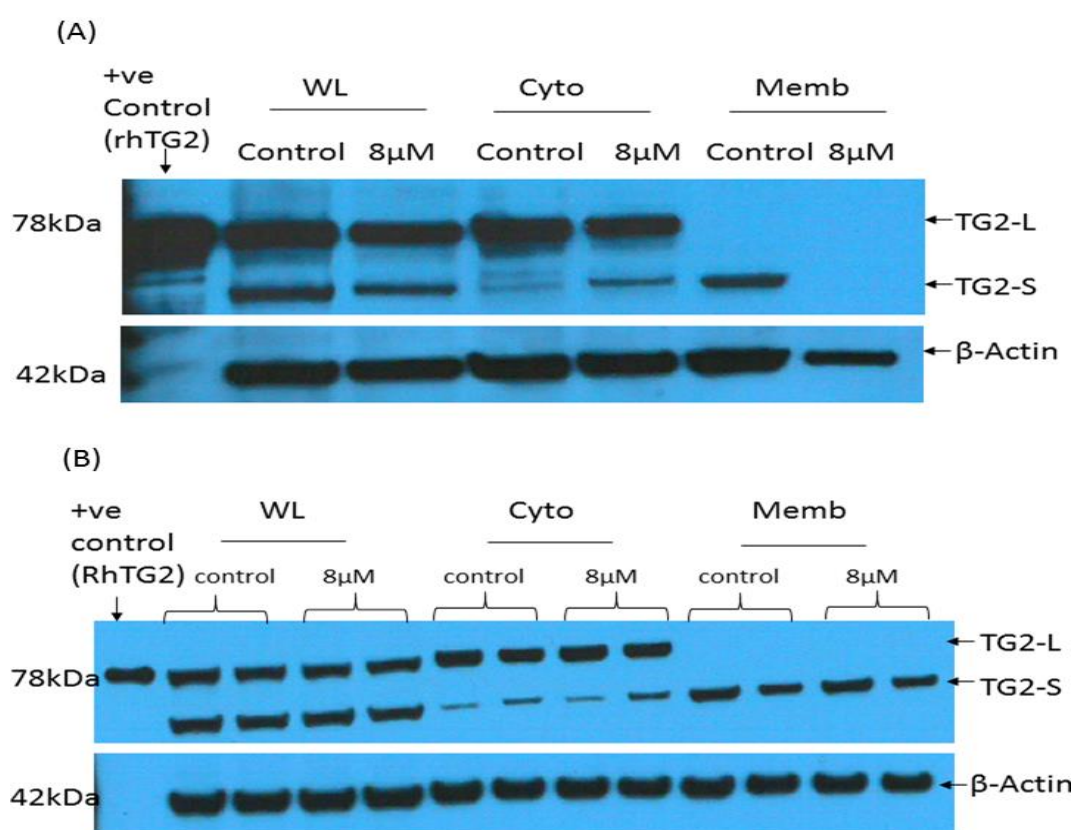
The pattern of TG2 expression during uptake of Alexa fluor 546-labelled cisplatin in both cell lines was observed by immunofluorescence analysis (see section 2.20). Results shown that there was no difference in TG2 expression at the protein level, consistent with the results shown in figure 5.8A (figure 6.2).



**Figure 6.2 Analysis of TG2 expression during cellular uptake of fluorescent cisplatin**

HepG2 and HepG2/cr cells were treated with Alexa fluor 546 labelled-cisplatin and the expression of TG2 in the cytoplasm and the nucleus were observed using an anti-TG2 antibody by immunofluorescence. The slides were observed using a DMR-X fluorescence microscope, at x200 magnification. Scale bar = 25  $\mu$ m.

To determine whether there was change in TG2 expression pattern, in different cellular compartments, cisplatin was applied to both cell lines. However, interestingly, Western blot analysis of separated cell fractions showed that treatment of normal and chemo-resistant cells with 8  $\mu$ M cisplatin for 24 hours caused alterations in the location of TG2 isoforms and the patterns of Alexa fluor 546-labelled cisplatin uptake. Initially, TG2-L was seen to be exclusively located in the cytoplasm and TG2-S was predominantly localised in the plasma membrane of both cell lines (figures 6.3A and B).



**Figure 6.3 Localisation of TG2 during cellular uptake of cisplatin.**

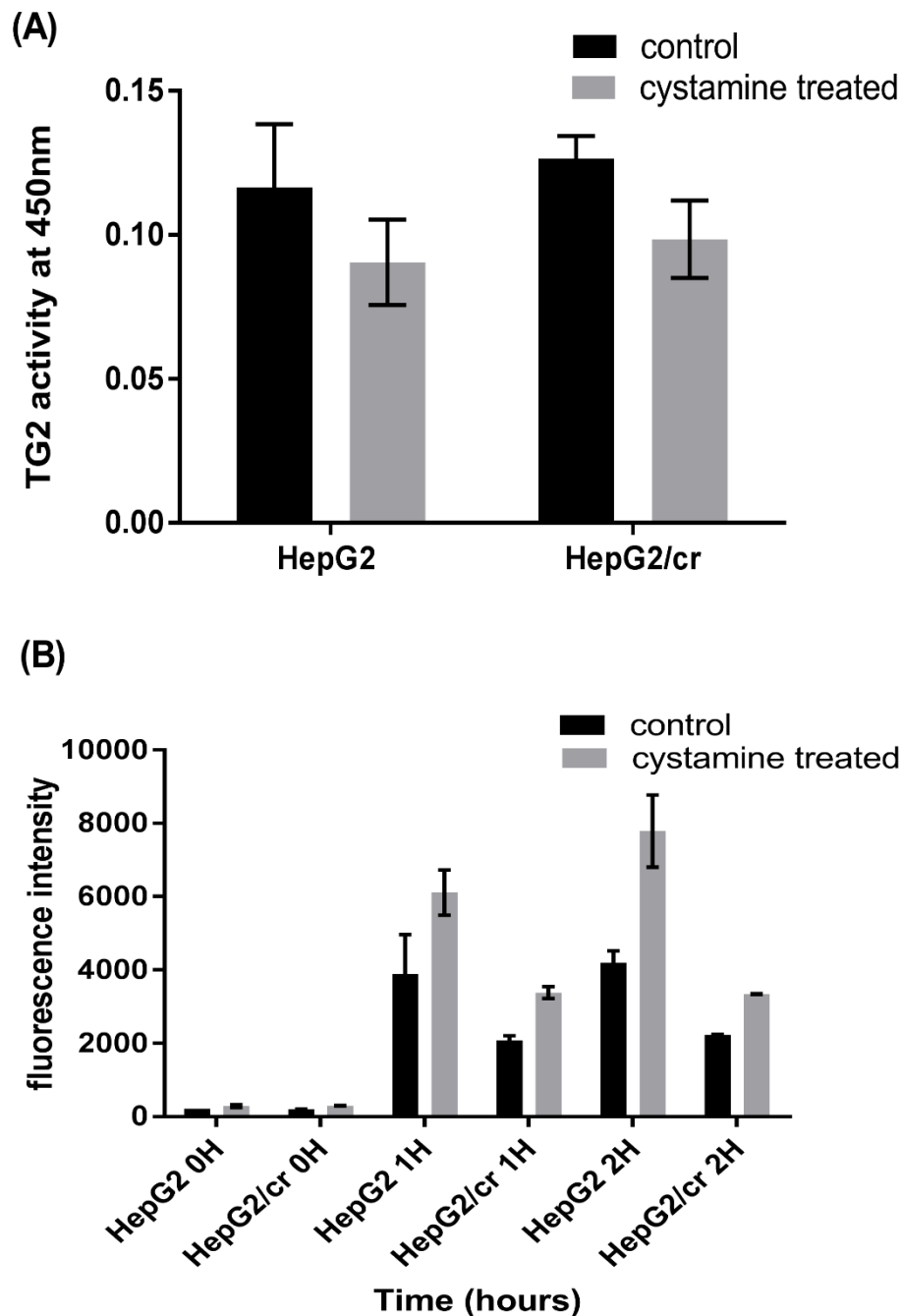
HepG2 and HepG2/Cr cells were treated or not-treated with 8  $\mu$ M cisplatin for 24 hours. Then cytoplasmic and membrane-associated proteins were extracted with a commercially-available membrane preparation kit. The TG2 expression was measured in (A) parental HepG2 and (B) chemoresistant HepG2/cr cells by Western blotting. WL = whole lysate, Cyto = Cytoplasm, Memb = Membrane. The anti-TG2 antibody (raised in mouse, cat. no. ab2368) used in this analysis was different to that used in chapter 4 and 5, as this antibody can bind to both TG2 isoforms.



Following cisplatin treatment of normal cells, TG2-S was lost from the plasma membrane and appeared to relocate to the cytoplasm (figure 6.3A); an effect not seen in chemo-resistant cells (figure 6.3B).

### **6.2.3 TG2 inhibition by cystamine increased uptake of Alexa fluor 488-labelled cisplatin**

As observed in Chapter 5, TG2 transamidation activity was increased in cisplatin-resistant cells compared to parental cells. To investigate whether inhibition of TG2 activity influences changes in cellular uptake of cisplatin, parental HepG2 and cisplatin-resistant HepG2/cr cells were pre-incubated with the TG2 inhibitor, cystamine, at a final concentration of 2 mM for 48 hours. Then, cells were incubated with 40 U/ml of Alexa fluor 488-labelled cisplatin for up to 2 hours and fluorescent uptake of cisplatin into cells was measured by flow cytometry.



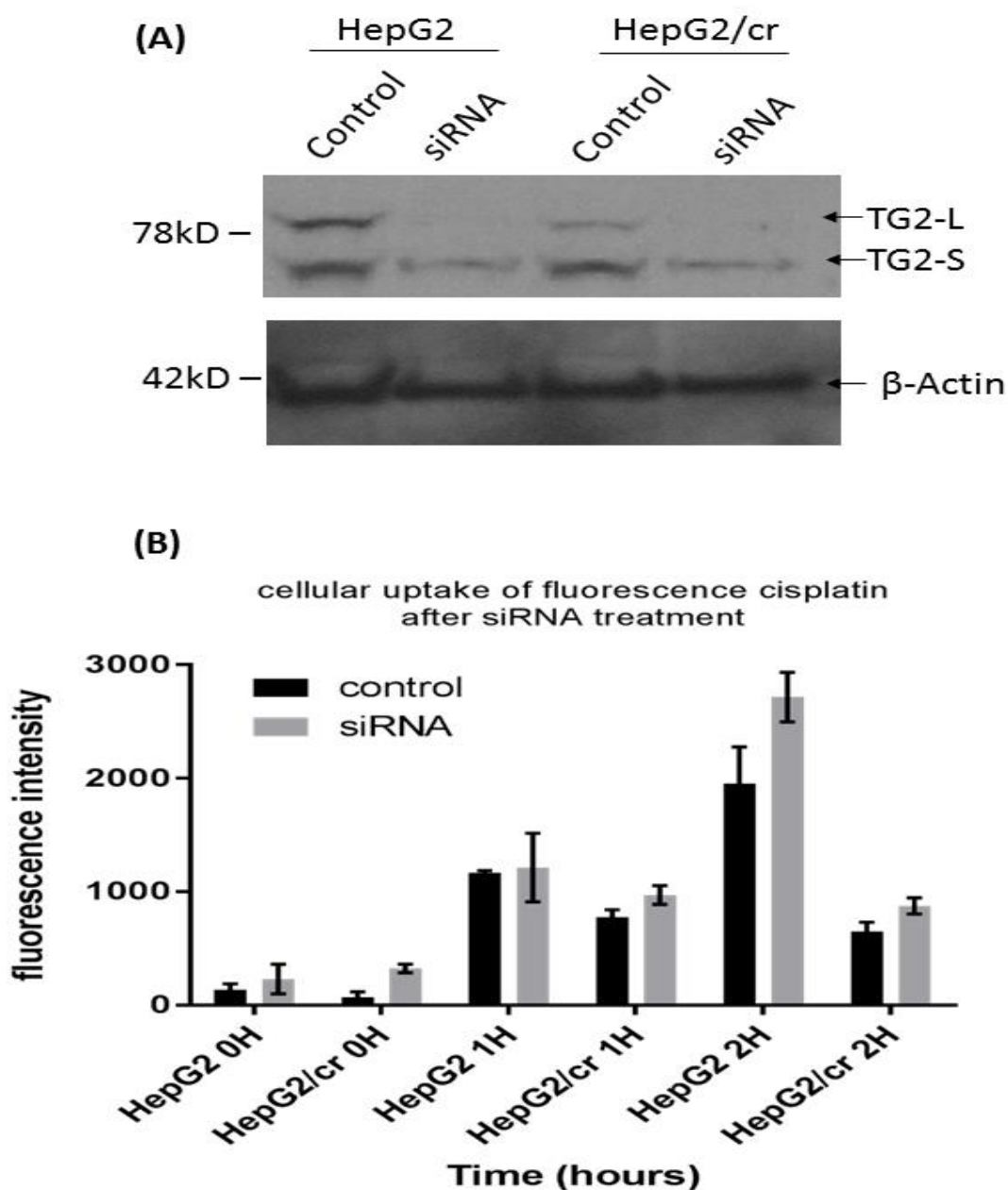
**Figure 6.4 Inhibition of TG2 activity increased the uptake of fluorescent cisplatin in both cell lines.**

(A) The parental HepG2 and cisplatin-resistant HepG2/cr cells were treated or not-treated with the TG2 inhibitor, cystamine, at a final concentration of 2 mM for 48 hours. TG2 activity was measured by TG2-specific colorimetric assay kit. (B) The cystamine pre-treated cells were incubated with Alexa fluor 488-labelled cisplatin to a final concentration of 40 U/ml for up to 2 hours. The cells were collected by centrifugation, washed with PBS and then fluorescence intensity was measured by flow cytometry with fluorescent intensity detector filter set at FL-1 (FITC). Results are mean  $\pm$ SD of triplicate experiments.

The results indicate that the cystamine treatment decreased the TG2 transamidation activity (figure 6.4A), and significantly increased the accumulation or uptake of fluorescent cisplatin in both cell lines (figure 6.4B). Quantitatively, the level of uptake of fluorescent cisplatin was increased by approximately 50% in cystamine-treated cells compared to non-treated cells. Cystamine treatment did not affect cell viability (see section 4.2.9).

#### **6.2.4 TG2 gene silencing increased uptake of Alexa fluor 488-labelled cisplatin**

To determine whether TG2 mRNA knockdown increases the uptake of fluorescently-labelled cisplatin, as observed in cystamine treatment, a TG2-specific siRNA oligomer was delivered using Lipofectamine2000 transfection reagent. After 48 hours, TG2 expression was measured by Western blotting, and remaining cells were incubated with Alexa fluor 488-labelled cisplatin at a final concentration of 200 U/ml for 1 hour, and 40 U/ml for up to 2 hours, for confocal microscopy and flow cytometry analysis, respectively. Figure 6.5A indicates that mRNA silencing was effective and TG2-L was effectively eliminated from both sensitive and chemo-resistant cells. The cellular uptake effect was time-dependent, with similar patterns at the two time points, but greater effect being seen after two hours compared to one hour incubation, in parental cells (figure 6.5B).

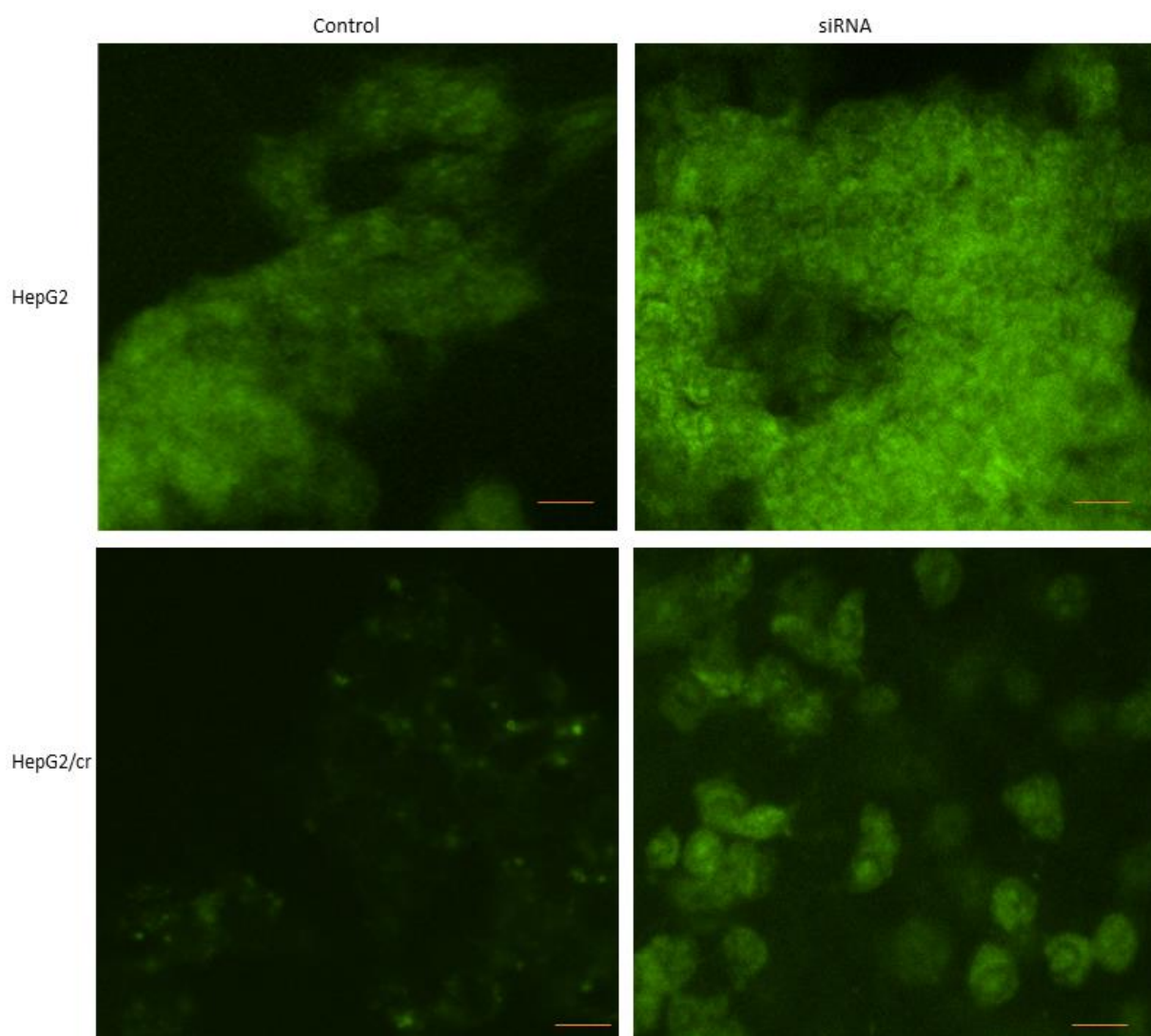


**Figure 6.5 Cellular uptake of Alexa fluor 488-labelled cisplatin after TG2 mRNA silencing measured by flow cytometry**

(A) Western blot showing reduction of TG2 expression after siRNA treatment for 48 hours. (B) Cellular uptake of fluorescent cisplatin after siRNA treatment. Parental and resistant cells were pre-treated with, or without siRNA specific for TG2 mRNA for 48 hours and then cells were incubated with Alexa fluor 488- labelled cisplatin to a final concentration of 40 U/ml for up to 2 hours. Then cells were harvested by trypsinisation, suspended in ice-cold PBS and fluorescence intensities were measured by flow cytometry with fluorescent detector set at FL-1 (FITC). The results are  $\pm$ SD of triplicate experiments.

The short form (TG2-S) however was not fully eliminated from cells, although its expression appeared much reduced in both cell lines, which showed only a trace of TG2-S (figure 6.5A). This incomplete removal of TG2-S from cells may explain the

pattern of uptake of cisplatin when control cells are compared to TG2 knockdown cells (figure 6.5B).



**Figure 6.6 Cellular uptake of Alexa fluor 488-labelled cisplatin after TG2 mRNA silencing analysed by confocal microscopy**

TG2 expression of HepG2 and HepG2/cr cells was reduced using specific TG2 siRNA oligomer for 48 hours and then cells were incubated with Alexa fluor 488-labelled cisplatin at a final concentration of 200 U/ml for 1 hour. Then cells were fixed in 70% ethanol at -20 °C and mounted in fluorescent mounting medium. The slides were observed under a laser scanning confocal microscope at x400 magnification with excitation/emission wavelengths set at 488/530 nm. Scale bar = 0.44  $\mu$ m.

The pattern of expression suggests that TG2-L knockdown increases cisplatin uptake in both cell lines, as the fluorescent intensity was greater in siRNA-treated cells

compared to non-treated (control groups) in both cell lines. In siRNA-treated cells, fluorescent cisplatin was visible in the nucleus of both cell lines, whereas fluorescent cisplatin was not seen in siRNA non-treated (control group) HepG2/cr cells (figure 6.6).

### **6.3 Conclusion**

These results suggest that the presence of TG2 either in the cell membrane or cytoplasm reduced the trafficking of anti-cancer drug cisplatin in cisplatin-resistant cancer cells and that its inhibition may provide a novel mechanism to overcome the defective uptake of anti-cancer drugs in other chemotherapy-resistant cancers. TG2 may possibly be of use as a biomarker of defective cellular uptake or endocytosis in clinical tumour chemotherapy.

## **CHAPTER 7:**

### **IMMUNOPRECIPITATION OF TRANSGLUTAMINASE 2**

### **SUBSTRATES DURING CELLULAR UPTAKE OF**

### **DANSYLCADAVERINE**

#### **7.1 Introduction**

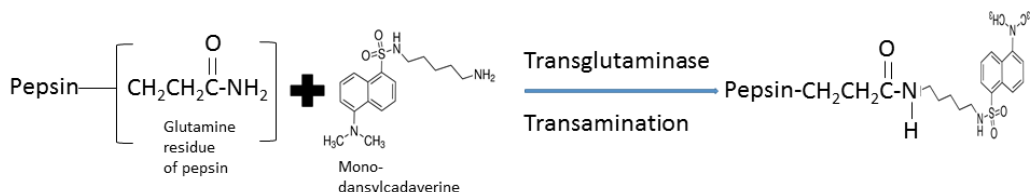
The purpose of this chapter is to validate anti-dansyl antibody so that this antibody can be used to immunoprecipitate DNC-labelled proteins, which have been modified by TG2 during cellular uptake of DNC in HepG2 cells, as discussed in chapter 3. For this purpose, first the catalytic action of TG2 was confirmed using pepsin as a model substrate for transamination reaction. Pepsin was used as it was commercially cheap, and its complete crystal structure was available, and modification sites are relatively easy to predict. Previously researchers have used pepsin for such kind of studies but using putrescine as an amine donor (Coussons, et al., 1992b). Also studies on pepsin modification will contribute to better understand TG2 substrate specificity. Once confirmed following pepsin modification study that DNC-labelled pepsin can be isolated and visualised by immunoprecipitation using anti-dansyl antibody, the same antibody was used to immunoprecipitate DNC-labelled proteins from cell lysate that had been treated with DNC.

TG2 has been implicated in the control of several physiological processes by modifying substrate proteins. As observed in chapters 3 and 6, the presence of TG2 has implications in the mechanism of cellular uptake of dansylcadaverine and cisplatin, respectively, in HepG2 cells. Therefore, the tendency of TG2 to modify proteins during cellular uptake of DNC was investigated. HepG2 cells were treated with DNC and the DNC-labelled proteins were then immunoprecipitated and characterised according to their molecular weight by SDS-PAGE. Initially,

immunoprecipitation and TG2 activity assays were developed using pepsin as a model substrate protein and DNC as an amine donor (see below).

### 7.1.1 Pepsin as a model substrate for TG2

Pepsin is a well-known gastric protease that catalyses the breakdown of the polypeptide chain of proteins into smaller peptides under physiological conditions. Detailed information is available on its complete structure (Tang, et al., 1973; Cooper, et al., 1990). Previously, Waelsch reported that pepsin can be a candidate substrate of transglutaminase (Mycek and Waelsch, 1960). With the help of identified predictor amino acids (Khew, et al., 2010), pepsin was examined and predicted as a substrate protein for transglutaminase and this was confirmed experimentally *in vitro*. The modification of pepsin by guinea pig liver transglutaminase is shown in figure 7.1.



**Figure 7.1 Illustration of the pepsin modification reaction catalysed by transglutaminase.**

The amine group of glutamine residue of pepsin may be replaced with dansylcadaverine by transglutaminase enzyme activity in the presence of  $\text{Ca}^{2+}$ . The product of this reaction is a dansylcadaverine-labelled glutaminy residue of pepsin.

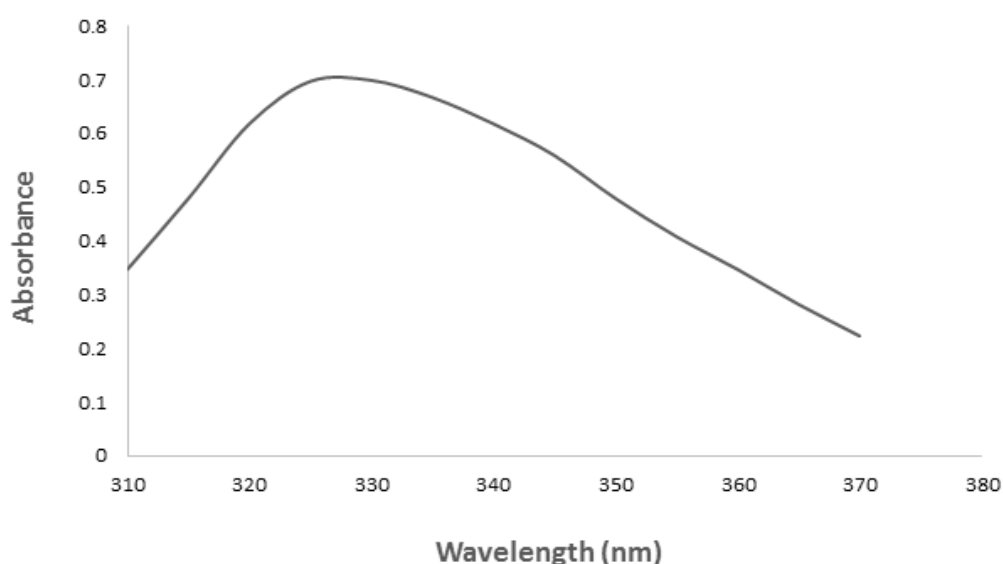
The action of TG2 activity was investigated using DNC as an amine donor and porcine pepsin as an amine acceptor, to establish the subsequent experiments that can be performed in cellular environment to characterise and isolate DNC-labelled proteins, which may have been modified by the action of TG2, during cellular uptake of drugs (i.e., DNC and cisplatin), to better understand the physiological roles of TG2 in the mechanism of membrane transport of compounds/drugs.



## 7.2 Results

### 7.2.1 Measurement of absorbance spectrum of dansylcadaverine

In order to quantitate DNC, the molar absorbance coefficient was determined. The absorbance maximum of dansylcadaverine (DNC) was identified by measuring the optical density of DNC in a 1 cm cuvette at various wavelengths. Figure 7.2 shows the absorbance spectrum of DNC; it indicates that the maximum fluorescence absorbance of DNC was obtained at a wavelength of 330 nm.



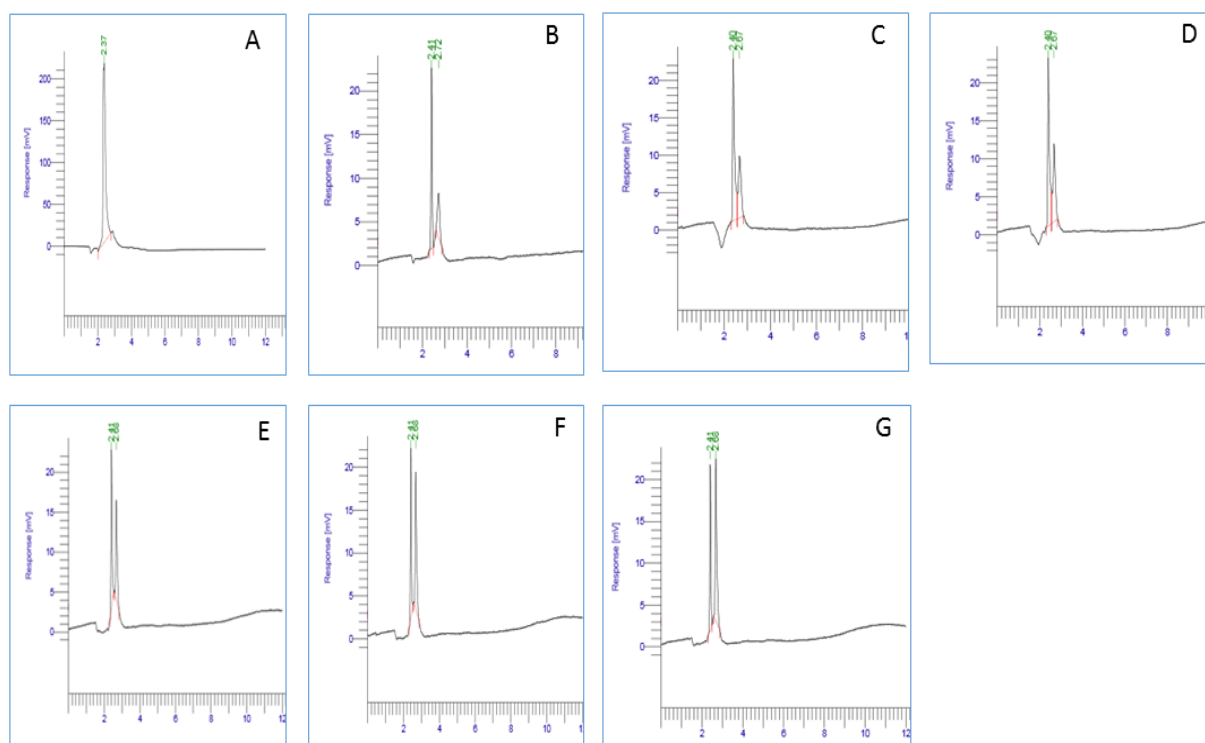
**Figure 7.2 Measurement of absorbance spectrum wavelength of DNC.** Absorbance of 1 ml of DNC (1 mM) in 1 cm cuvette was measured at wavelengths of 310-370 nm.

### 7.2.2 Analysis of DNC-labelled pepsin by HPLC

To characterise the TG2 activity for subsequent experimental groups, the susceptibility of porcine pepsin to the action of guinea pig liver transglutaminase was tested by measuring the  $\text{Ca}^{2+}$ -dependent incorporation of the primary amine dansylcadaverine (DNC). Pepsin was deactivated by incubating the samples at pH 8.0 and the stock solution was stored at 4 °C for 24 hours, at pH 8.0 prior to the modification reaction. The deactivated pepsin was then incubated with DNC and  $\text{Ca}^{2+}$

in the presence or absence of guinea pig liver transglutaminase for up to 24 hours at RT.

The modification of pepsin was first confirmed by isolating samples by HPLC using a C18 column (see section 2.26). The samples with modified pepsin were collected and measured using a UV detector set at 215 nm. As the incubation time was increased, more pepsin was labelled with DNC. In figure 7.3B, the second peak represents non-modified pepsin, whereas in figures 7.3C-G, the second peak represents the modified pepsin with DNC. Results indicate that as incubation time increased, the second peak increased in size (figures 7.3C-G).

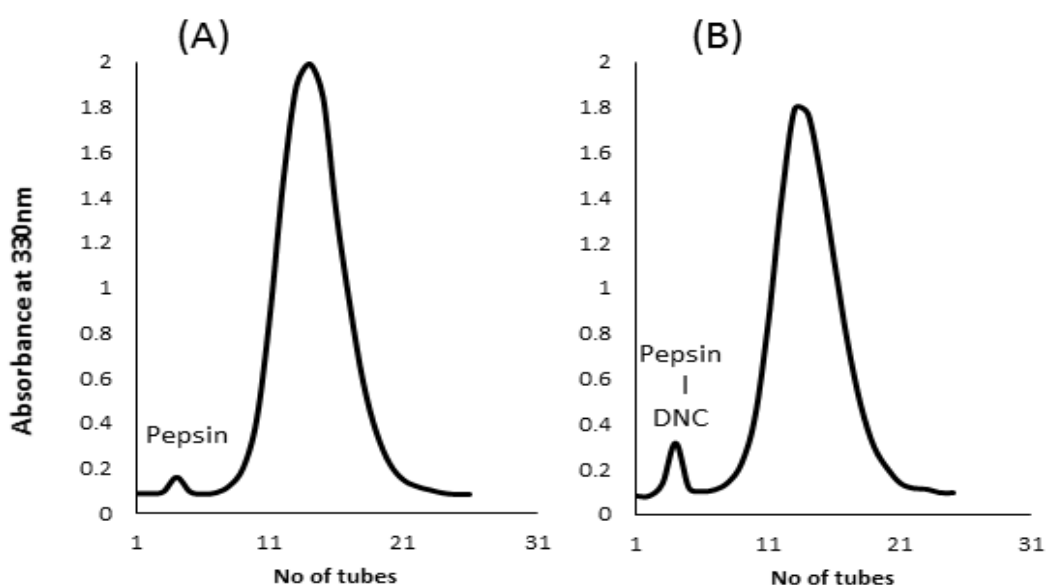


**Figure 7.3 Elution profile of dansylcadaverine-labelled pepsin by HPLC.**

Pepsin was incubated with dansylcadaverine in the presence or absence of guinea pig liver transglutaminase for different time intervals, and 100  $\mu$ l from reaction mixtures were taken out at different time points and separated by HPLC. (A) Blank reaction (no pepsin and no transglutaminase), (B) control (only pepsin), (C) 1 hour, (D) 2 hour, (E) 5 hours, (F) 10 hours, and (G) 24 hours. All the graphs (except A and B) shown here are products of reaction in presence of transglutaminase and absence of EDTA.

### 7.2.3 Analysis of TG2 activity by DNC-incorporation into pepsin by gel filtration chromatography

Following TG2 catalysed modification of pepsin (see section 2.24), the dansylated-pepsin was isolated by gel filtration chromatography using a PD-10 sephadex G-25 column. Then, absorbance of eluted samples was measured at 330 nm, at which the dansyl group gives maximum fluorescence.



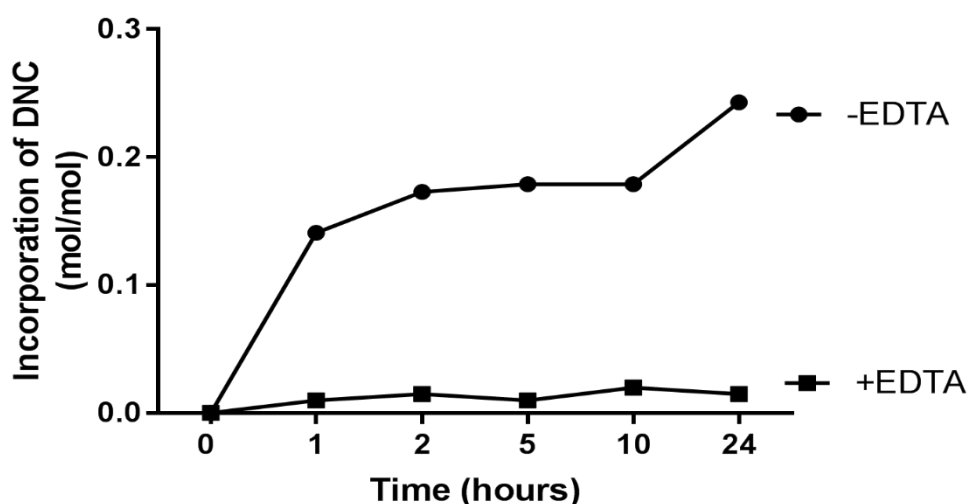
**Figure 7.4 Elution profile of dansylcadaverine labelled pepsin of gel filtration on PD-10 Sephadex G-25 columns.**

The samples (A) in the absence of transglutaminase, (B) in the presence of transglutaminase were prepared and the reaction mixtures after 24 hours of incubation were separated by gel filtration chromatography. The absorbance of eluted samples (1 ml each in separate tube) was measured at 330 nm. No of tubes on x-axis indicates the 1 ml samples in each single tube. Results are representative of triplicate experiments.

The first peak in figure 7.4B indicates that the DNC-labelled pepsin has increased absorbance at  $A_{330}$  compared to non-modified pepsin (figure 7.4A). These results confirm earlier reports that porcine pepsin can be modified by transamination reaction by transglutaminase enzyme (Coussons, et al., 1992b). The second major peak represents non-covalently bound, i.e., free DNC.

#### 7.2.4 Time-dependent incorporation of dansylcadaverine into pepsin by guinea pig liver transglutaminase

The quantitative analysis of DNC-labelled pepsin was performed by measuring the optical density of eluted samples at 330 nm at various time intervals.



**Figure 7.5 Time dependent incorporation of dansylcadaverine into pepsin.**

Time-dependent incorporation of dansylcadaverine into pepsin by guinea pig liver transglutaminase (gpTgase). Pepsin was incubated with gpTgase in the presence or absence of EDTA for various time points.

Figure 7.5 shows that as the incubation time increased, more DNC was incorporated into pepsin. In the presence of EDTA no DNC was incorporated. The results show that 0.25 mol of DNC was incorporated per 34 kDa subunit of pepsin. Previous studies, using the less bulky substrate putrecine, suggest that up to 1 mol/mol incorporation is possible in non-reduced sample of pepsin, suggestive of a single reactive strand in the protein (Coussons, et al., 1992b). Evidence of high molecular weight ( $M_r$ ) material in Coomassie stained gels indicate that cross-linking of pepsin occurs, which probably explains  $< 1$  mol/mol DNC-pepsin incorporation (see figure 7.7).

### 7.2.5 Western blotting showed pepsin labelled with dansylcadaverine

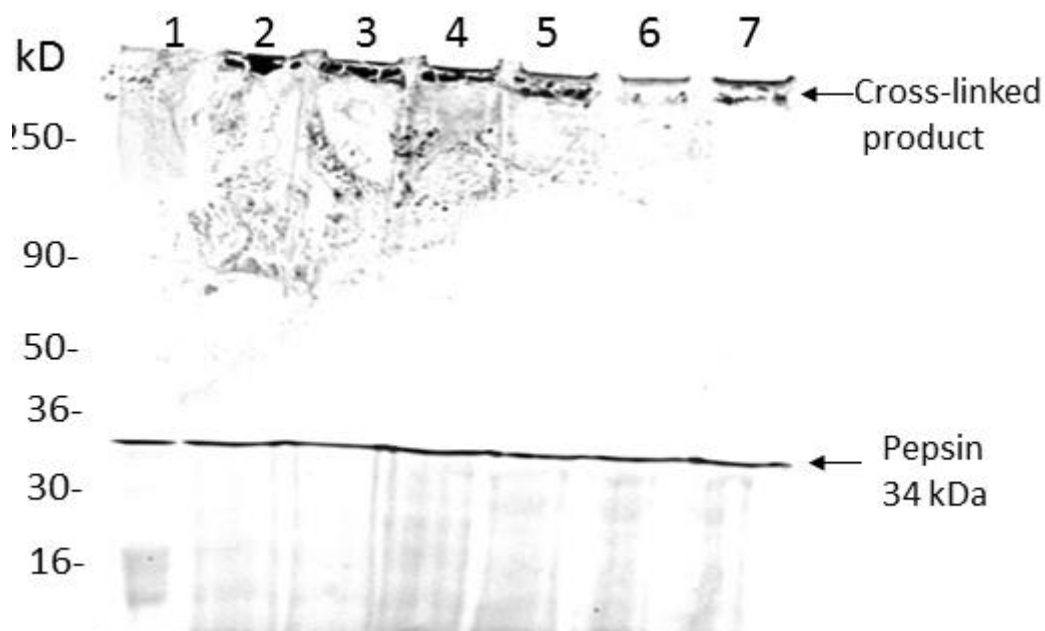
The pepsin was labelled with DNC by Tgase for up to 24 hours (section 7.2.2, 7.2.3, 7.2.4) and the resulting products were analysed using SDS-PAGE and by inspecting gels under ultraviolet fluorescent (UV) light (figure 7.6A).



**Figure 7.6 Western blot of dansylcadaverine-labelled pepsin.**

(A) Inspection of a gel in UV fluorescent light before transferring to nitrocellulose membrane, (B) Western blot of modified pepsin detected by anti-dansyl antibody. Lanes 1-7 represent the samples in the presence of Tgase incubated for different period: Lane 1 - Negative control (EDTA was added to reaction mixture; sample loaded is from 24 hours incubation); lane 2 - 0 hour; lane 3 - 1 hour; lane 4 - 2 hours; lane 5 - 5 hours; lane 6 - 12 hours; lane 7 - 24 hours; lanes 8-13 represent the samples in the absence of Tgase, lane 8 - 0 hour; lane 9 - 1 hour; lane 10 - 2 hours; lane 11 - 5 hours; lane 12 - 12 hours; lane 13 - 24 hours. Lanes described represents both diagrams. Results are representative of three independent experiments.

The pepsin became increasingly fluorescent as a function of incubation time (figure 7.6A, lanes 1-7), whereas no visible bands were observed in the presence of EDTA and/or when Tgase was omitted from mixtures (figure 7.6A, lanes 8-13).



**Figure 7.7 SDS-PAGE of modified pepsin.**

Only DNC-labelled pepsin samples were separated on 12% SDS-PAGE gel, as apposed to non-labelled pepsin samples. Lanes represent the samples taken at different periods of incubation. Lane 1 - control (only pepsin); lane 2 - 0 hours; lane 3 - 1 hour; lane 4 - 2 hours; lane 5 - 5 hours; lane 6 - 10 hours; lane 7 - 24 hours.

The Coomassie staining of this gel showed effectively no changes of the protein band pattern of labelled pepsin (figure 7.7), in contrast to the bands observed under UV. Western blot analysis confirmed that there was increasing pattern of pepsin modification (figure 7.6B).

### **7.2.6 Preliminary structural characterisation and identification of pepsin as a candidate protein substrate**

In order to investigate the substrate preference of TG2, pepsin was used as a model substrate protein, as its pure form is commercially available in relatively large quantities at reasonable cost and because the complete crystal structure is known (Tang, et al., 1973; Cooper, et al., 1990). The online ExPASy bioinformatics search engine was used to access the amino acid sequence of porcine pepsin and thus develop a model for prediction of available modification sites. The available scientific research articles were used to find out the complete structure of pepsin and the

TRANSDAB wiki ([http://genomics.dote.hu/wiki/index.php/Main\\_Page](http://genomics.dote.hu/wiki/index.php/Main_Page)) (Csosz, Mesko and Fesus, 2009) tool was used to find out the list of existing Tgase substrates. The primary amino acid sequence of pepsin and alignment of glutamine residues is shown in figure 7.8.

| Residue no |   | glutamine |   | Residue no |   |   |   |   |   |   |   |     |
|------------|---|-----------|---|------------|---|---|---|---|---|---|---|-----|
|            | ↓ |           | ↓ |            |   |   |   |   |   |   |   |     |
| 19         | I | G         | T | P          | A | Q | D | F | T | V | I | 30  |
| 49         | C | S         | D | H          | N | Q | F | N | P | D | D | 61  |
| 63         | F | E         | A | T          | S | Q | E | L | S | I | T | 75  |
| 84         | G | Y         | D | T          | V | Q | V | G | G | I | S | 96  |
| 93         | I | S         | D | T          | N | Q | I | F | G | L | S | 105 |
| 137        | D | N         | L | W          | D | Q | G | L | V | S | Q | 149 |
| 142        | Q | G         | L | V          | S | Q | D | L | F | S | V | 154 |
| 185        | V | E         | G | Y          | T | Q | I | T | L | D | S | 197 |
| 205        | C | S         | G | G          | S | Q | A | I | V | D | T | 217 |
| 227        | I | A         | I | N          | I | Q | S | D | I | G | A | 240 |
| 249        | C | S         | S | I          | D | Q | L | P | D | I | V | 261 |
| 261        | T | I         | D | G          | V | Q | Y | P | L | S | P | 273 |
| 272        | S | A         | Y | I          | L | Q | D | D | D | S | C | 284 |
| 303        | D | V         | F | I          | R | Q | Y | Y | T | V | F | 315 |

**Figure 7.8 Prediction of glutamyl residues in primary structure of pepsin.**

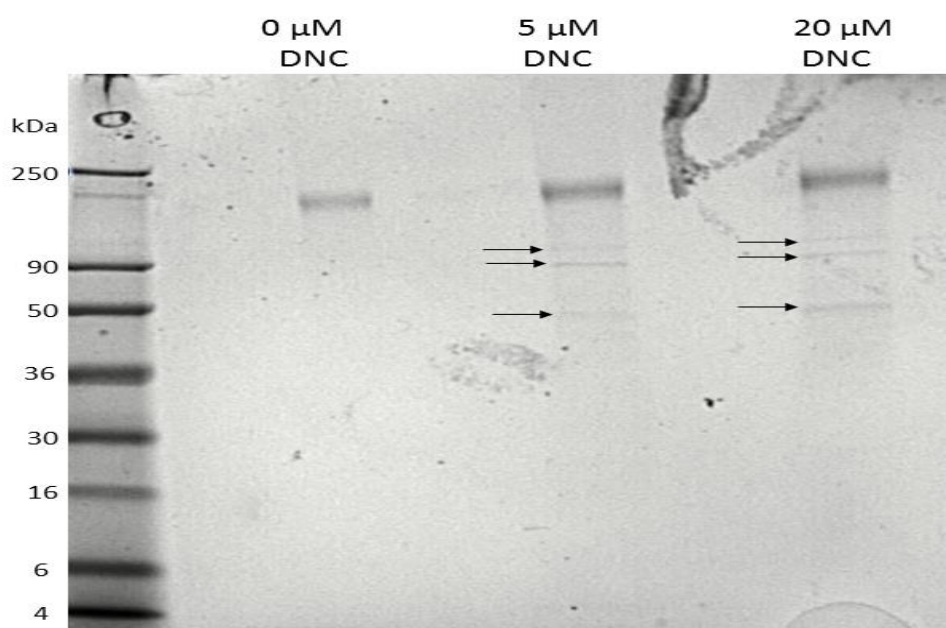
Possible reactive glutamine residues (shown in red) for transamidation reaction extracted from the primary structure of pepsin. Out of these, two glutamine residues (Gln-278 and Gln-309) were selected by applying the rules described in Table 1.1.

On the basis of rules as described in Table 1.1 in the introduction (see section 1.5), two short peptides of amino acid residues SAYILQDDSDSC (Gln-278) and DVFINQYYTVF (Gln-309) were predicted as possible Tgase modification sites in pepsin (figure 7.8). The predicted glutamine residues were from the C – terminus end of pepsin.

### 7.2.7 Characterisation of cellular proteins during cellular uptake of dansylcadaverine in HepG2 cells

It was confirmed from the Western blot of DNC-labelled pepsin that DNC groups can cross-react with anti-dansyl antibody (figure 7.6), before using the same antibody to immunoprecipitate DNC-labelled protein from cellular lysates. HepG2 cells were treated and total proteins were isolated following cell lysis; non-DSC treated cells were tested in parallel as controls (section 2.28). The results obtained showed that a

limited number of proteins were labelled with DNC following cellular uptake (figure 7.9).



**Figure 7.9 Immunoprecipitation of DNC-labelled proteins from HepG2 cells**

HepG2 cells were treated with different concentrations of DNC for 24 hours. Then, cell lysates were prepared using cell lysis RIPA buffer. To the 200  $\mu$ l of cell lysates, 5  $\mu$ g of anti-dansyl antibody were added and DNC-labelled proteins were isolated by immunoprecipitation. The DNC labelled proteins are indicated by the arrows.

Following 24 hours' incubation of cells with DNC, three DNC-labelled bands were observed with molecular weights of 102 kDa, 90 kDa, and 46 kDa. One of the bands, at 46 kDa, resembles the known molecular weight of TG2 substrate aldolase A, which has been previously identified as an intracellular substrate (Lee, et al., 1992). Aldolase A is known to generate extra energy for cancer cells; this drives cells towards cell proliferation and chemoresistance. If the modified protein is aldolase A, then TG2 may possibly inhibit energy generation in cancer cells by modifying aldolase and this could also impact on the inhibition of cellular uptake of drugs. The other two bands were not close to the molecular weight of other identified intracellular TG2 substrates, suggesting some new substrates of TG2 and these substrates may



possibly be involved during cellular uptake of drugs. Sequence analysis will be required to confirm whether the 46 kDa band found was actually aldolase A.

### **7.3 Discussion**

The specificity of transglutaminase (Tgase) towards substrate proteins depends partly on the reaction conditions of the cellular environment. So far, more than 155 substrates of TG2 have been identified (see appendix II) but none of these substrates give a clear consensus sequence regarding the substrate specificity of TG2.

From the primary structure of pepsin, short peptides containing glutamine (fig 7.8) were analysed for possible reactive glutamine substrate of TG2. This was done by applying the predictive model, which distinguishes both encouraging and discouraging factors in potential substrates (Table 1.1). From the crystal structure and primary structure of pepsin (Tang, et al., 1973; Cooper, et al., 1990), it was clear that out of 14 glutamine residues, two glutamines were selected as possible reactive glutamines. The side chains of Gln-143, Gln-148 and Gln-255 were not significantly exposed to the solvent. By applying the charge rules, one of the discouraging characteristics for transglutaminase substrates specificity (Coussons, et al., 1992a), showed that the Gln-55 (Aspartate at -3, +4, +5), Gln-69 (Glutamate at -4), Gln-90 (Aspartate at -3), Gln-99 (Aspartate at -3), Gln-191 (Aspartate at -4, Glutamate at -4), Gln-211 (Aspartate at +4), and Gln-267 (Aspartate at -3) would not act as substrates for modification because these peptides contain one of the amino acids that discourage enzyme specificity towards substrate. The residues Gln-25, Gln-233 and Gln-278 were ruled out as these residues contain more than one aspartic acid. Gln-278 (SAYILQDDDSC) and Gln-309 (DVFINQYYTVF) (shown in 3D structure in figure 7.10) were considered as possible reactive glutamine residues for modification by guinea pig liver transglutaminase, as these residues were exposed to the solvent and have accessibility of 0.67 which is a minimal requirement for transglutaminase-catalysed modification. The two predicted reactive glutaminy residues were from C-

terminal sequences, and contained features that resemble other studies (Khew, et al., 2010).



**Figure 7.10 Prediction of glutamyl residues in three-dimensional structure of pepsin.**

Position of possible glutamine residues (indicated by arrows) in three-dimensional structure of pepsin. These two glutamine residues were selected by applying the substrate characteristics in primary structure of pepsin and are shown at particular positions (yellow arrow indicates Gln-278, and blue arrow indicates Gln-309). The three dimensional structure was created in ExPasy bioinformatics online tool.

Quantitative analyses of DNC incorporated by transglutaminase indicate that pepsin has glutaminyl residues that are reactive for the transglutaminase reaction, and thus, further analysis by endoproteinase digestion and mass spectrometry of peptidyl fragments of DNC-peptides compared to control fragments would be required to validate these peptides as reactive residues. Also, further studies are needed to investigate whether glutaminyl residues of pepsin are an *in vivo* substrates and if so, what the role of TG2 in the regulation of pepsin's functions? The three DNC-labelled proteins isolated by immunoprecipitation following cellular uptake of DNC also need more analysis, e.g., by performing Mass spectrometric analysis.

In conclusion, the data presented here suggest that pepsin acts as a substrate of TG2 and the discouraging factors described by Coussons et al (1992) and other researchers are somehow essential for the substrate specificity of TG2. The peptide residues predicted in this study will contribute to better understanding of *in vivo* Tgase transamidation activity and substrate specificity, and can be used as suitable probes to assess the endogenous activity of TG2, which is associated with various diseases including cancer. Three DNC-labelled proteins observed as TG2 substrates may serve very important functions during cellular uptake of anti-cancer drugs such as cisplatin in chemoresistant cancer cells.

## **CHAPTER 8:**

### **Discussion and future work**

#### **8.1 Discussion**

Cisplatin, the so-called the “penicillin” of cancer (Cisplatin.org, 2017), remains an effective and extensively applied drug that is used to treat several types of cancer. Unlike other anti-cancer drugs, cisplatin is not restricted to licencing, and so is easily producible by any appropriately-validated company. However, chemoresistance to cisplatin, and toxicities to normal cells are major problems, but if this can be overcome it could again find a wider applicability. Thus, increasing cisplatin efficiency in cancer treatment could potentially save billions of pounds, and potentially many lives, which is worthy of investigation and this is the focus of the current study.

During cisplatin therapy, patients are injected intravenously, with a bolus dose of 75-100 mg/m<sup>2</sup> of cisplatin every 3-4 weeks, or on a monthly basis, in multiple cycles (often 5 cycles), depending on the type of cancer (McDermott, et al., 2014). Cisplatin is often used in combination with other antineoplastic drugs, to achieve maximum effects. Some patients may respond very well to this therapy, whereas some may respond well at first, but relapse after subsequent treatment. In other patients, resistance may start after just an initial dose. Consequently, development of such resistance may require administration of higher dosages, which can lead to severe multiorgan toxicities. Results presented here are consistent with the clinical scenario, and show that a single shock treatment of the hepatocellular carcinoma HepG2 cell line with 8  $\mu$ M cisplatin (relatively close to therapeutic plasma concentration, i.e. <2  $\mu$ g/ml (6.6  $\mu$ M) 2 hours postadministration (McDermott, et al., 2014)), continuously for 4 days, developed resistance to cisplatin (figures 5.2 and 5.3). The findings could be illustrative of how patients can develop cisplatin resistance after initial treatment, and

shows how a cancer cell can become aggressively more resistant following multiple cycles of chemotherapeutic treatment.

It is well known that TG2 levels are raised in many cancers (Eckert, et al., 2015), though the specific effects that this has on neoplastic cells are not fully understood. In recent years there has been a suggestion that, in addition to roles in apoptosis and cell adhesion, TG2 may contribute in some way to chemotherapeutic resistance, as described in detail in the introduction (section 1.9.4.1), and this contention is the main theme for the current investigation. It has long been known that TG2 may be involved in endocytosis (Davies, et al., 1980) and so any alterations in this process could easily explain reduced uptake of chemotherapeutic drugs (Mellman and Yarden, 2013), if they were to enter cells by this route. The results herein show that TG2 transamination activity is increased in cisplatin-resistant HepG2 cells (figure 5.7). This increased TG2 enzymatic activity suggests that TG2 may possibly cross-link cellular proteins and so inhibit apoptosis, and in that way may contribute to the development of drug resistance. This is in contrast to early studies on TG2, where researchers showed that extensive intracellular protein cross-linking by TG2 is required for induction of apoptosis (Fesus and Piacentini, 2002): this effects may be dependent on differences in cellular conditions and types of cell studied.

A series of experiments (see sections 4.2.7-4.2.9) that investigated the relationship between TG2 levels and cell survival confirmed the generally held view, that increased TG2 levels appears protective to cancer cells and that decreased TG2 levels are associated with poorer survival of cancer cells. This was done using retinoic acid to increase TG2 levels and cystamine to decrease TG2 levels. The results consistently support a role for TG2 in cell survival (figures 4.8 and 4.9). The findings reported here therefore suggest a potentially novel mechanism of action of cisplatin-induced cytotoxicity in relation to apoptosis or protection of cells from cisplatin via

modulation of TG2 expression (sections 4.2.2-4.2.9), and possibly by direct inhibition of enzyme activity (section 4.2.10).

Cellular uptake studies using DNC (which is a competitive inhibitor of TG2) showed that decreased TG2 levels increased uptake of fluorescent probe (presumably by endocytosis), both in HepG2 and CAK12 cell lines (figures 3.3 and 3.5). This was also seen using a fluorescently-labelled analogue of cisplatin (figure 6.6). Western blot analysis confirmed that loss of TG2-L was consistent with these effects, as siRNA knockdown did not significantly affect TG2-S levels, which showed some traces of TG2-S in both cell lines (figure 6.5). This incomplete removal of TG2-S explains the pattern of uptake of cisplatin in chemoresistant cells (figure 6.5B). Western blot analysis showed that cisplatin treatment caused loss of TG2-S from the plasma membrane and relocation to the cytoplasm (figure 6.3). This may explain the induction of apoptosis in these cells, as TG2-S is associated with proapoptotic activity (the reverse of TG2-L's role) (Antonyak, et al., 2006). Interestingly, no such relocation was seen in chemoresistant cells, where TG2-S remained in the plasma membrane following cisplatin treatment (figure 6.3). These results indicate that the presence of TG2 isoforms in the plasma membrane of chemo-resistant cells may affect the trafficking of cisplatin, and therefore may also be responsible for driving cancer cells towards chemo-resistance. To the best of my knowledge, I believe this is the first time it has been shown that TG2 isoforms change their sub-cellular location upon cisplatin entry. The two isoforms identified in this study were not directly dissected by specific analysis, e.g., Mass spectrometric (MS)-analysis, but conclusions are aided by published data from other studies (Antonyak, et al., 2006; Tee, et al., 2010).

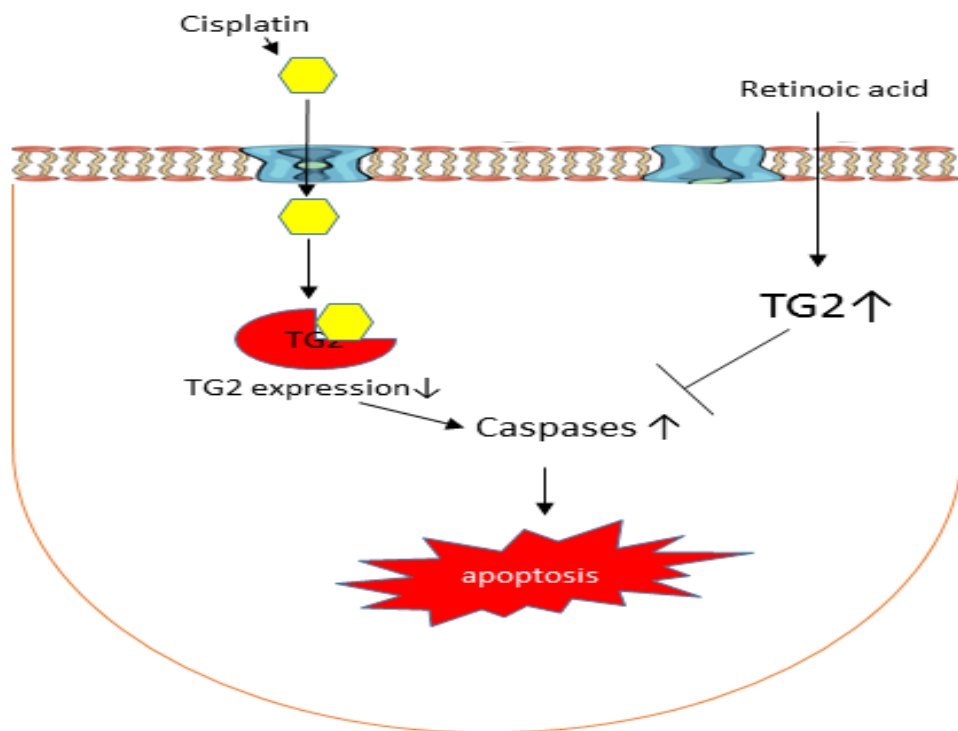
Collectively, the results suggest that TG2 may act by blocking uptake of DNC and cisplatin in hepatocarcinoma cells (HepG2) and may possibly involved in other cell types. Unblocking of this activity via inhibitors may therefore offer a strategy for increasing susceptibility of these cells to chemotherapeutic drugs. Interestingly,

cystamine derivatives are well tolerated clinically (Fujisawa, et al., 2012) and may offer a route to cisplatin/cystamine co-treatment of patients with hepatocellular carcinoma.

It is known from several studies that the reduced uptake of cisplatin in various cisplatin-resistant cells has been linked to various transporter proteins. These include ATP7B and ATP7A (Katano, et al., 2004; Safaei, et al., 2005), Claudin-4 (Yoshida, et al., 2011), TMEM205 (Shen, et al., 2010), and ABC transport proteins MRP1 and MRP2 (Kool, et al., 1997; Borst, et al., 2000) - suggesting important, but not necessarily exclusive, roles for such transporters during influx and efflux of drugs in drug-resistant cells. The current data suggest a reasonably strong connection between TG2 and cisplatin uptake, leading to an increase in cell death by apoptosis in tumour cells - but does not yet offer a mechanistic explanation. Thus, on the basis that TG2 substrate may contribute to the effects observed, TG2 substrates that had been modified by DNC during uptake experiments were immunoprecipitated and analysed by SDS-PAGE. The results show that a protein of approximately the same molecular weight as aldolase (46 kDa) was modified during DNC treatment (figure 7.8). Increased expression of this glycolytic enzyme has been associated with cancer cells and it has previously been identified as a substrate for TG2 in HT29 colon cancer cells (Lee, et al., 1992). As cancer cells are essentially glycolytic in their energy production, it would be interesting if ATP-dependent endocytic events were modified (i.e. blocked) downstream by TG2 modification of a key glycolytic enzyme such as this. Also, other two DNC-labelled bands found were unknown to previously identified substrates, suggesting some new substrates of TG2 and these substrates may possibly suggest a connection between TG2 and cellular uptake of drugs.

Finally, it may be significant that *in vitro* experiments presented here show that as well as affecting gene expression of TG2, cisplatin can directly inhibit TG2 (figure 4.10). This suggests a secondary route to potentially blocking TG2 activities by

cisplatin and thus inducing cell death, by a hitherto undocumented mechanism. Therefore, the current study speculates that this inhibition of TG2 by cisplatin may be involved in the stimulation/regulation of other pro-apoptotic/pro-survival proteins involved in apoptotic/cell survival pathways in addition to its direct involvement, in apoptotic body formation in HepG2 cells (figure 8.1).



**Figure 8.1 Proposed mechanism of cisplatin-induced apoptosis or cell survival by TG2.**

In HepG2 cells, cisplatin inhibited the expression and activity of TG2. Once TG2 is inhibited, it may increase the expression of caspases and trigger apoptosis (Tatsukawa, et al., 2011). On the other hand, overexpression of TG2 by retinoic acid may inhibit caspases and thus protect cells from cisplatin toxicity.

Several questions arise as a consequences of this work: 1) Is TG2 alone responsible for transport of cisplatin? Or 2) Does it regulate other drug transporters? These questions need further analysis.



## 8.2 Conclusions

The induction of apoptosis in hepatocarcinoma cancer cells by cisplatin appears not only to be dependent on drug-dependent DNA modification, but may also be due to functional damage caused by this drug to known protein modulators of apoptosis, e.g., TG2. *In vitro* results from this study show that there may be some direct effect of cisplatin on TG2 activity, possibly caused by cisplatin competing for intracellular substrates at the active (lysyl) binding site of TG2. Collectively, these results may explain why increased TG2 expression in cancer cells contributes to the development of their resistance to chemotherapeutic drugs such as cisplatin; higher doses may be expected to be required to reduce TG2 expression to levels consistent with non-resistance. These observations also support the contention that there may be potential for improving cisplatin-based cancer treatment by enhancing the effectiveness of patient-tolerable cisplatin doses, especially if cisplatin chemotherapy is combined with relatively non-toxic doses of specific TG2 inhibitors, such as DON compounds (6-diazo-5-oxo-L-norleucine containing peptides). Such treatment strategies for patients might also include avoidance of rich nutritional sources of retinoids, e.g., carrots, which have been counter-indicated in several epidemiological nutritional studies, where recipients of these supplements became more prone to development of cancer(s) than those consuming placebos (Omenn, et al., 1996). Collectively, these results indicate that the level and location of TG2 activity in hepatocellular carcinoma prevents or limits the entry of anti-cancer drugs, and thus inhibits apoptosis. Thus, TG2 may represent a novel biomarker and therapeutic target for overcoming the chemotherapeutic resistance of hepatocarcinoma.

### 8.3 Future work

Several future studies are suggested from the findings of this thesis; these may include the following:

- The investigation of cisplatin/TG2 interactions, including bioinformatic modelling to demonstrate the feasibility of binding of cisplatin at the catalytic site;
- Experiments described herein (sections 4.2.6, 4.2.9 and 6.2.3) should be repeated using more specific inhibitors of TG2, e.g., DON compounds (6-diazo-5-oxo-L-norleucine containing peptides);
- The potential compensation by TG1 and other Tgases should be investigated in cell death, as this study detected induction of TG1 expression during cell death;
- Given the protective effects of TG2 against cisplatin seen herein on high TG2 expressing system like Hepatocarcinoma, it would be interesting to test to see if the same effects could be seen in low TG2 expressing systems;
- It would be a large step forward to test to see if these cisplatin-resistant HepG2 cells can survive as implants in mouse models (He, et al., 2015; Rao, et al., 2016) and to compare the effects of modulation of TG2 expression on cellular uptake mechanisms as observed in this study;
- It would be useful to investigate the effects of cisplatin on other modulators of apoptosis, e.g., caspases, both *in vitro* and *in vivo* in order to help further understand the mode of action of cisplatin on downstream elements of apoptotic pathways;
- It would also be interesting to investigate the involvement of TG2 during cellular uptake of other anti-neoplastic/cisplatin drugs in HepG2/other cell lines, respectively;

- Identify the immunoprecipitated protein in both parental and chemoresistance cells using MS-analysis, western blotting and endoproteinase digestion and investigate their link with TG2 in the mechanism of drug trafficking. This should help clarify whether TG2 alone is a drug transporter or it is just an activator/deactivator and/or blocker of other drug transporters;

Collectively, such future studies will help to illustrate further the mechanisms involved in cancer development, and will help to develop novel strategies to design drugs and increase efficiency of cisplatin chemotherapy.

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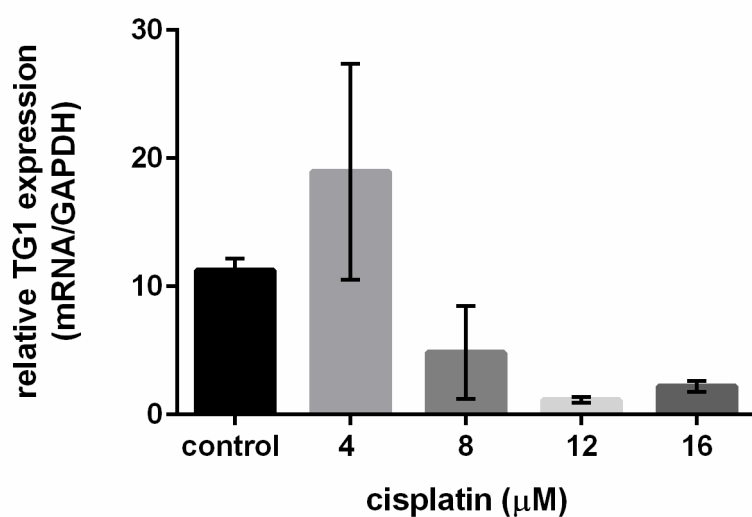
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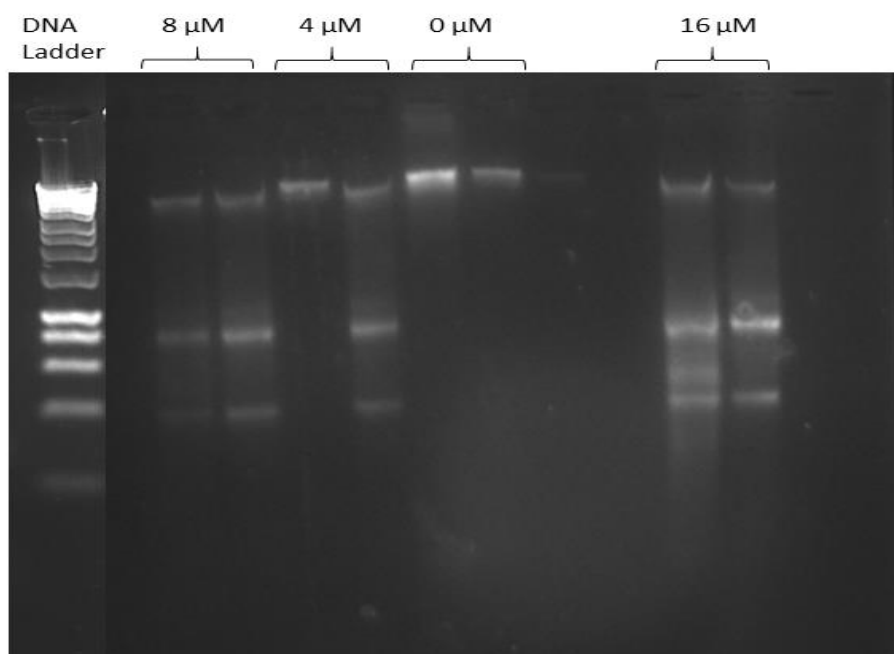
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## Appendix I: Supplementary data for chapter 4, 5 and 6



**Figure I.1 Effects of cisplatin on TG1 gene expression**

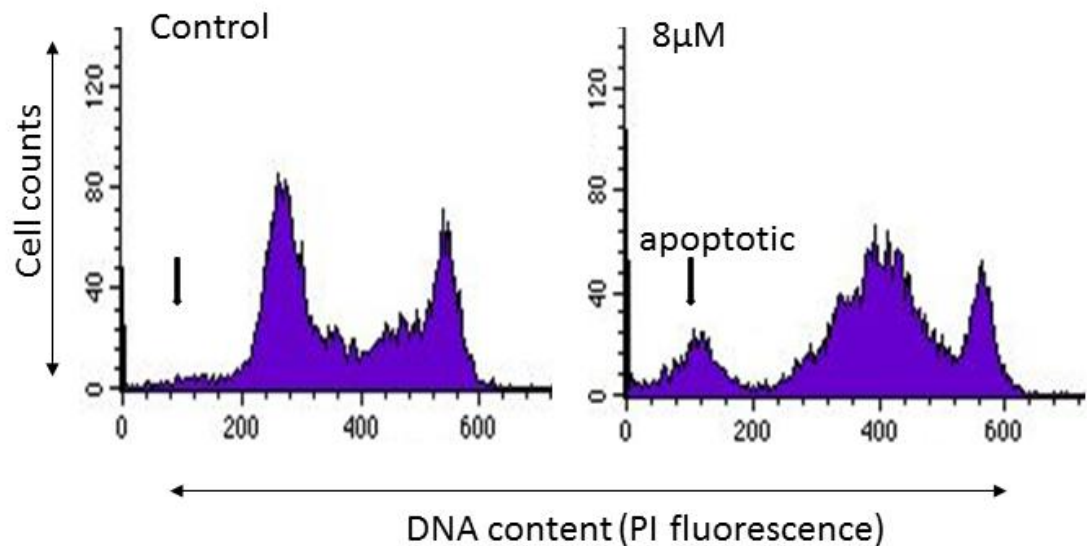
TG1 mRNA expression measured by RT-PCR. HepG2 cells were incubated with 0-16 μM cisplatin for 24 hours.



**Figure I.2 Confirmation of apoptosis by DNA fragmentation analysis.**

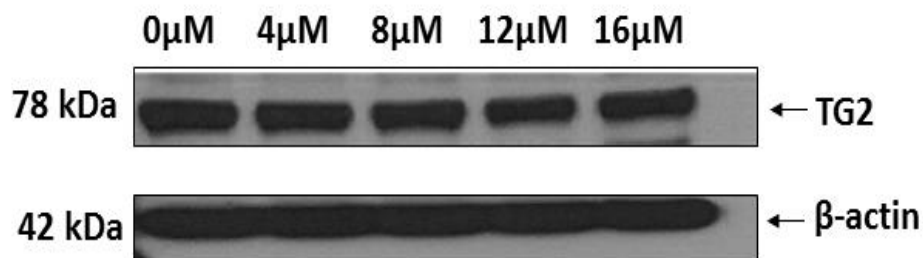
HepG2 cells were treated with 0-16 μM cisplatin for 24 hours. DNA was isolated and separated on 1.2% agarose gel electrophoresis.





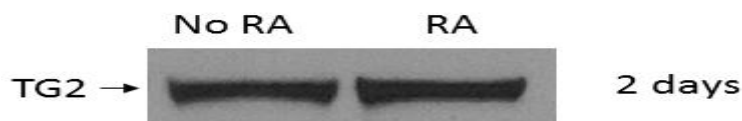
**Figure I.3 Apoptosis induction by cisplatin treatment after 48 hours of incubation.**

HepG2 cells were treated or not treated with 8  $\mu$ M cisplatin for 48 hours. Apoptosis induction was analysed by flow cytometry using propidium iodide (PI) staining.



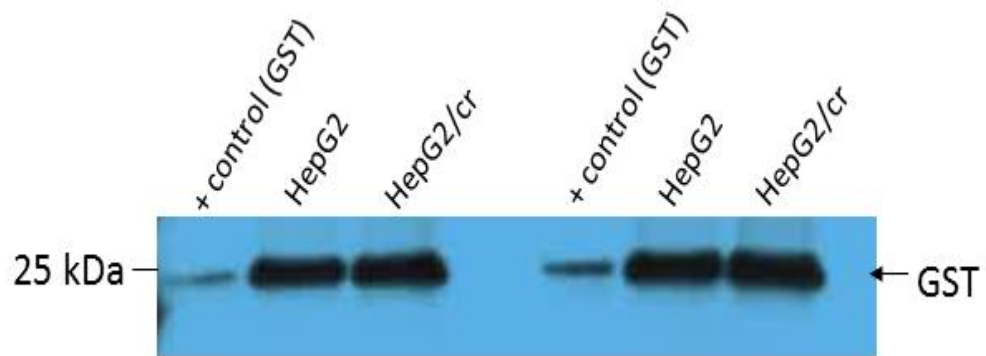
**Figure I.4 Effects of cisplatin on TG2 protein expression after 6 hours of treatment.**

HepG2 cells were treated with 0-16  $\mu$ M cisplatin for 6 hours. TG2 protein expression was measured by Western blotting.



**Figure I.5 TG2 expression after retinoic acid treatment for 2 days.**

HepG2 cells were treated with 10  $\mu$ M retinoic acid for 2 days. TG2 protein expression was measured by Western blotting.



**Figure I.6 Comparison of glutathione-s-transferase (GST) expression in HepG2 and HepG2/cr cells.**  
Western blot showing the bands for GST in HepG2 and HepG2/cr cells.

## Appendix II: List of TG2 substrate proteins

| Substrate   | Subcellular Localization                       | Reactive glu/lys                |
|---|--|---------------------------------|
| <b>40S ribosomal protein SA</b>                         | Cytosol  | -                               |
| <b>Acidic proline rich protein</b>                      | Extracellular; enamel pellicle in oral cavity  | -                               |
| <b>Aconitase</b>  | Mitochondria                                   | -                               |
| <b>ACTH</b>   | Plasma   | -                               |
| <b>Actin(TG2)</b>                                       | Cytoskeleton                                   | Q41/K113                        |
| <b>Aldolase A</b>                                       | Cytosol  | -                               |
| <b>Alpha lactalbumin(TG2)</b>                           | Extracellular; milk                            | Q54/K16,K93,K122                |
| <b>Alpha synuclein</b>                                  | Cytosol  | Q79,Q99/K80                     |
| <b>Alpha-2 macroglobulin receptor</b>                   | Plasma membrane                                | -                               |
| <b>Alpha-2 plasmin inhibitor</b>                        | Extracellular; plasma                          | Q2                              |
| <b>Alpha-2-HS-glycoprotein</b>                          | Extracellular; mineralized compartment of bone | -                               |
| <b>Alpha-ketoglutarate dehydrogenase</b>                | Mitochondrial matrix                           | -                               |
| <b>Amyloid beta A4 peptide</b>                          | Extracellular; Cerebrospinal fluid             | Q15/K16,K28                     |
| <b>Androgen receptor</b>                                | Nucleus  | K311                            |
| <b>Angioidin</b>  | Cytosol extracellular matrix                   | -                               |
| <b>Ankyrin</b>  | Erythrocyte cytoskeleton                       | -                               |
| <b>Annexin I</b>  | Cytosol; plasma membrane                       | Q19/K29                         |
| <b>Arginase I</b>                                       | Cytosol  | -                               |
| <b>AT-rich interactive domain-containing protein-1A</b> | Nucleus  | Q82,Q84,Q98<br>(Q465,Q467,Q481) |
| <b>Ataxin-1</b>   | Nucleus  | -                               |

|   |  |   |
|---|--|---|
| <b>ATP synthase</b>                             | Mitochondrial inner membrane                   | -   |
| <b>Band 3 anion transport protein</b>           | Erythrocyte membrane                           | Q30   |
| <b>Band 4.1 protein</b>                         | Erythrocyte cytoskeleton                       | -   |
| <b>Bcr-Breakpoint cluster region</b>            | Cytosol  | -   |
| <b>Beta casein</b>                              | Extracellular; milk                            | Q167/Q182,Q194  |
| <b>Beta endorphin</b>                           | Extracellular; secreted                        | Q11/K29   |
| <b>Beta tubulin(TG2)</b>                        | Cytoskeleton                                   | -   |
| <b>Beta-2-microglobulin</b>                     | Cell surface                                   | -   |
| <b>Betaine-homocysteine S-methyltransferase</b> | Cytosol  | Q384,Q385,Q392,Q393                                       |
| <b>BiP protein</b>                              | Endoplasmic reticulum lumen                    | -   |
| <b>Bone sialoprotein</b>                        | Extracellular; mineralized compartment of bone | -   |
| <b>C-CAM</b>                                    | Plasma membrane                                | Q496,Q497   |
| <b>C1 inhibitor</b>                             | Extracellular; plasma                          | Q453  |
| <b>Calbindin</b>                                | Extracellular; secreted signal molecule        | -   |
| <b>Calpain</b>                                  | Cytosol  | -   |
| <b>Caspase-3</b>                                | Cytosol  | -   |
| <b>Cathepsin D</b>                              | Cytosol  | -   |
| <b>CD38</b>                                     | Plasma membrane                                | -   |
| <b>Clathrin heavy chain</b>                     | Intracellular; vesicle membrane                | -   |
| <b>Collagen(TG2)</b>                            | Cytosol/extracellular                          | III.-Q14<br>VII.-Q2796,Q2800,<br>Q2805,Q2807              |
| <b>Crystallin</b>                               | Cytoskeleton/plasma membrane,                  | $\beta$ B2-Q9, $\beta$ B3-Q21,<br>$\beta$ A3-Q23/24/ K33, |

|   |                                      |                                   |
|---|--------------------------------------|-----------------------------------|
|   | cytosolic/nuclear localization       | $\alpha$ B-K175                   |
| <b>Cyclic Thymosin beta 4</b>                         | Cytoplasm                            | Q36/K16                           |
| <b>Cytochrome C</b>                                   | Mitochondria                         | Q42                               |
| <b>Deoxyribonuclease Y</b>                            | Nucleus                              | -                                 |
| <b>Dihydropyrimidinase-like 2 protein</b>             | Cytosol                              | -                                 |
| <b>Dual leucine zipper-bearing kinase(DLK)</b>        | Cytosol/membrane associated          | -                                 |
| <b>EGF receptor</b>                                   | Plasma membrane                      | -                                 |
| <b>Elafin(TG2)</b>                                    | Cornified envelope                   | Q2, Q59/K6                        |
| <b>Elongation factor 1<math>\alpha</math></b>         | Cytosol                              | -                                 |
| <b>Elongation factor 1Y</b>                           | Cytosol                              | -                                 |
| <b>Enolase</b>  | Cytosol                              | -                                 |
| <b>Envelope glycoprotein gp120</b>                    | HIV exterior membrane                | Q265                              |
| <b>Envelope glycoprotein gp41</b>                     | HIV exterior membrane                | Q51,Q52,Q66/K77                   |
| <b>Ephrin A</b>                                       | Plasma membrane, extracellular space | -                                 |
| <b>Eukaryotic initiation factor 4F(eIF-4F)</b>        | Cytosol                              | -                                 |
| <b>Exendin 4</b>                                      | Excreted                             | K12,K27                           |
| <b>Ezrin-Radixin-Moesin binding phosphoprotein 50</b> | Cytosol/plasma membrane              | -                                 |
| <b>F-box only protein</b>                             | Cytosol                              | -                                 |
| <b>Fatty acid synthase</b>                            | Cytosol                              | -                                 |
| <b>Fibrinogen alpha chain</b>                         | Extracellular; plasma                | Q328,Q366/K148,<br>K230,K303,K413 |
| <b>Fibrinogen gamma chain</b>                         | Extracellular; plasma                | Q398/K406                         |
| <b>Fibronectin</b>                                    | Extracellular; plasma                | Q3,Q32                            |
| <b>Filamin 1</b>                                      | Cytoskeleton                         | -                                 |

|   |  |  |
|---|--|--|
| <b>Fructose 1,6-bisphosphatase</b>              | Cytosol  | -  |
| <b>Galectin 3</b>                               | Nuclear  | Q47,Q56/K114   |
| <b>Gliadin</b>                                  | Vacuole  | Q72  |
| <b>Gliadoralin A</b>                            | Secreted   | K60  |
| <b>Glucagon</b>                                 | Extracellular; plasma  | Q3,Q20   |
| <b>Glutathione S-transferase</b>                | Cytosol  | Q51  |
| <b>Glyceraldehyde-3-phosphate dehydrogenase</b> | Mainly cytosolic associated to cytoskeleton and membranes, nucleus | K191,K268,K331   |
| <b>Hepatitis C virus core protein</b>           | Hepatitis C virus  | -  |
| <b>Histamine</b>                                | Extracellular; secreted  | -  |
| <b>Histatin</b>                                 | Extracellular; enamel pellicle in oral cavity                      | -  |
| <b>Histone H1</b>                               | Nucleus  | -  |
| <b>Histone octamer -H2A, -H2B, -H3, -H4</b>     | Nuclear  | H2A-Q24,Q104,Q112<br>H2B-Q95,Q22/K116,K125<br>H3-Q125,Q5,Q19<br>H4-Q27,Q93 |
| <b>HIV-1 aspartyl protease</b>                  | HIV virus  | -  |
| <b>Hsp 27</b>                                   | Plasma membrane, cytoskeletal element                              | Q31,Q190/K209  |
| <b>Hsp 60</b>                                   | Mitochondria   | -  |
| <b>Hsp70</b>                                    | Cytosol  | -  |
| <b>Hsp70/90 organizing protein</b>              | Mitochondria   | -  |
| <b>Hsp 90</b>                                   | Cytosol  | -  |
| <b>Human clara-cell 10kDa protein</b>           | Extracellular; secreted  | -  |
| <b>Huntingtin</b>                               | Cytosol  | -  |
| <b>Hyphal wall protein-1</b>                    | Germ tube and true hyphae surface of C. albicans                   | -  |

|   |   |   |
|---|---|---|
| <b>Ig kappa chain C region</b>                                  | Extracellular; secreted                   | -   |
| <b>Importin <math>\beta</math>1 subunit</b>                     | Cytosol, nuclear envelope                 | -   |
| <b>Template: infobox</b>  | Cytoskeleton                              | Q41   |
| <b>Insulin</b>  | Extracellular; plasma                     | Chain A-Q5,Q15<br>Chain B-Q4  |
| <b>Insulin-like growth factor-binding protein-1</b>             | Extracellular fluid                       | Q66,Q67   |
| <b>Insulin-like growth factor-binding protein-3</b>             | Extracellular fluid                       | -   |
| <b>Inter-alpha-inhibitor</b>                                    | Extracellular space                       | HC1-Q8,Q319,Q468<br>HC2-Q90,Q203,Q204,Q299,<br>Q315,Q420,Q431,Q438<br>Bikunin-Q74 |
| <b>Lamin A, C</b>   | Nuclear lamina                            | -   |
| <b>Latent transforming growth factor beta binding protein 1</b> | Extracellular matrix                      | Q374  |
| <b>Lipoprotein A</b>  | Extracellular; plasma/extracellular space | -   |
| <b>Low-density lipoprotein receptor-related protein6</b>        | Membrane, endoplasmic reticulum           | K1403   |
| <b>Melittin</b>   | Bee venom                                 | Q25   |
| <b>Microfibril-associated glycoprotein(MAGP)</b>                | Extracellular matrix                      | Q20   |
| <b>Midkine</b>  | Extracellular, plasma                     | Q42,Q44,Q95/K63   |
| <b>Myelin basic protein</b>                                     | Myelin membrane                           | Q74,Q122,Q146,Q149  |
| <b>Myosin</b>   | Cytosol                                   | -   |
| <b>Neurofilament protein</b>                                    | Neuronal cytoskeleton                     | -   |
| <b>Neuropeptide Y</b>   | Excreted                                  | Q34   |
| <b>NF-kappa-B inhibitor <math>\alpha</math></b>                 | Cytosol                                   | Q266,Q267,Q313/<br>K21,K22,K177   |
| <b>Nidogen</b>  | Extracellular; basement membrane          | Q726  |

|   |  |                |
|---|--|----------------|
| <b>Nuclease sensitive element binding protein1</b>              | Nucleus  | -              |
| <b>Nucleophosmin</b>  | Nuclear  | -              |
| <b>Orexin B</b>   | Excreted                                       | Q8,Q12         |
| <b>Osteonectin</b>  | Extracellular                                  | Q3,Q4          |
| <b>Osteopontin</b>  | Extracellular; mineralized compartment of bone | Q34,Q36        |
| <b>Parkin</b>   | Cytosol  | K49            |
| <b>Periphilin</b>   | Nuclear membrane, cornified envelope           | -              |
| <b>Periplakin</b>   | Cornified envelope                             | -              |
| <b>Phosphoglycerate dehydrogenase</b>                           | Cytosol  | -              |
| <b>Phospholipase A2</b>   | Cytosol , venom                                | Q4/K10         |
| <b>Phosphorylase kinase</b>                                     | Cytosol  | -              |
| <b>Plasminogen</b>  | Extracellular;secreted                         | Q322/K298      |
| <b>Plasminogen activator inhibitor-2</b>                        | Cytosol, plasma                                | Q83,Q84,Q86    |
| <b>Plasmodium falciparum liver stage antigen-1</b>              | Cytosol  | -              |
| <b>Procarboxypeptidase B/U</b>                                  | Extracellular; secreted                        | Q2,Q5,Q292     |
| <b>Prohibitin</b>   | Mitochondria                                   | -              |
| <b>Protein kinase C delta type</b>                              | Cytoplasm, cell membrane                       | Q545           |
| <b>Protein synthesis initiation factor 5A</b>                   | Cytosol  | K(Hypusine 50) |
| <b>RAP-<math>\alpha</math>-2 macroglobulin related protein</b>  | Cytosol  | Q261           |
| <b>Retinoblastoma protein</b>                                   | Nucleus  | -              |
| <b>Rho associated, coiled coil, containing protein kinase 2</b> | Cytoskeleton                                   | -              |
| <b>RhoA</b>   | Cytoskeleton                                   | Q52,Q63,Q136   |
| <b>S100 calcium-binding protein A10</b>                         | Cytosol, cornified envelope                    | Q4             |



|   |   |  |
|---|---|--|
| <b>S100 calcium binding protein A11</b>     | Cytosol, keratinocyte cornified envelope      | Q102/K3  |
| <b>S100 calcium-binding protein A7</b>      | Cytosol, cornified envelope                   | Q5/K88   |
| <b>Seminal vesicle secretory protein IV</b> | Extracellular; rat seminal fluid              | Q9,Q86/K2,K4,<br>K59,K78,K79,K80   |
| <b>Serotonin</b>                            | Extracellular; secreted signal molecule       | -  |
| <b>SNAP-25</b>                              | Plasma membrane                               | -  |
| <b>SP-1 transcription factor</b>            | Nucleus                                       | -  |
| <b>Spectrin</b>                             | Cytoskeleton, plasma membrane                 | -  |
| <b>Statherin</b>                            | Extracellular; enamel pellicle in oral cavity | -  |
| <b>Substance P</b>                          | Extracellular; secreted                       | Q5   |
| <b>Suprabasin</b>                           | Cornified envelope                            | -  |
| <b>Synapsin 1</b>                           | Synaptic vesicle membrane                     | -  |
| <b>Synapsin I</b>                           | Bound to synaptic vesicle membrane            | Ia-Q665,Q685   |
| <b>T-complex protein 1ε subunit</b>         | Cytosol                                       | -  |
| <b>Tau protein</b>                          | Cytoskeleton                                  | Q6,Q88,Q124,Q244,QQ276,<br>Q288,Q351,Q424/K24,K163,<br>K174,K180,K190,K225,K234,<br>K240,K383,K385 |
| <b>Thymosin beta 4</b>                      | Cytosol , plasma                              | Q23,Q36/K38  |
| <b>Thyroglobulin</b>                        | Extracellular; follicle lumen                 | -  |
| <b>Troponin</b>                             | Cytoskeleton                                  | TnT-Q191<br>TnI-K193   |
| <b>Tumour rejection antigen1</b>            | Endoplasmic reticulum lumen                   | -  |
| <b>Ubiquitin</b>                            | Cytosol                                       | K29,K48  |
| <b>Uteroglobin</b>                          | Extracellular; secreted                       | -  |

|   |                          |                                  |
|---|--------------------------|----------------------------------|
| <b>UV excision repair protein<br/>RAD23 homolog B</b> | Nucleus                  | -                                |
| <b>Valosin</b>  | Nucleus, cytosol         | -                                |
| <b>Vasoactive intestinal<br/>peptide</b>              | Extracellular; secreted  | Q16/K20,K21                      |
| <b>VEGFR-2</b>  | Plasma membrane, nucleus | -                                |
| <b>Vigilin</b>  | Nucleus, cytosol         | -                                |
| <b>Vimentin</b>                                       | Lens cytoskeleton        | Q453,Q460/<br>K97,K104,K294,K439 |
| <b>Vitronectin</b>                                    | Cytoskeleton             | Q93                              |
| <b>Y-box binding protein</b>                          | Cytoplasm, Nucleus       | -                                |

## **Appendix III: General solutions**

### **1) SDS-PAGE**

#### **Laemmli 2x sample buffer:**

4% SDS  
10% 2-mercaptoethanol  
20% glycerol  
0.004% bromophenol blue  
0.125 M Tris-HCl, pH 6.8

#### **1x MOPS Buffer:**

20 mM 4-morpholinopropanesulfonic acid, pH 7.0  
8 mM sodium acetate  
1 mM EDTA

#### **1x MES Buffer:**

20mM 2-morpholinoethanesulfonic acid, pH 7.0  
8 mM sodium acetate  
1mM EDTA

#### **1x Tris-Glycine buffer:**

25 mM Tris-Base, pH 8.3  
192 mM Glycine  
0.1% SDS

#### **Coomassie stain:**

0.25% coomassie brilliant blue R-250 in a mixture of water:acetic acid:methanol (40:10:50)

#### **Coomassie stain remover:**

A mixture of 60% water, 10% acetic acid, and 30% methanol.

### **2) Western blotting**

#### **1x TBS:**

50 mM Tris Base, pH 7.6  
0.20 M NaCl

#### **Transfer Buffer:**

25 mM Tris  
192 mM glycine  
20% Methanol

#### **1x TBST:**

1 mL Tween-20 in 1 lit 1x TBS

#### **1x PBS:**

10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4  
2 mM KH<sub>2</sub>PO<sub>4</sub>  
137 mM NaCl  
2.7 mM KCl

**Blocking reagent:**

- 1) 5% non-fat dry milk in TBST
- 2) 3-5% BSA in TBST

**Ponceau S Red:**

0.5 g in 100 mL 1% acetic acid

Stain removal- 10% isopropyl alcohol/10% acetic acid

Steps: stain blot for 2-5 min, rinse in water, if not then in stain removal.

**Antibody removal solution:**

0.5 M Tris-HCl, pH 6.7, 0.7%  $\beta$ -mercaptoethanol, 10% SDS

Steps: incubate blot in antibody removal solution for 15 minutes at 65° C, then wash membrane with 1x PBS or TBST for 2 hours to overnight, air dried and then reuse.

**3) Agarose gel electrophoresis****Agarose gel:**

1.2% agarose in 1x TBE buffer

**1x TBE:**

45 mM Tris-base

45 mM boric acid

2 mM EDTA, pH 8.0

**4) Flow cytometry****Binding buffer:**

0.1 M HEPES, pH 7.4

1.4 M NaCl;

25 mM  $\text{CaCl}_2$