ANGLIA RUSKIN UNIVERSITY

INVESTIGATION OF THE EFFECTS OF PHYTOCHEMICAL INDUCED CHANGES IN TG2 ACTIVITY ON THREE CELL LINE MODELS OF CANCER CHEMOTHERAPY

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A thesis in partial fulfilment of the requirements of Anglia Ruskin University for the degree of Doctor of Philosophy

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A Little learning is a dangerous thing;
Drink deep or taste not the Pierean springs:
There shallow draughts intoxicate the brain,
And drinking largely sobers us again.

Alexander Pope

ANGLIA RUSKIN UNIVERSITY
ABSTRACT

INVESTIGATION OF THE EFFECTS OF PHYTOCHEMICAL INDUCED CHANGES IN TG2 ACTIVITY ON THREE CELL LINE MODELS OF CANCER CHEMOTHERAPY

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The use of Phytochemicals as chemotherapeutic agents is a growing area of research, with biologicaly active agents such as the flavonoids, curcumin, resveratrol and berberine all having proven to be both anti-tumourgenic and well tolerated compounds. As such agents are easily purified from plant sources, require no NICE/FDA approval for use and generally exhibit favourable pharmakokinetic behavior, continued research in this area is suggested. Transglutaminase 2 (TG2) is an enzyme that is abnormally expressed in a variety of cancers with retinoic acid (a TG2 activity modulator) having been shown to increase suceptability of a variety of cancer models to chemotherapeutics. Some research has been conducted into the use of Phytochemicals to modulate TG2 activity/levels/localization in an effort to sensitise cancers to other therapies, but there are at the time of writing large gaps in the current body of work.

This work compared and contrasted how the phytochemicals resveratrol and berberine affect cancer cell model systems and the activity of the enzyme TG2. The aim was to use established cancer cell lines to test the effectiveness of the phytochemicals in isolation and in combination as chemotherapeutics and to determine how they influence cell viability, cellular adhesion/migration and the cell cycle and if this relates to TG2 activity. The model systems used in this study involved 3 cancer cell lines; these were HepG2 (a human hepatocarcinoma model system), Caki-2 (human renal carcinoma model system) and 1321N1 (a human astrocytoma model system). The results of this study would determine whether resveratrol and berberine have the potential to be used at clinically-achievable doses to treat cancer and whether the mechanism by which they induce any chemotherapeutic effects involves the modulation of TG2. This research is important as existing cancer therapies show limited effectiveness and are in general not well tolerated, finding and evaluating new therapeutics is therefore highly desirable.

Analyses of cell viability was performed using CCK-8 and flow cytometry. Cell cycle and TG2 enzymatic activity were analysed using flow cytometry. Analyses of cellular adhesion and metastasis were performed using scratch test assays and a series of microscopic observations. mRNA levels were measured using qPCR, and inhibition of TG2 employed the TG2 competitive inhibitor cystamine.

A model combined drug therapy was developed that modulated TG2 activity. The phytochemical berberine increased TG2 enzymatic activity in both Caki-2 and HepG2 cells and induced cell death in the HepG2 model. Resveratrol treatment inhibited both the cell cycle and cellular migration and also increased the strength of cellular adhesion in both cell lines. Potential binding sites for resveratrol on the TG2 enzyme were determined using the SwissDock software.

The well-tolerated phytochemicals berberine and resveratrol in combination proved effective at inducing cell death, inhibiting the cell cycle and preventing metastasis at clinically-achievable doses in the HepG2 model system. Resveratrol alone proved affective at inhibiting the cell cycle and inhibiting migration at clinically-achievable doses in the Caki-2 model system. These results show the potential for development of a combined Resveratrol/Berberine therapy to treat human Hepatocarcinoma.

Key words: Cancer, Transglutaminase 2, apoptosis, cell cycle, cellular adhesion, cellular migration, Caki-2, HepG2, 1321N1, Model system

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Lastly, thanks to all my friends for putting up with me whilst I tackled this project.

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Chapter One. Introduction

In multi-cellular organisms, there is a constant effort to maintain a homeostatic balance between the number of new cells that are generated via mitosis and the number of damaged or unrequired cells that are removed from the body. This constant turnover of cells is necessary for the development of structures such as the fingers and toes, which arise from the initially webbed limbs found in the human foetus (Zakeri, 1994). The mechanisms by which animals regulate mitosis, detect cellular abnormalities and initiate the method of programmed cell death known as apoptosis (Lowe, 1999) involves a large number of regulatory genes. Some of these regulatory genes act to stimulate mitosis, whilst others inhibit mitosis or initiate apoptosis. Uncontrolled cellular proliferation can result in the development of diseases such as cancer that can result in death of the organism.

Although there is a large and complex web of interacting gene products involved in the cell cycle and cell death, from the 1990's onwards certain receptors, enzymes and regulatory proteins have emerged as key regulators in these processes. These regulators when abnormally expressed or when mutated can have a direct effect on the machinery of the cell cycle, with some, for example the Bcl-2 family of enzymes (Czabotar, 2013) and transglutaminase 2 (Budillon, 2013) either stimulating apoptosis or inhibiting it depending on their expression profile, localization or conformation. This inhibition of apoptosis can directly influence the susceptibility of a cancer to chemotherapeutic drugs, in many cases, resulting in drug resistance. Increased transglutaminase 2 expression has for example been implicated with a poor prognosis in acute myeloid leukemia (AML) and it has been observed (Lancelot, 2013) that elevated levels of transglutaminase 2 expression are found in patients during an AML relapse as compared with the initial levels when first diagnosed.

The body of knowledge in the field of cancer biology is increasing at an ever faster rate since early studies into cell signaling and apoptosis in the 1980's; particularly with regards to the involvement of the cell cycle and apoptosis as regulators of both the severity, and susceptibility to drugs of cancers. This knowledge does however still contain many gaps, for example there are thought to be ~140 genes involved in tumourgenesis (Vogelstein, 2013) however the relationship and interactions between the products of these genes and how targeting these interactions might affect tumougenesis/drug susceptibility is still very unclear. For example the enzyme Transglutaminase 2 (TG2) is known to be aberrantly expressed/activated in many cancers (Gundemir, 2012) and some evidence suggests that

modifying its expression/activity may alter the effectiveness of chemotherapy in certain cancers (Herman et al., 2006); however the details of how best to target TG2 and what suitable and clinically tolerable compounds to use is not clear. With the number of patients diagnosed with cancer increasing throughout the world over recent decades (WHO, 2015) and the large cost to society, both economically and emotionally, of this group of diseases, it is clear that further clarification of the mechanisms involved in carcinogenesis is required.

1.1 Cell death

In order to develop effective treatments for cancer, an understanding of the differing ways that cells can lose viability and eventually die is necessary. The differing mechanisms of cell death will be discussed thoroughly in the proceeding sections, however broadly speaking cell death occurs in either a controlled (programmed) way involving a secries of biochemical and molecular events or alternatively in a poorly controlled way resulting in the spilling of the cellular contents into surrounding tissues. The controlled method of cell death is generally termed “apoptosis” from the Greek denoting “falling off” as in the leaves falling from a tree, whilst the uncontrolled form of cell death is usually refered to as “necrosis” from the Greek denoting “to kill.” Many of the most effective currently available chemotherapeutic drugs rely to one extent or another on initiating cell death, usually by apoptosis, however the non-specific initiation of cell death that most of these drugs induce often results in the death of non-cancerous cells together with the death of the tumourgenic tissue. The need for the development of more targeted drugs to initiate cell death is therefore key to producing more effective clinical treatments for cancer.

1.1.1 Introduction to apoptosis

The word apoptosis was first used in a 1972 paper by Kerr, Wyllie, and Currie to describe a morphologically distinct type of cell death (Kerr et al., 1972). Apoptosis is the process by which a cell ceases to grow and divide and instead enters a process that ultimately results in the controlled death of the cell without spillage of its contents into the surrounding environment. Apoptosis is also sometimes referred to as programmed cell death (or more colloquially 'cellular suicide'). The initiation of apoptosis is dependent on the activation of a series of cysteine-aspartic proteases known as caspases. There are two categories of caspases, the initiator caspases and the executioner caspases (Elmore, 2007). Once cell damage is detected, the initiator caspases (caspases 8 and 9) are activated from inactive procaspases and go on to activate the executioner caspases (caspases 3, 6 and 7). The activation of the

executioner caspases initiates a cascade of events that results in DNA fragmentation from activation of endonucleases, destruction of the nuclear proteins and cytoskeleton, crosslinking of proteins, the expression of ligands for phagocytic cells and the formation of apoptotic bodies (Poon, 2014 and Martinvalet, 2005). For a full discussion of how apoptosis can be distinguished from necrosis see section 2.3.4. Broadly speaking however, apoptosis can be distinguished from the other major type of cell death – Necrosis, both visually under the microscope and via a number of molecular biology techniques; including flow cytometry with Annexin V staining and DNA fragmentation assays. In the former, the apoptotic bodies containing the contents of the dead cell can be phagocytosed by surrounding cells, although this behavior is observed primarily in cell culture (Elmore, 2007), *in vivo* cells such as macrophages often remove apoptotic cells before they fragment. This results in a containment of the injured tissue and as a result, reduces the risk of collateral damage to surrounding cells.

The process of apoptosis is highly conserved within multi-cellular organisms and is genetically controlled (Lockshin, 2004). Apoptosis can be initiated by the cell itself when it detects damage via a number of intracellular sensors; a mechanism known as the intrinsic pathway. Alternatively, it can result from the interaction between a cell of the immune system and a damaged cell, which is known as the extrinsic pathway of apoptosis (Oppenheim, 1990 and Oppenheim, 2001). In the human body, it is estimated that approximately 1×10^9 cells undergo apoptosis per day (Elliott, 2010). Both the intrinsic and extrinsic pathways of apoptosis work synergistically to ensure that multi-cellular organisms remain healthy and defective cells are removed from the body. Failure to regulate apoptosis can result in the pathologies exhibited in many diseases. For example, in degenerative diseases such as Alzheimer's where neuronal death appears to be initiated by the activation of caspases; a key group of enzymes involved in apoptosis (Dickson, 2004). Too little apoptosis, however, can result in the uncontrolled growth and division of cells that is observed in cancer. See Figure 1.1 for a summary of the intrinsic and extrinsic pathways of apoptosis.

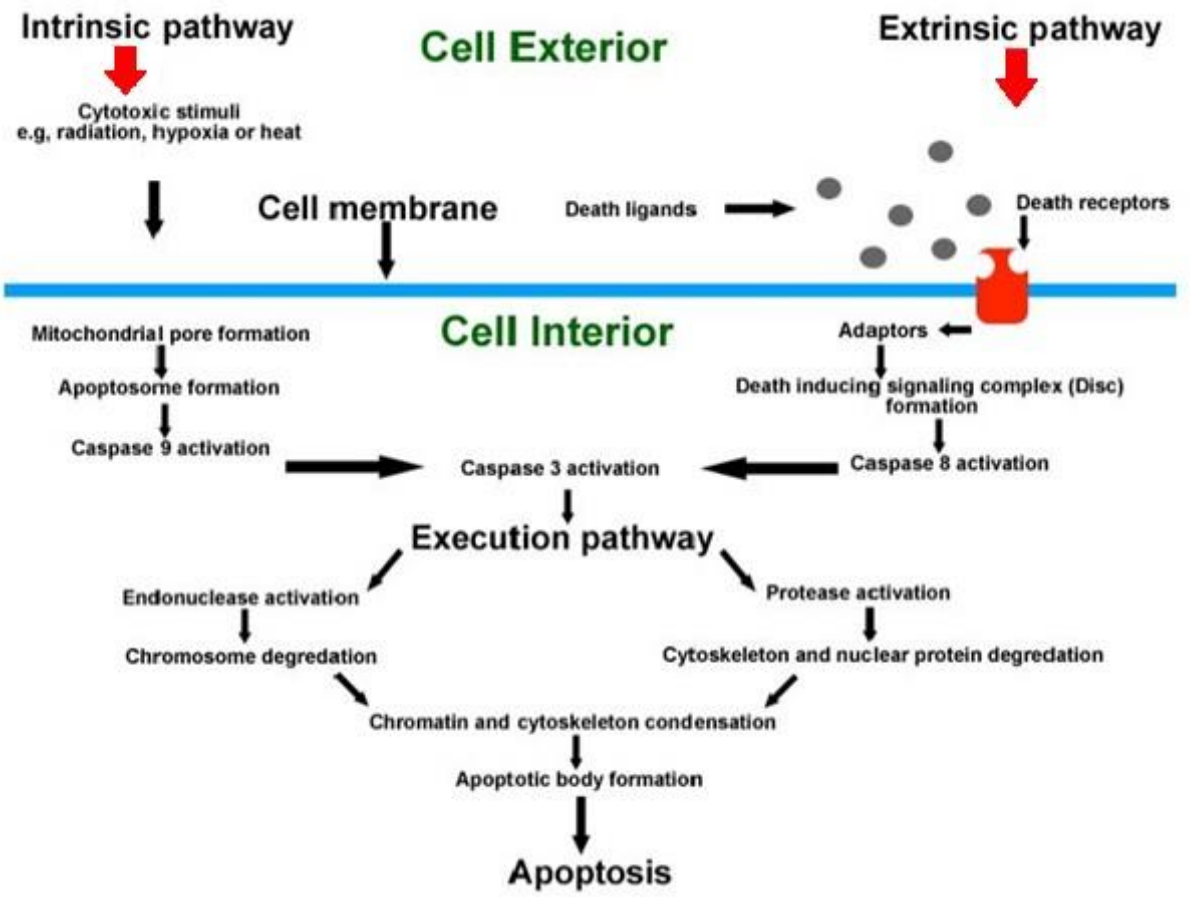

Figure 1.1. A summary of the major components of the intrinsic and extrinsic pathways of apoptosis

The intrinsic pathway of apoptosis is initiated by the cell itself in response to damage. The extrinsic pathway is initiated via death receptors stimulated by cells of the immune system. Both pathways converge when caspase 3 is activated, resulting in cell death.

1.1.2 Intrinsic pathway of apoptosis

The intrinsic pathway, also known as the mitochondrial pathway of apoptosis (Igney and Krammer, 2002) involves variety of stimuli that act on multiple targets within the cell. This form of apoptosis is dependent on factors released from the mitochondria and is initiated either from a positive or negative pathway. Negative signals arise from the absence of cytokines, hormones and growth factors in the immediate environment of the cell. Without these pro-survival signals, pro-apoptotic molecules within the cell, such as puma, noxa and bax that are normally inhibited become active and initiate apoptosis. Other factors that initiate apoptosis are positive in nature and include exposure to hypoxia, toxins, radiation, reactive oxygen species, viruses and a variety of toxic agents (Brenner and Mak, 2009) although in the case of some cells, such as neutrophils, hypoxia can promote cell survival (Walmsley, 2005). The initiator caspase that controls the intrinsic pathway of apoptosis is caspase 9, which is able to bind to adapter protein apoptotic protease activating factor 1 (APAF1) following exposure of

its caspase recruitment domain (CARD domain). APAF1 in a non-apoptotic cell is usually folded in such a manner that its CARD domain is blocked and procaspase 9, which also contains a CARD domain is unable to bind to it. When apoptosis is induced either by positive or negative stimuli, changes are triggered in the mitochondrial membrane, the result of which is opening of the mitochondrial permeability transition (MPT) pore. Once the MPT pore is open, pro-apoptotic proteins, (including cytochrome c, Smac/Diablo and HtrA2/Omi) are able to leak from the mitochondria into the cytoplasm and activate apoptosis (Cain, 2002). Cytochrome c induces apoptosis by binding to the WD domain of APAF1 monomers, which results in a conformational change in APAF1 exposing a nucleotide binding and oligomerization domain that is able to bind deoxy ATP (dATP). This binding induces an additional conformational change in APAF1, exposing both its CARD and oligomerization domains, thus allowing several APAF1's to assemble into a complex known as an apoptosome (Acehan, 2002). The apoptosome contains in its open centre several exposed CARD domains, which then recruit and activate several procaspase 9 proteins. These activated caspase 9 enzymes are able to activate the executioner procaspase 3, which in the form of active caspase 3 can fully induce apoptosis (Cain, 2002). Smac/Diablo and HtrA2/Omi help initiate apoptosis by inhibiting inhibitors of apoptosis proteins (IAPs), although without the release of cytochrome c, inhibiting IAPs alone is insufficient to initiate apoptosis (Ekert and Vaux, 2005).

1.1.3 Extrinsic pathway of apoptosis

The extrinsic pathway, also known as the death receptor pathway of apoptosis (Igney and Krammer, 2002) is initiated by patrolling NK cells or macrophages when they produce death ligands, which upon binding with death receptors(DR's) in the target cell membrane induce the extrinsic pathway via the activation of procaspase 8 to caspase 8 (Kang, 2004). DR's are members of the TNF (tumor necrosis superfamily) and includes several members, (Bossen, 2006) with each DR having a corresponding death ligand (see Table 1.1). To activate caspase 8 a death ligand must bind to a DR, resulting in recruitment of monomeric procaspase 8 via its death inducing (DED) domain to a death-inducing signal complex (DISC) located on the cytoplasmic domain of the ligand-bound DR. The DISC also includes either an adaptor protein known as FAS-associated death domain (FADD) or TNFR-associated death domain (TRADD) which facilitate the interaction of procaspase 8 to the DISC (Kang, 2004). The recruitment of several procaspase 8 monomers to the DISC results in their dimerization and activation, with the resultant caspase 8 able to induce apoptosis via one or the other of two distinct sub-pathways. The particular sub-pathway that is induced depends on whether the cells are classed

as type I or type II cells (Samraj, 2006). In type I cells, caspase 8 directly cleaves executioner caspases and therefore directly initiates apoptosis. In type II cells, IAPs inhibit direct caspase 8 activation of the executioner caspases, unless the IAPs are inhibited by proteins released from the mitochondria (Spencer, 2009 and Jost, 2009). The important role of caspase 8 in controlling the extrinsic pathway of apoptosis (see Figure 1.1) has been observed in caspase 8 deficient mice which show no response to DR ligands (Varfolomeev, 1998).

Table 1.1. Human death receptors and their corresponding ligands

Death receptor (DR)	**Death ligand**
TNF receptor-1 (TNFR1)	TNF
CD95 (also known as Fas and APO-1)	CD95-ligand (CD95-L, also known as Fas-L)
Death receptor 3 (DR3)	TLIA
TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1, also known as DR4)	TRAIL (also known as Apo2-L)
TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R2, also known as DR4)	TRAIL (also known as Apo2-L)

Whether or not apoptosis is triggered by the extrinsic or the extrinsic pathways, its tight regulation is essential and failure to regulate it effectively can have dire consequences. In cancer for example, the cell fails to initiate apoptosis due to mutations in the various mechanisms of initiation. If this occurs in tandem with the cell failing to respond to external signals that would normally provoke the extrinsic pathway or inhibit proliferation, then this causes the cell to grow and divide uncontrollably, resulting in the formation of either a benign tumour or in cancer (Philchencov, 2004).

1.1.4 Pyroptosis. Programmed cell death with collateral damage

Classic apoptosis (both the intrinsic and extrinsic pathways) are characterized by the compartmentalization of intracellular components of the cell and removal of cellular debris without any collateral damage occurring to nieghbouring healthy cells (see section 1.1). An alternative form of apoptosis has been identified (Boise, 2001) that although following a programmed series of caspase-dependent events, is pro-inflammatory. This form of cell death termed “pyroptosis” (Fink, 2005) has been identified in macrophages infected with *Salmonella* or *Shigella* and is not observed in caspase-1 deficient cells (Allen, 1995). Once

activated by a pathogen, caspase-1 processes the pro-forms of the inflammatory cytokines IL-1β and IL-18 into their active forms, resulting in apoptosis of the cell, but with a concurrent release of inflammatory cytokines into the environment (Hersh, 1999). Since its discovery, pyroptosis has been observed in the central nervous system (Liu, 1999) and the cardiovascular sysem (Kolodgie, 2000), suggesting that this form of cell death is biologically significant.

1.1.5 Autophagy. A mechanism for both protecting and killing stressed cells

Autophagy is a process where cellular components such as macroproteins or even whole organelles are sequestered into lysosomes for degredation (Klionsky, 2000). The lysosomes are then able to digest these substrates, the components of which can either be recycled to create new cellular structures and/or organelles or alternatively can be further processed and used as a source of energy. Autophagy can be initiated by a variety of stressors, most notably by nutrient deprivation (caloric restriction) or can result from signals present during cellular differentiation and embryogenesis and on the surface of damaged organelles (Mizushima, 2008). Autophagy has also been shown to be involved in both the adaptive and the innate immune system where it may degrade intracellular pathogens and deliver antigens to MHC class II holding compartments and initiate the transportation of viral nucleic acids to Toll-like receptors (Levine, 2007). Although autophagy is often used to recycle cellular components, it can result in destruction of the cell and in this way has been linked to removal of senescent cells from aged tissues and destruction of neoplastic lesions (Mizushima, 2008). Failure of autophagy as well as potentially allowing the development of cancer has also been associated (particularly in aged organisms) with the accumulation of protein aggregates in the neurons and the development of neurodegenerative conditions including Alziemers disease (Nixon, 2011).

To date 3 distinct forms of autophagy have been identified - macroautophagy, microautophagy and selective autophagy (Cuervo, 2004). In the most described form of autophagy "macroautophagy" whole regions of the cell are enclosed in double-membrane vesicles refered to as autophagosomes. These autophagosomes then fuse with lysosomes to become autophagolysosomes and the contents are then degraded by proteases present therein. In microautophagy the cargo (organelles or regions of the cytosol) directly interact with and fuse with the lysosomes (Li, 2012). Microautophagy is more specific than macroautothagy and can be triggered by signaling molecules present on the surface of damaged organelles such as mitochondria or peroxisomes resulting in specific fusion of

lysosomes with these organelles. Depending on which organelle is targeted, the resultant autophagic vesicle is refered to by a specific name, for example if a mitochondria, the term used is mitophagy or for a peroxisome the term peroxophagy is used. In selective autophagy, also known as chaperone-mediated autophagy (CMA) proteins within the cytoplasm are targeted for fusion with lysosomes by a cytosolic chaperone through interaction between the chaperone and a pentapeptide present within the amino acid sequence of the substrate. Substrate proteins then bind to a lysosomal receptor LAMP-2A and are carried into the lysosome for degredation (Dice, 2007).

1.1.6 Necrosis. Cell death with collateral damage

Unlike apoptosis, necrosis is an alternative uncontrolled form of cell death that is induced by external injury, such as hypoxia or inflammation (Elmore, 2007). This process often involves upregulation of various pro- inflammatory proteins and compounds, such as NF-κB resulting in the rupture of the cell membrane causing spillage of the cell contents into surrounding areas, resulting in a cascade of inflammation and tissue damage. In contrast to apoptosis, necrosis is an energy independent form of cell death, where the cell is damaged so severely by a sudden shock (radiation, heat, chemicals, hypoxia etc) that it is unable to function. The cell usually responds by swelling, (a process known as oncosis) as it fails to maintain homeostasis with its environment.

The above definition of necrosis as a counterpoint to apoptosis is a useful concept, however as necrosis is usually observed as an endpoint state in cell culture by the presence of cellular fragments in the media, what is described in many cases could simply be the remains of late apoptotic cells the apoptotic bodies of which have lost integrity. *In vivo* these apoptotic bodies would be phagocytosed by patrolling white blood cells, however in the single cell population environments found in culture this does not occur, which can confuse the diagnosis of the exact mechanism of cell death. A more accurate counterpoint to controlled cell death is oncosis (described in the proceeding section).

1.1.7 Oncosis. Cell death with collateral damage

The term oncosis is derived from the Greek word “onkos,” which means swelling and is best thought of as a prelethal pathway that ultimately leads to cell death (Levin, 1998). Its main features are the swelling of both the cell generally and the organelles of the cell specifically, together with an increase in membrane permeability. As oncosis progresses, there is a depletion of intracellular energy stores and an eventual failure in the ionic pumps of the

plasma membrane (Majno, 1995). The result of oncosis is a leakage of cellular debris into surrounding tissues and consequently damage to surrounding cells (inflammation). Oncosis can be induced by all of the methods previous described in section 1.1.5, or in some cases by infection of the cell by pathogens such as Rotavirus (Perez, 1998).

1.2 Cancer

Healthy cells are in a state of constant communication with their environment. They receive signals via cytokines and a variety of ligands from surrounding cells, inducing them to synthesise, and in many cases secrete substances such as growth factors or pro-apoptotic ligands. Compounds present in the local environment signal cells to grow, divide or to enter a state of cellular senescence or apoptosis. When this constant two way communication between groups of cells and their environment is maintained, the environment of the local tissues is kept in a state of homeostasis and the normal function of the cells, tissues, organs and indeed the whole body is maintained (Bertram, 2000). When a cell stops responding to the signals produced by its neighbours, the result can be uncontrolled growth, division or even migration of the cell and its daughters to other parts of the body. Collectively, this group of aberrant behaviours is known as cancer.

1.2.1 Resistance to apoptosis in cancer

The reasons why cells stop responding to environmental signals and thus exhibit the cancerous phenotype are varied. For example, damage to a single base pair of DNA in the promoter of a proto-oncogene can cause a proto-oncogene to transform into an over expressed oncogenic form (Bertram, 2000). Once activated, oncogenes lose their ability to respond to intracellular signals that would normally retard their expression and instead produce abnormally high levels of mRNA for making proteins whose functions are to stimulate cell growth, division and angiogenesis. Mutations in tumour suppressor genes can also prevent expression of genes whose products recognise cellular damage, stimulate cells to pause the cell cycle and give cells time to repair damage; the failure of these mechanisms can ultimately result in a failure to induce apoptosis. This disruption of cellular homeostasis can result in the cancerous phenotype (Baylin and Ohm, 2006). Together with an increase in the severity of a cancer is often observed both a decrease in apoptosis and an increase in transglutaminase 2 expression (Park, 2010).

1.2.2 Epigenetics of cancer

Although genetic changes underpin development of several types of cancer, since the early 2000's it has become apparent that in many cancers, epigenetic changes also play important roles in the progression of the disease (Baylin and Ohm, 2006; Feinberg et al., 2006; Feinberg and Tycko, 2004; Jones and Baylin, 2002; Lund and van Lohuizen, 2004; Herman and Baylin, 2003).

Examples of such changes include alterations in patterns of DNA methylation, histone hypoacetylation, repressive histone methylation status and promoter DNA hypermethylation. Since all of these alterations can affect gene expression, epigenetic change can result in drug resistance or metastasis.

Unlike genetic changes where a gene is permanently transformed, epigenetic changes can in some cases be reversed, which offers a huge potential for future cancer therapies. One such therapy currently under investigation involves the use of histone deacetylase inhibitors (HDACIs).

Inhibitors of DNA methyltransferases also show great promise in this area (Bolden et al., 2006; Yoo and Jones, 2006).

1.3 Transglutaminases

Transglutaminases (TGs) are a pleiotropic family of enzymes capable of diverse biological functions, being differentially expressed in a variety of organs and cells. Their activity was initially reported in guinea pig liver (Clarke, 1957), however the term transglutaminase was not used until 1959 when Mycek *et al.* observed that TG catalysed an acyl transfer reaction between a γ- carboxamide group of peptide-bound glutamine and an ε-amino group of peptide-bound lysine (polyamine), which resulted in the formation of a ε-(γ-glutamyl)lysine isopeptide bond. In 1959, Waelsch determined that this transamidase- mediated incorporation of primary amines into proteins required metal ions. Folk and Chung (1973) then moved the field forward by determining the metal ions that TG2 needed to perform its activities; calcium (Ca^{2+}) being found to be the most effective, with the ions Sr^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} having the ability to act as less reactive substitutes for Ca^{2+}. Folk's team (1980) also determined that divalent heavy metals inhibited the transamidation reactions. Since then, it has been confirmed that transglutaminases post-translationally modify proteins by forming ε(γ-glutamyl)lysine covalent linkages (folk, 1980). By inducing covalant crosslinking of proteins,TGs produce highly stable products that resist proteolytic degradation and take part in several biological processes, including apoptosis (Chau et al., 2005).

All of the transglutaminases apart from erythrocyte 4.2 are catalytically active and possess a similar gene structure and primary structure. TGs also have a similar tertiary structure and catalytic mechanism which indicates that they evolved via gene duplication from the same ancestral progenitor gene (Grenard 2001). The genes are found on 5 chromosomes and show homology with the papain-like cysteine proteases, which share the same conserved catalytic triad (Cys-His-Asp or Cys-His-Asn) as the transglutaminases (Griffin, 2002).

The transglutaminase family currently comprises of nine members in mammals (see Table 1.2 below for a full description of all family members). TG1 is the keratinocyte transglutaminase, TG2 is tissue transglutaminase, TG3 is epidermal transglutaminase, TG4 is prostate transglutaminase, TG5-7 (also known as TGX, TGY and TGZ), factor XIIIa (found in plasma) and erythrocyte 4.2 (Griffin, 2002).

Table 1.2. The transglutaminase family of proteins

TG Protein	Gene and location	Cell types expressed in	Functions	Reference
Factor XIIIA	F13A1 Found on chromosome 6 p24-p25	Platelets Dentritic cells Astrocytes Chondrocytes	Involved in blood clotting, wound healing, ECM stabilization growth	(Griffin, 2002)
TG1	TGM1 Found on chromosome 1414 q11.2	Keratinocytes	Differentiation of epidermal keratinocytes	(Griffin, 2002)

TG2	TGM2 Chromosome 20 q11.2-q12	Ubiquitous	Cellular adhesion and differentiation, apoptosis, drug resistance in certain forms of cancer, membrane signalling, cell migration	(Gundemir, 2012) and (Griffin, 2002)
TG3	TGM3 Chromosome 20 q11.2-q12	Squamous epithelium	Hair follicle maturation	(Griffin, 2002)
TG4	TGM4 Chromosome 3 p22-p21.33	Prostate cells	Semen coagulation	(Griffin, 2002)
TG5	TGM5 Chromosome 15 q15.2	Epidermal cells	Involved in normal skin structure.	(Szczecinska, 2014)
TG6	TGM6 Chromosome 20 q11-15	Epidermal cells	Motor function And bone development	(Fukui, 2013)
TG7	TGM7 Chromosome 15 q15.2	Ubiquitous	Unknown	(Grenard, 2001)
Band 4.2	EPB42 Chromosome 15	Erythrocytes	Maintenance of membrane integrity	(Satchwell, 2009)

1.3.1 Transglutaminase 2 (TG2)

In a review by Gundemir (2012) TG2 was memorably described as a "molecular Swiss army knife." This description seems apt as TG2 is the most widespread and versatile member of the transglutaminase family, with the majority of cell types in the human body expressing TG2 to some extent. In addition to transamidase, TG2 exhibits protein disulphide, isomerase, guanine and adenine nucleotide binding and hydrolyzing activities. TG2 in some cases also acts in a supportive role within the cell by cross-linking proteins into a scaffold. Although primarily a cytosolic protein, TG2 is also sometimes localised in the nucleus or can associate with membrane structures, including both the inner and outer cell membrane and the mitochondrial membranes (Tee, et al 2009). External to the cell, TG2 can be found as a component of the extracellular matrix where Griffin (2002) describes TG2 and all transglutaminases as "natures glues." The multiple roles of the transglutaminases make it difficult to be clear as to the specific involvement of TG2 in apoptosis and in cancer, but it seems that its role is dependent on several factors. These include, but are not exclusive to, the cell type, the environment that the cell is exposed to; for example, whether the environment is hypoxic or subject to inflammatory cytokines (Monsonego et al., 1997), the conformational state of the protein and the location within the cell of the enzyme (membrane-bound or cytosolic). In Figure 1.2 below, there is an overview of the primary functions of TG2.

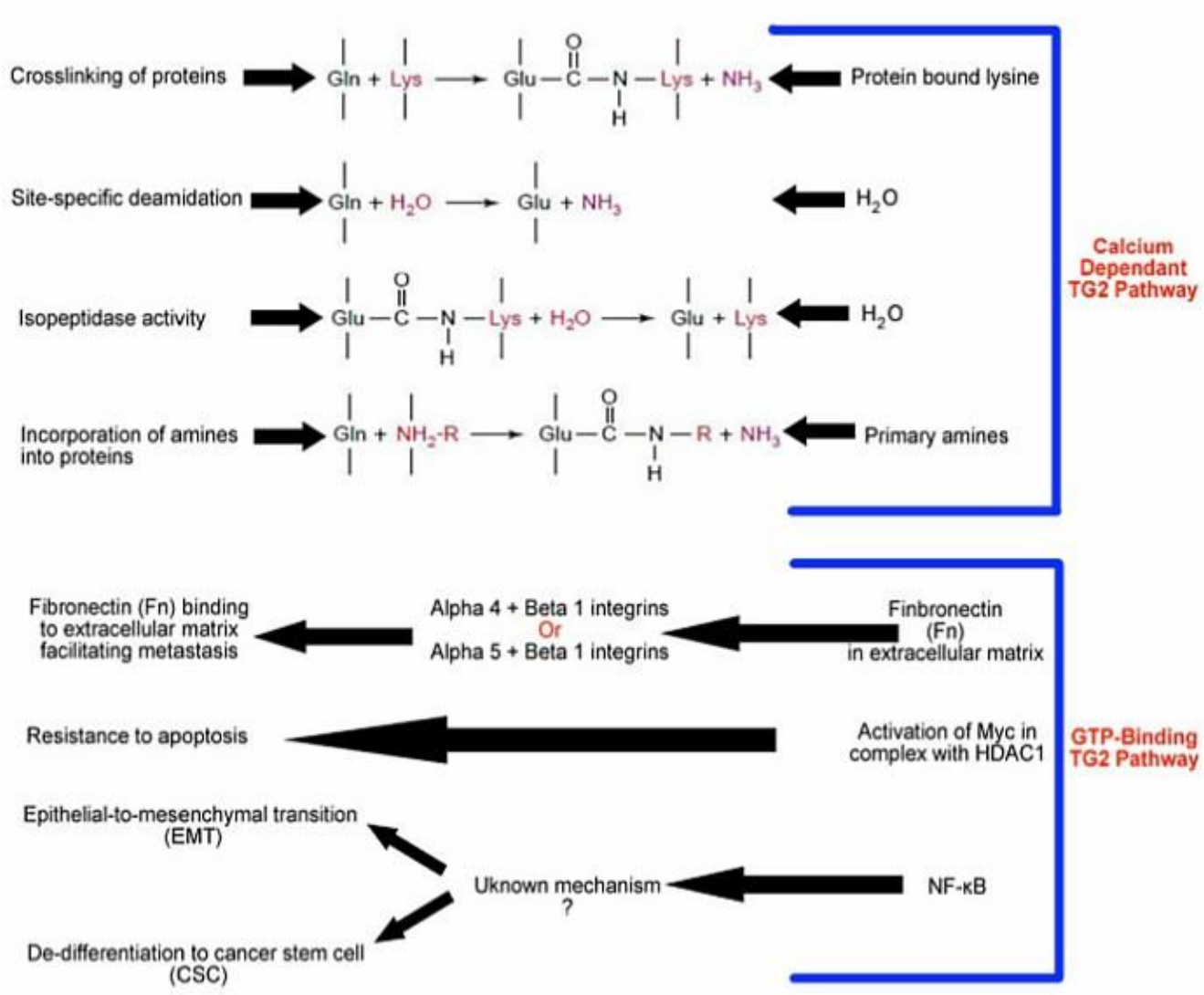

Figure 1.2. TG2 mechanisms of action (self-produced image)

2A calcium-dependent pathway. In its open form, TG2 catalyzes the acyl-transfer reaction between the γ-carboxamide group of a glutaminyl protein residue and the -amino group of a lysyl residue, which results in the formation of an isodipeptide N -(γ -glutamyl)-lysine. In moderately acidic conditions, TG2 can deaminate peptide-bound glutanyl residues using H2O as an acyl-acceptor to form a glutamyk residue. TG2 can form an isopeptide bond and crosslink proteins when the ε-group of a peptide bound lysine is the acyl-acceptor. When a primary amine is used as an acyl- acceptor, the glutanyl residue is post-translationally modified.

2B GTP-binding pathway. In its closed form, the catalytic triad of TG2 is blocked, leaving the GTP-binding site exposed. TG2 is then able to interact with the alpha and beta integrins to bind with fibronectin in the extracellular matrix and thus promote cellular adhesion.

1.3.1.1 TG2 structure

Transglutaminise 2 is ~80kDa in weight, contains approximately 685 amino acids (see Figure 1.3 forfull sequence) and in humans is encoded by the TGM2 gene on chromosome 20q11-12 (Grenard

et al., 2001). Consisting of 13 exons and 12 introns the TGM2 gene encodes 4 domains in the 3D structure of its translated protein product, shown in Figure 1.4. These are a N-terminal β-sandwich domain (residues 1-138), which includes the integrin and fibronectin binding sites, the protein core domain (139-471) containing several α-helices and β-sheets, which includes the substrate binding pocket and the catalytic triad domain 'Cys277-His335-Asp358' which mediates acyl-transfer reactions. TG2 also contains a tryptophan essential for catalytic activity and two C-terminal β-barrel domains; one containing a binding pocket for GTP and interaction sites with the α1B adrenergic receptor, and the other containing a phospholipase Cδ1 interaction site (Chen and Mehta, 1999).

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         10         20         30         40         50
MAEELVLERC DLELETNGRD HHTADLCREK LVVRRGQPFW LTLHFEGRNY
         60         70         80         90        100
EASVDSLTFS VVTGPAPSQE AGTKARFPLR DAVEEGDWTA TVVDQQDCTL
        110        120        130        140        150
SLQLTTPANA PIGLYRLSLE ASTGYQGSSF VLGHFILLFN AWCPADAVYL
        160        170        180        190        200
DSEEERQEYV LTQQGFIYQG SAKFIKNIPW NFGQFEDGIL DICLILLDVN
        210        220        230        240        250
PKFLKNAGRD CSRRSSPVYV GRVVSGMVNC NDDQGVLLGR WDNNYGDGVS
        260        270        280        290        300
PMSWIGSVDI LRRWKNHGCQ RVKYGQCWVF AAVACTVLRC LGIPTRVVTN
        310        320        330        340        350
YNSAHDQNSN LLIEYFRNEF GEIQGDKSEM IWNFHCWVES WMTRPDLQPG
        360        370        380        390        400
YEGWQALDPT PQEKSEGTYC CGPVPVRAIK EGDLSTKYDA PFVFAEVNAD
        410        420        430        440        450
VVDWIQQDDG SVHKSINRSL IVGLKISTKS VGRDEREDIT HTYKYPEGSS
        460        470        480        490        500
EEREAFTRAN HLNKLAEKEE TGMAMRIRVG QSMNMGSDFD VFAHITNNTA
        510        520        530        540        550
EEYVCRLLLC ARTVSYNGIL GPECGTKYLL NLNLEPFSEK SVPLCILYEK
        560        570        580        590        600
YRDCLTESNL IKVRALLVEP VINSYLLAER DLYLENPEIK IRILGEPKQK
        610        620        630        640        650
RKLVAEVSLQ NPLPVALEGC TFTVEGAGLT EEQKTVEIPD PVEAGEEVKV
        660        670        680
RMDLLPLHMG LHKLVVNFES DKLKAVKGFR NVIIGPA
```

Figure 1.3. Amino acid sequence of human TG2. (Bayardo, 2004)

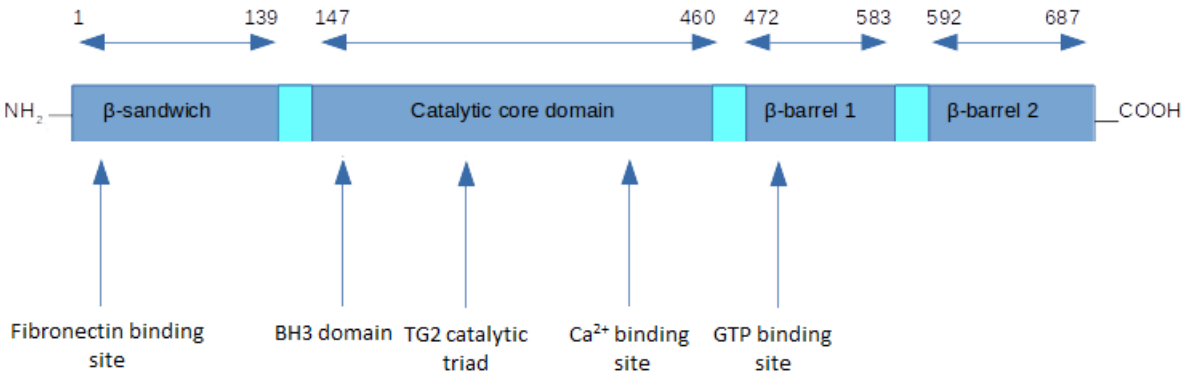


Figure 1.4. Schematic protein domain structure of TG2

1.3.1.1.1 Conformations of TG2

There have been a number of x-ray crystallography resolutions of TG2 in its open and closed forms. Figure 1.5 shows the open form and the closed GDP and GTP bound conformations. Note that it was observed (Liu, 2002) that TG2 forms dimers in its GDP bound configuration.

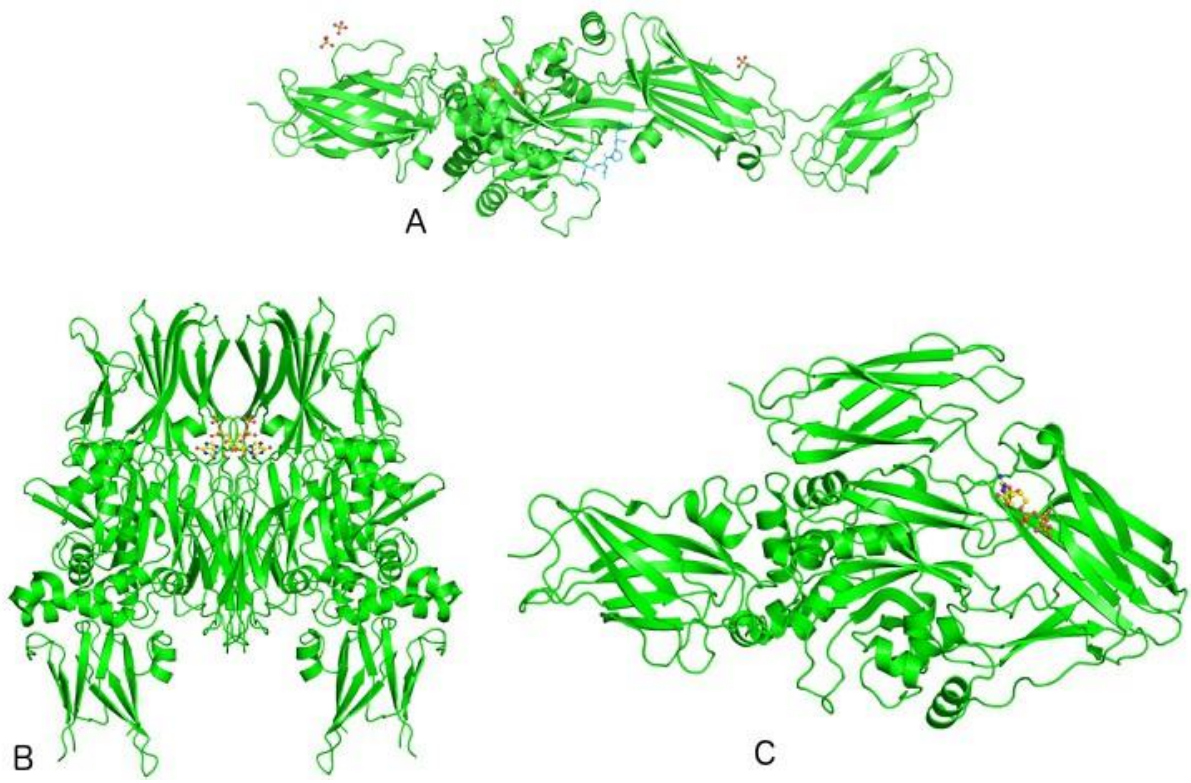


Figure 1.5. The different conformations of TG2

1.5A shows TG2 in its open conformation. Sulphate ions are used to stabilise the conformation (Pinkas, 2007 – PMID: 18092889). **1.5B** shows a dimeric assembly of TG2 with 6 GDP molecules bound to the dimer (Liu, 2002 – PMID: 11867708). **1.5C** shows monomeric TG2 with 3 GTP residues bound to it (Jang, 2014 – PMID: 25192068).

1.3.2 TG2. A multi-functional enzyme

TG2 functions primarily along two distinct pathways, i.e. the GTP-binding and the Calcium-dependant transamidation pathway (Fesus and Piacentini, 2002). These pathways influence each other, with either pathway usually becoming dominant within the cell (Tee, 2009). The structural basis for the multifunctional nature of TG2 was clarified (Liu, 2002) when the crystal structure of TG2 was analysed in its GDP-bound closed configuration and its GTP-bound open configuration. In its open configuration, the transamidation active site is exposed, whilst in its closed configuration, it is blocked (Pinkas, 2007). It is thought that when Ca^{2+} binds to TG2, this facilitates transamidation, whereas when guanine binds, TG2 assumes its closed GDP-bound configuration and by doing so blocks transamidation (Begg, 2006). It should be noted that in healthy cells, the GDP-bound configuration is dominantly expressed and the open configuration is rarely seen, being observed primarily in apoptotic or differentiating cells.

In contrast to the majority of the other TG family members, TG2 binds and hydrolyses GTP in a magnesium-dependent mechanism (Lee, 1989). When bound to GTP (as discussed further in section 1.3.2.2), TG2 has an involvement in signal transduction and acts as a G-protein. TG2 has also been shown to participate in cellular adhesion, apoptosis, drug resistance and even to interact with the α1B adrenergic receptor, which allows the transduction of signals to phospholipase Cδ1 (Chen and Mehta 1999).

TG2 has also been observed to act as a protein kinase (Mishra, 2007) and a protein disulphide isomerase (Hasegawa, 2003). Finally, TG2 has been shown to interact directly with other proteins without any clear enzymatic activity, displaying the properties of a protein scaffold (Akimov, 2001). Taken together, the large variety of ways that TG2 can interact with other molecules in the cell show that this enzyme is truly multi-functional.

1.3.2.1 Transamidation. How TG2 cross-links proteins

The calcium dependant transmidation pathway is controlled via the transamidase active site, which contains the catalytic triad Cyseine-277, Histidine-355, and Aspartate-358 (Lorand et al., 2003). These reactions primarily catalyze the modification of proteins via the Ca^{2+} dependant crosslinking of N-ε (γ-glutamyl) and lysine. The cross-linked, transamidation products of TG2 are particularly apparent in cells that are challenged by hypoxic or inflammatory environments. Transamidation reactions become increasingly prevalent when cells enter an apoptotic state and evidence is emerging that the transamidation of proteins by TG2 is a key component of apoptosis (Tee et al., 2009).

1.3.2.2 GTP-binding. How TG2 effects cellular adhesion and metastasis

Herman and colleagues reported in Oncogene (Herman et al., 2006) that the GTP-binding component of TG2 was essential for metastasis of the breast cancer cell line MCF-7. This GTP-

binding component of TG2 also resulted in a substantially increased resistance to drug induced apoptosis. They concluded that drug resistance in the MCF-7 strain of breast cancer is induced by TG2 binding with both fibronectin (Fn) in the extracellular matrix (ECM) and with beta and alpha integrins. Alpha5/beta1 integrins (α 5β1) combined as a heterodimer is associated with the activation of Bcl2, a pro-survival (anti-apoptotic) mediator. This TG2/integrin/Fn binding was shown in this cell line to activate focal adhesion kinase (FAK) a non-receptor protein tyrosine kinase, which induced survival processes. Activation of α 5β1 integrin was shown previously to activate the mitogen-activated protein kinase (MAPK) pathway (Roovers et al., 1999) and also to induce cyclin D1 expression. It was noted in this paper that Fn interaction with TG2/α5β1 results in the activation of Cdc42 and Rac like GTPases, both of which contribute to cell attachment, motility, and metastasis.

1.3.2.3 Localisation of TG2

Although TG2 can become externalised into the cell matrix in association with β-integrin (Akimov, 2001), the majority of TG2 is intracellular and predominantly cytosolic (Collighan and Griffin, 2009). Lacking a mitochondrial targeting sequence, TG2 is nonetheless observed in the mitochondrial outer membrane and the inner membrane space (Piacentini, 2002). TG2 has been shown to translocate via importin-α-3 to the nucleus from the cytoplasm where it associates directly chromatin (Lesort, 1998), dissociating from its transporter once inside the nucleus (Peng, 1999). Nuclear TG2 can either function as a G-protein or if Ca^{2+} is present, it is able to transamidate transcription factor SP1 and histones, thus providing a direct mechanism for the regulation of gene expression (Keresztessy, 2006).

1.3.3 Regulation of TG2

As previously stated in section 1.3.2.1, TG2 transadmidation of specific substrates is controlled by the catalytically active regulatory triad Cys (C277), His H335), Asp (D358), however a fourth conserved residue of tryptophan (W241) is also required for catalytic activity, stabilizing the active site, the experimental removal of which disables the protein (Murthy, 2002).

Activity of TG2 is regulated both by the intracellular levels of Ca^{2+} and by the levels of GDP/GTP (Verderio, 2004). The transdamidating activity of TG2 is increased as levels of Ca^{2+} increase, which (*in vivo*) usually results from a failure of the cell to maintain homeostasis, with a healthy cell actively pumping Ca^{2+} into the extracellular fluid (Pinkas, 2007). The levels of Ca^{2+} and GTP in the cell determine whether TG2 assumes an open configuration, which exposes its catalytic triad for reactivity or its closed configuration, which blocks the triad. In a healthy cell, the TG2 assumes a closed configuration as Ca^{2+} levels are low – 50-100nM as compared to 2.5mM in the blood plasma

(Bronner, 2001) and GTP/GDP is bound to the protein (Liu, 2002). When Ca^{2+} influx to the cell occurs, the TG2 then assumes an open configuration exposing the Cys277, which is then able to form a thioester bond and initiate transamidation of proteins (Chen and Mehta, 1999). GTP/GDP non-competitively inhibits transamidation by controlling whether the TG2 is in its open or closed configuration. When GDP bound, TG2 compacts into its closed configuration with the two C-terminal β-barrels overlapping a large proportion of the catalytic triad, which blocks substrates from accessing that region. When a high Ca^{2+} influx to the cell occurs, the Ca^{2+} binds to TG2 and changes its configuration to the open form, exposing the triad, allowing substrate reactivity (Begg, 2006). As TG2 has a higher affinity for guanine than it does for Ca^{2+} (Datta, 2006) it is the guanine that is primarily responsible for regulating transamidation, not the Ca^{2+}, which is low under normal conditions.

1.3.4 TG2 has both pro and anti-apoptotic roles

TG2 has been implicated both in promoting and inhibiting apoptosis; whether TG2 is pro-apoptotic or anti-apoptotic is dependent on a number of interacting and only partially understood factors. A key factor seems to be whether TG2 is in its GTP-binding or transamidation configuration (Park, 2010). Overexpression of TG2 has however been shown to be induced during apoptosis (Szegezdi, 2000). A loss of Ca^{2+} homeostasis following the initiation of damage to the cell membrane causes an influx of Ca^{2+} into the cytoplasm, which activates transamidation. Once activated TG2 catalyses the formation of protein-protein scaffolds, which function to stabilise the integrity of the cell membrane and help to form the self-contained vesicles around intracellular debris that are characteristic markers of apoptosis and which prevent potentially damaging material from entering the extracellular matrix. Fesus (1992) had observed that TG2 expression appeared to be primarily associated with an increased propensity towards apoptosis, as did Fok and Mehta (2007) who observed that TG2 is associated with apoptosis inducing factor (AIF) released from the mitochondria, which lead to its translocation to the nucleus, the result of which was apoptosis in this pancreatic ductal adenocarcinoma model. Yoo et al (2012) also observed that TG2 promoted apoptosis via the calpain/Bax protein signalling pathway.

1.3.4.1 TG2 as an inducer of apoptosis

Overexpression of TG2 sensitises cells to apoptosis and inhibition of TG2 expression tends to reduce the sensitivity of cells to apoptosis. For example, Melino (1994) observed that overexpression of TG2 in the SK-N-BE(2) neuroblastoma cell line resulted in a 4-5 fold increase in the rate of apoptosis as compared to wild type cells. One explanation for this involves the BH3 domain of TG2, which shares a 70% homology with the BH3 domain of the Bcl-2 family of apoptotic regulators. It has been reported that peptides mimicking this sequence are able to stimulate apoptosis by inducing a conformational change in the pro-apoptotic Bcl-2 member Bax,

which then translocated to the mitochondrial membrane, facilitating the release of cytochrome c into the cytoplasm and thus stimulating apoptosis (Rodolfo, 2004). It was also observed that TG2 itself can directly interact with Bax, but not with the anti-apoptotic Bcl-2 family members once bound to Bax, TG2 can facilitate polymerisation of Bax via its transamidation activity and facilitates the translocation of Bax to the mitochondria (Rodolfo, 2004). Following the initiation of apoptosis, the cell undergoes a change in structure and shape as the cytoskeleton loses cohesion. It is therefore significant that cytoplasmic actin is an abundant glutaminyl substrate for TG2 when in its open configuration and it has been observed that TG2 interacts with and transamidates the actin cytoskeleton in HL-60 and U937 cells during apoptosis (Nemes, 1997).

1.3.4.1.1 Effects of TG2 on NF-κB

TG2 has been observed to aid apoptosis via its GTP-binding, which results in an increase in the activity of the pro-apoptotic inflammatory regulator NF-κB. Kim et al (2006) determined in several strains of breast cancer cell line that there is a correlation between TG2 and IκBα levels. In TG2 transfected cells, an approximate 2-fold increase in NF-κB activity was recorded. When however TG2 was inhibited, his team observed up to a 3-fold decrease in NF-κB activity. This correlation provides evidence that TG2 triggers the activation of NF-κB. These findings were further supported by Lee et al. (2004) whose experiments with BV-2 Microglia identified that TG2 causes the polymerisation of IκBα which causes the inactivation of IκBα, preventing it from binding to NF-κB. This means that IκBα is no longer able to inhibit NF-κB and therefore NF-κB becomes active.

Further research by Kim et al. (2010) involved the use of TG2 knockout mice. Kim's team exposed cells from these mice to TNF-α induced hypoxia. The results of this exposure showed no increase in the production of inflammatory mediators such as COX-2 over control levels. TG2 has also been reported to increase in expression over 24 hours, the period of time in which increased activation of NF-κB is sustained in the presence of TNF-α (Park, et al., 2011). The pathway linking TG2 and NF-κB may form a positive feedback loop, as the gene promoter region of the TG2 gene contains a binding site for NF-κB (Mirza, et al., 1997). This may explain how the levels of TG2 are able to increase so dramatically following the activation of TG2.

1.3.4.2 TG2 inhibits apoptosis and promotes drug resistance

Hermans et al, (2006) determined that β1, α4 and α5 integrins were expressed at higher levels in doxorubicin resistant cells than in drug sensitive cells. TG2 surface expression was expressed at higher levels on both of the drug resistant cell lines being slightly higher in MCF-7/RT than in MCF-7/Dox. They also noted that TG2 closely associated with β1 and β5 integrins on the outer membrane of drug-resistant MCF-7/Dox and MCF-7/RT cells. The incubation of TG2-expressing

drug-resistant MCF-7 cells on Fn-coated surfaces was found to activate FAK. Inhibition of TG2 expression using SiRNA, resulted in Fn-mediated cell survival mechanisms in drug-resistant MCF-7 cells was also inhibited. Surface expression of β1 integrin on MCF-7/RT and MCF-7/Dox cells (both of which were shown to be TG2-positive) was found to be three to four-fold higher than in drug-sensitive TG2-negative MCF-7/WT cells. β5 integrin expression was high in all cell lines. The expression of α 4 and α 5 integrins was found to be low in drug-sensitive MCF-7/WT cells but high in drug-resistant MCF-7/RT and MCF-7/DOX cells. FAK levels increase with TG2 expressing MCF-7 cells, when grown on Fn. alpha 4 integrin was shown to be high in drug resistant MCF-7 cells, but low in drug sensitive cells. Alpha 5 integrin was observed to be high in drug resistant cells and low in drug sensitive cells. Finally, they found that beta 1 integrin levels were higher in drug resistant MCF-7 cells - 3 times which observed in drug sensitive cells. This study suggests that TG2 in its GTP-bound configuration is able to promote cell survival and inhibit the pro-apoptotic transamidating role of the enzyme.

A more recent study into the pro-survival role of TG2 (Kumar et al., 2012) showed that a catalytically inactive form of TG2 (TG2-C277S) is as effective at inducing epithelial-to-mesenchymal transition (EMT) as wild- type TG2 (TG2-WT) and in promoting cancer stem cell like behaviour (CSC) in mammary epithelial cells. The same study found that overexpression of a GTP-binding- deficient mutant (TG2-R580A) failed to result in the same behaviour. Additionally, the activation of the pro-inflammatory transcription factor NF-κB by TG2 was essential for initiating this EMT-CSC behaviour in mammary epithelial cells. This paper supports the hypothesis that transamidation is in some scenarios pro-apoptotic, whereas GTP-binding by TG2 is anti-apoptotic. It also helped to build on previous studies that showed a relationship between TG2 and NF-κB.

1.3.4.3 The TG2-L and TG2-S isoforms

It was observed as early as 1997 that there are two forms of TG2, a long primary form and a shorter mutant form. Both of these isoforms can be induced in rat brain by the inflammation-associated cytokines interleukin-1β and also to a reduced extent by the tumour necrosis factor-α (Monsonego et al., 1997). The shorter form (now known as TG2- S) was discovered and found to lack the GTP-binding C terminus residue that the longer TG2-L form expresses. The body of work relating to TG2-S and its relationship with TG2-L is limited, leaving the exact mechanisms and relationships between the isoforms unclear. Antonyak and his team were the first to discuss how these two isoforms might have opposing effects (Antonyak et al., 2006) and that TG2-L and TG2-S were actually alternatively spliced transcripts of a primary TG2 mRNA, now known as TG2-T (TG2

total). The TG2 isoforms are formed through exon skipping and intron retention. Following translation, both TG2-S and TG2-L express the same 538 N-terminal amino acids. TG2-S however contains 10 amino acids that are not translated in the TG2-L form and TG2-L contains 149 unique amino acids at its C terminus. The TG2-S protein lacks the GTP-binding Arg-580 residue that is essential for many of TG2-L's functions. The major commonality between the isoforms is that they both contain the transamidase active site, which contains the catalytic triad Ca^{2+}. When bound to TG2, Ca^{2+} was found to initiate transamidase activity and to suppress GTP binding; however when bound to guanine (the dominant state) transamidation is suppressed. This mutual suppression of either GTP-binding or transamidation goes some way to explaining the opposing effects of the two TG2 variants, with high TG2-S expression being associated with apoptotsis and high TG2-L levels being found in drug resistant cancer cell lines (Tee, et al 2009).

1.3.4.4 TG2 in inflammation and cancer

The inflammatory response and cancer formation/progression show many similarities to each other, with a number of inflammatory regulators playing roles also in cancer development (Mehta, 2010). As early as 1863 Rudolf Virchow observed the presence of leucocytes in neoplastic tissues and proposed a connection between the inflammatory response and cancer. His suggestion was that "lymphoreticular infiltrate" at the sites of chronic inflammation reflected the origin of cancer at those sites. Since Virchows' initial observation, it has since been noted that the inflammatory microenvironment of tumours is infiltrated with leukocytes in the supporting stroma of cancerous tissue and in the surrounding tissues. In ovarian tumours for example analyses of 20 parafin-embedded biopsies revealed a leukocyte infiltrate composed primarily of CD68+ macrophages, together with CD8+/CD45RO+ T cells with median values of 3700 cells/mm^3 and 2200 cells/mm^3 respectively. In this same infiltrate natural killer cells, B cells and mast cells were also present, but at far lower numbers – 0-200 cells/mm^3 (Negus, 1997). Of note is that in areas of necrosis the only form of leukocyte observed in significant numbers were macrophages. Tumour-associated macrophages (TAM) which are derived from circulating monocytes are a large component of the majority of tumours (Mantovani, 1992) and are attracted to the site of a tumour by the presence of chemokines. When activated correctly by the presence of lipopolysaccharides (LPS) and interferon (IFN)-y *in vitro* TAMs are induced to the M1 sub-type and are able to kill tumour cells (Sica, 2006). In the environment of a tumour, TAM's are however not usually exposed to M1 inducing compounds and instead encounter various cytokines and chemokines including interleukin (IL)-1, IL(6), (IL)-10 and TNF which promote the TAMs to differentiate to the M2 sub-type; In a tumour M2 is associated with the secretion of growth and angiogenic factors together with protease

enzymes that actively degrade the extracellular matrix, which can result in tumour cell proliferation, angiogenesis of the tumour and and initiation of invasion/metastasis of cancer cells. The presence of high levels of TAMs at the site of a tumour also inhibits the adaptive immune system and correlates with poor patient prognosis (Sica, 2006). More recently it was revealed that inflammatory mediators are also responsible for random accumulation of genetic alterations in cancer cells.

Of the inflammatory regulators involved in both inflammation, cancer and in the immune system, NF- κβ is a major player with its abnormal regulation being associated with many disperate forms of cancer (Karin, 2006). In both neoplastic cells and in inflammatory cells NF- κβ is activated downstream of the toll-like receptor (TLR)-MyD88 pathway and by the cytokines IL-1β and TNF-α. NF- κβ can become activated in hypoxic environments (Rius, 2008) and once activated induces the expression of adhesion molecules, inflammatory cytokines and enzymes involved in the prostaglandin synthase (pro-inflammatory) pathway (COX-2). NF- κβ is also a potent inducer of antiapoptotic genes such as Bcl2 that promote cell survival and help neoplastic cells to resist chemotherapy (Karin, 2006). It is significant that as tumours increase in size, the environment within the tumour mass becomes increasingly hypoxic, which may lead to a positive feedback loop linking activation of the inate immune system with inflammation further progression of the tumour.

One common result of cancer proliferation and invasion and the consequent inflammation, is some loss of homeostasis of cells in the area of the tumour and often in cells of the tumour itself. The result of this can be an influx of Ca^{2+} into the cell, or liberation of Ca^{2+} from the mitochondria and the endoplasmic reticulum, which results in both TG2 overexpression and a change in conformation of TG2 into the open transamidating configuration; the result of this is an increase in transamidation and the stimulation of apoptosis which can be greatly increased by further loss of Ca^{2+} homeostasis, comparable to that which is induced by radiation or chemotherapy (Kumar, 2013).

To summarise, overwhelmingly the weight of experimental data supports the hypothesis that transamidation is generally pro-apoptotic, whilst GTP binding is usually pro-survival, with transamidation crosslinking and stabilising proteins within apoptotic bodies (Fesus and Szondy, 2005) and GTP binding and closing off access to the catalytic triad and therefore blocking transadmidation. GTP binding is also known to activate FAK and P13K/Akt1, which are known to protect cells from apoptosis (Verna aMehta, 2007). It is now clear that whether TG2 is pro-apoptotic or pro-survival is dependent on a number of factors, including binding to co-factors, interactions with other proteins and cellular localization (Zemskov, 2006, Lorand, 2003 and Griffin, 2002). For example if a cell or organelles such as the mitochondria and endoplasmic reticulum

become permeable to Ca^{2+}, then Ca^{2+} is free to accumulate in the cytoplasm to the levels required for it to bind to TG2 and to induce TG2 to alter its configuration to the open (transamidating) conformation. This can allow TG2 to migrate to the mitochondira and to polymerise BAX, potentially resulting in the induction of apoptosis. TG2 can even be induced to migrate to the nucleus, translocating via importin-α-3 to the nucleus from the cytoplasm where it associates with chromatin activating apoptotic regulatory genes (Lesort, 1998).

1.3.5 TG2 as a chemotherapeutic target

There are a number of methods for inhibiting TG2 *in vitro*, many of which have been used experimentally to determine the role of TG2 in the cell. These include gene knockout of TG2 and point mutations of its active site, however these methods have drawbacks as they often have off target effects on the behaviour of the cell. For example, using liposomes to transfect a cell can result in the cell becoming more sensitive to apoptosis, which would mean transfecting SiRNA for TG2 or a mutated TG2 gene could skew any results from experiments investigating apoptosis and the cell cycle as TG2 is known to be involved in both of these processes. Many researchers therefore choose to use competitive inhibitors of TG2 enzyme activity such as cystamine and monodansylcadaverine to elucidate the role of TG2 (Karpuj, 2002). These compounds compete with the amine substrates present in the cell (for example lysine) and so inhibit the activity of TG2. It will be shown in the results of this thesis that in high-expressing TG2 cell lines, this inhibition of enzyme activity results in apoptosis, whereas in low-TG2 expressing lines, inhibition has little effect on viability (discussed further in section 4.3).

1.4 Phytochemicals

Phytochemicals are non-nutritive components of various fruits, vegetables and other plants, such as grapes, some of which show significant anticarcinogenic and antimutagenic properties (Young-Joon Surh, 2003). Naturally occurring compounds are found in a large amount of currently used medicines, including in anti-cancer drugs. For example, in 2002 24% of new drugs that were developed were derived from or originated from natural compounds (Chin, 2006). Many phytochemicals are included in the human diet and in some regions of the world in traditional medicine. There are also a large variety of phytochemicals being used in traditional medicines or have/are being evaluated in clinical trials to determine their effectiveness and mode of action, such as curcumin (Aggarwal, 2003), genistein (Swami, 2009), berberine (Mantena, 2006) and resveratrol (Zhang, 2013). Work in this area has led to and is continuing to lead to the development of treatments for a varied number of diseases, including cancer, cardiovascular disease and intestinal

disorders, making this area of research a vibrant and dynamic one.

1.4.1 Phytochemicals as chemotherapeutic tools

Epidemiological studies support the anti-cancer properties of phytochemicals, indicating that individuals who have a diet rich in them have a markedly reduced incidence of cancer (Liu, 2004). Phytochemicals from plants and herbs have been shown to inhibit cancer formation, progression and metastasis by means of their anti-inflammatory and anti-oxidant effects (Holloway, 2016, Islam, 2014, Leiherer, 2013 and Lim, 2010). The exact pathways that different phytochemicals such as resveratrol, berberine and curcumin effect carcinogenesis are varied, but include modulating inflammatory cytokines such as NF-κB in the case of berberine or modulating the cell cycle and the rate of angiogenesis in the case of resveratrol (Kimura, 2001).

1.4.2 Berberine as a apoptotic regulator and chemotherapeutic agent

Phytochemiclas such as berberine have been observed to be effective against prostate cancer cell models (DU145, PC-3 and LNCaP) (Mantena, 2006). In an *in vitro* study Mantena's team determined that berberine inhibited cell proliferation and induced cell death in a dose and time dependant manner (24-72 hours at 10-100 umol/L). Mantena also treated non-neoplastic human epithelial prostate cells (PWR-1E) with identical concentrations of berberine under the same experimental conditions, but observed no significant effect on cell viability. Berberine has also been found to induce cell death in several oesophageal cancer cell lines (Yes-1 to Yes-6) and to reduce the rate of proliferation (Lizuka, 2000). Insight into the mechanisms by which berberine induces apoptosis came when berberine was found to induce apoptosis in the HepG2 (human hepatocarcinoma) cancer cell line by increasing the expression of the pro-apoptotic Bcl-2 protein Bax which then activated the intrinsic pathway (Wang, 2010). See Figure 1.6 for the molecular structure of berberine.

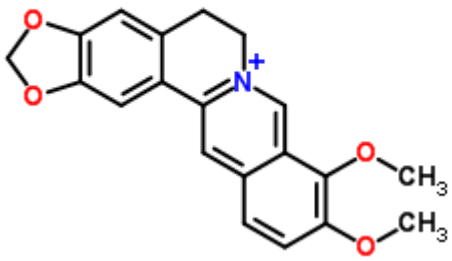


Figure 1.6. The molecular structure of berberine (Chemspider, 2016)

1.4.3 Resveratrol as a apoptotic regulator and a chemotherapeutic agent

Resveratrol - 3,4',5-Trihydroxystilbene (see Figure 1.7), belongs to a family of polyphenols called viniferines and is a phytoalexin produced in plants such as grapes and is catalysed by the enzyme stilbene synthase (Lekli, Ray and Das, 2010). Found in 2 isomoers, cis- (Z) and trans- (E), resveratrol was first identified in hellebore roots. Resveratrol has been shown to inhibit tumour formation in breast cancer (Venugopal and Liu, 2012) and also to induce apoptosis in several cancer cell lines, including the HL60 human leukemia and the T47D breast cancer cell lines (Clement, 1998). Clement *et al*, observed that increasing doses of resveratrol resulted in the externalisation of inner membrane phosphatidylserine (PS) and cellular DNA. This was indicative of loss of membrane asymmetry. The team also exposed primary peripheral blood lymphocytes (PLB's) to resveratrol at the same concentrations for up to 72 hours and observed no deterioration in cell viability. A more recent study (Zhang, 2013) showed that resveratrol in a dose dependant manner exerts antitumor affects via induction of autophagy by an unknown mechanism. The team observed that resveratrol inhibited proliferation of A549 lung cancer cells and induced cell death. By observing morphological changes over 24 hours, and finding numerous autophagosomes containing damaged organelles, they concluded that the cell death was via autophagy and demonstrated to be directly related to the accumulation of Ca^{2+} in the cytosol.

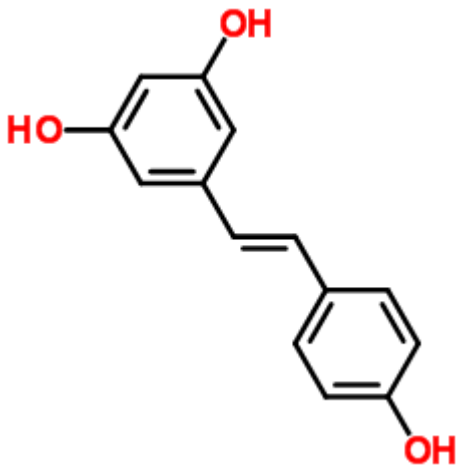


Figure 1.7. The molecular structure of resveratrol (Chemspider, 2016)

1.4.3.1 Berberine and resveratrol as a modulators of TG2

No work has yet been published on the effects of berberine on TG2 in cancer, however in 2011, Campisi *et al*, observed that berberine counteracted the upregulation evoked by glutamate of TG2 in primary rat astroglial cell cultures, restoring TG2 to control levels. Work on berberine has since primarily focussed on its anti-oxident and its use in neurodegenerative disorders.

A strong link was established between resveratrol and TG2, with Kumar *et al* (2014) observing a reduction in the migration of TG2-containing cells (SHYTG2 and Panc-28 cancer cells) when treated with resveratrol (0-10 uM). In migratory cells, they showed that resveratrol increases Ca^{2+} levels and transforms TG2 into its open extended form. This transformation of TG2 is sensitive to Ca^{2+} levels, and the open extended form is exclusively present in the plasma membrane fraction of migratory SHYTG2 and Panc-28 cells. They concluded that Ca^{2+}-mediated TG2, in association with the cell membrane is responsible for resveratrol's anti-metastatic effects.

1.5 Research aims and Objectives

1.5.1 Research questions

Currently there is a shortage of effective cancer therapies that are well tolerated by pateints. There are also no clinically available chemotherapeutics that are used specifically to target the enzyme TG2, a target that previous research shows is a potentially effective target for treating cancer. As phytochemicals have been shown to induce cell death in several cancer model systems, with the phytochemicals resveratrol and berberine showing promise as potential chemotherapeutics, the questions that this work focused on answering were:

Question 1: Can a novel phytochemical drug therapy be developed that effectively kills neoplastic cells in any or all of the cancer cell models used in this research? The models studied represented 3 disperate forms of cancer; these were liver cancer (using the HepG2 cell line as a model), renal cancer (using Caki-2) and a form of brain cancer known as astrocytoma (using 1321N1).

Question 2: If a phytochemical drug therapy can be developed and is proven to induce a substantial and statistically significant degree of cell death in one or all of the model systems, can the cell death be induced at clinically achievable doses?

Question 3: Do the phytochemical treatments tested exert their effects on cancer by modulating TG2 expression and/or activity? If so, then what are the possible mechanisms by which this modulation might be occurring?

Hypothesis 1: A novel phytochemical drug treatment can be developed to effectively induce cell death in one or more of the cancer cell models used in this project.

Hypothesis 2: The phytochemical treatment/treatments developed in this project induce their effects in the cancer cell model/models by modifying the behavior and/or the activity of the enzyme transglutaminase 2.

Hypothesis 3: The phytochemical treatments developed in this project induce their effects at doses that are clinically achievable.

1.5.2 Objectives

The specific objectives of this project were:

1. To use the previously established HepG2, Caki-2 and 1321N1 cancer cell line model systems to test the effectiveness of the phytochemicals resveratrol and berberine in isolation and in combination as chemotherapeutics
2. To measure the effects of the candidate phytochemicals on cell viability generally and apoptosis in particular
3. To determine the effects of the phytochemicals on the cell cycle
4. To characterise the effects on TG2 expression and enzymatic activity of the phytochemicals
5. To determine how the phytochemicals effect cellular adhesion and migration
6. To analyse the effect of TG2 inhibitors on the potency of the phytochemicals in order to determine if the chemotherapeutic effects of the compounds involve TG2 directly or if TG2 modulation is merely an off-target consequence of the treatment

The results of these studies will dissect the role of TG2 in cancer and to provide a greater knowledge of how phytochemicals can be used to treat cancer via the modulation of TG2. This work is of significance as existing cancer therapies show limited effectiveness and are in general not well tolerated so new therapeutic models need to be constantly developed. The evaluation of the combined effects of berberine and resveratrol represents a novel contribution to the subject area.

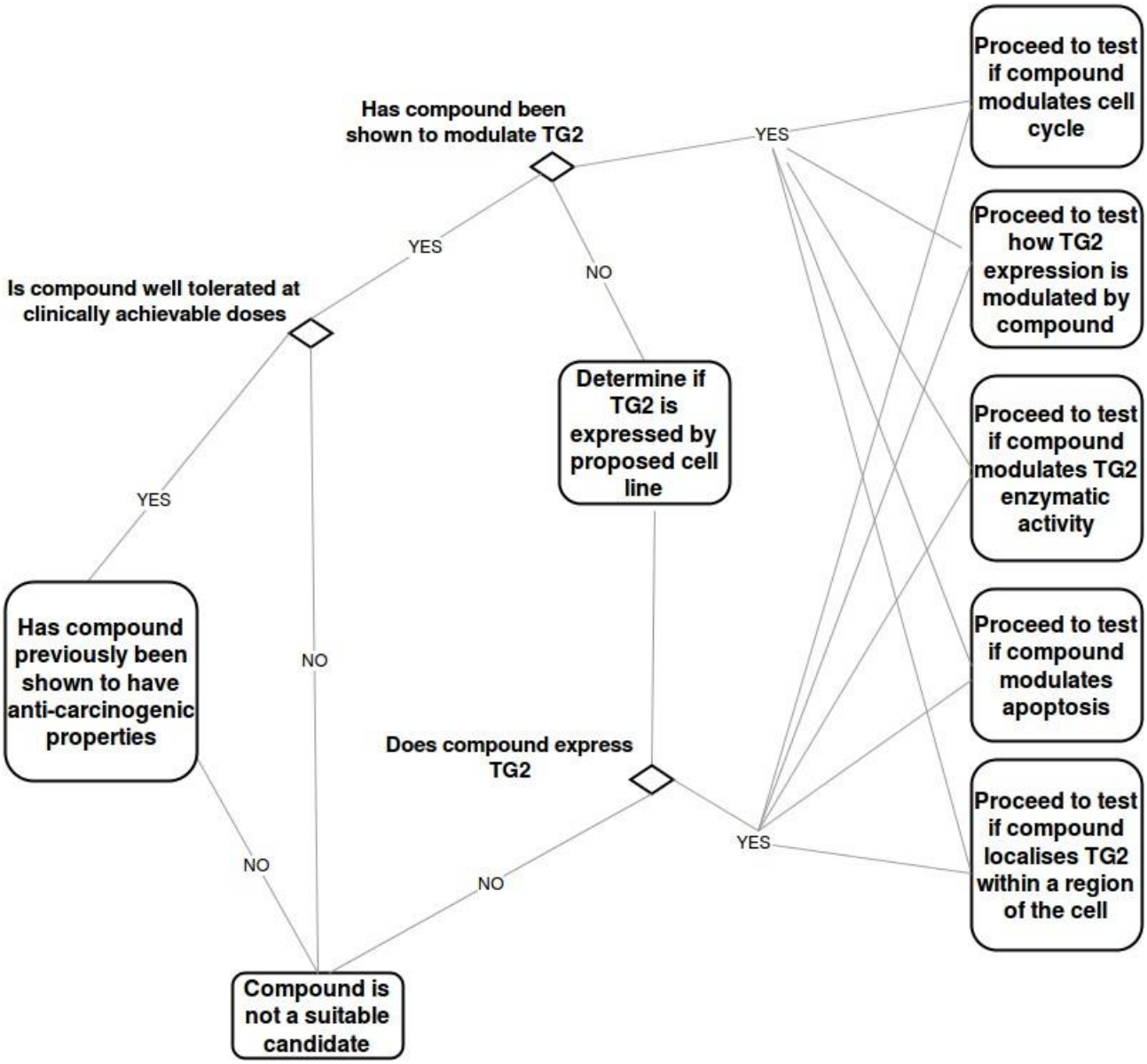


Figure 1.8. A decision tree showing how the shortlist of candidate compounds to be used in later experiments was arrived at.

1.5.3 Choice of phytochemicals

As phytochemicals are well tolerated and as many have already shown some promise as chemotherapeutic agents, several were chosen as candidate drugs for testing in this project. The decision to use the compounds resveratrol and berberine as experimental compounds in this project was arrived at following a review of the current literature (section 1.1-1.4) that facilitated a decision tree (see Figure 1.8). Using decision trees allowed for a logical progression of experiments to be performed and inappropriate candidate compounds and cell lines to be discarded, which greatly refined the final model. The final choice of compounds - resveratrol and berberine – was made as these candidates showed a high likelihood of both modulating TG2 and effecting the behaviour of the *in*

vitro cancer model used.

Chapter 2. Materials and methods

2.1. Introduction

A bank of techniques was used to profile the effects of the candidate compounds on transglutaminase 2 activity, cell viability, cell cycle, cell adhesion and cell migration. Each technique has its own bene fits and drawbacks, for example cell viability can be measured by trypan blue staining, the CCK-8 assay, DNA fragmentation and flow cytometry. Some of these techniques are quick to perform, but lack accuracy e.g, CCK-8, whereas others are very accurate but use expensive reagents and are (relatively speaking) time consuming. In order to ensure that the correct methodologies were used and in the correct order a decision tree was therefore created (see Figure 2.1).

2.2. Materials

All plastics were obtained from Thermo-Fisher Scienti fic. All reagents (unless otherwise stated) were obtained from Sigma Aldrich and all antibodies from Abcam. The cell lines used are specified in section 2.2.3.

2.2.1 Equipment

All flasks, pipette tips and other hard consumables unless otherwise stated were purchased from Fisher Scienti fic UK Ltd, Bishop Meadow Road, Loughborough. These included:

- T-25, T-75 and T-150 cell culture flasks
- 96, 24, 12 and 6 well plates
- 2.5 cm2 petri dishes
- P2, P10, P200 and P1000 auto pipettes (Gilson)
- 1ml, 5ml, 10ml and 25ml serological pipette tips
- 10ul, 200ul and 1000ul pipette tips
- Class III microbiological safety cabinet (Envair)
- Trinocular Inverted Phase Contrast Microscope (Nikon TMS-F)
- Gloves
- Lab coat
- Protective eye wear (goggles)

- AccuSpin 1R centrifuge
- Spectrophotometer, Nanodrop ND1000 (ThermoScientific)
- Sunrise plate reader (Tecan)
- QBA1 heat block (Grant)
- Vortex mixer (Stuart)
- Accuri C6 flow cytometer (BD Biosciences)

2.2.2 Equipment

All flasks, pipette tReagents and kits

- RNeasy ® Mini Kit (50) (Qiagen)
- FITC Annexin V Apoptosis detection kit I (BD Pharmigen)
- iScript one-step RT-PCR kit with Syber green, cat. No. 170-8893 (Bio-rad)
- QuantiTect ® Reverse Transcription kit (Qiagen)
- Berberine Chloride Hydrate (C 20 H 18 ClNO 4)
- Reveratrol (C14H12O3)
- Hydrogen Peroxide solution
- McCoy's 5A Medium
- Roswell Park Memorial Institute Medium (RPMI)
- Dulbecco's Modified Eagle's Medium (DMEM)
- Hank's Balanced Salt Solution (HBSS)
- Penicillin-Streptomycin
- Glutamine
- Foetal Calf Serum
- Dimethyl Sulphoxide (DMSO)
- Trypsin/EDTA

2.2.3 Cell lines

2.2.3.1 Caki-2

Human kidney carcinoma (American Type Culture Collection, ATCC). Obtained from Sigma, this cancer cell line was derived from a 69 year old Caucasian male. It contains multilaminar bodies and micro filiments. Caki-2 cells are tumourgenic in nude mice and grow in a monolayer.

2.2.3.2 HepG2

Human liver carcinoma (American Type Culture Collection, ATCC). Obtained from Sigma, this cancer cell line was derived from a 15 year old Caucasian male. HepG2 is a well differentiated hepatocellular carcinoma, responds well to growth hormone stimulation and grows in clumps.

2.2.3.3. 1321N1

Human astrocytoma (Glial cell) cancer cell line (American Type Culture Collection, ATCC). Obtained from Sigma, this cell line was derived from a sub-clone of the 1181N1 cell line, which had itself been derived from a sub-culture of the U-118 cell line in 1975.

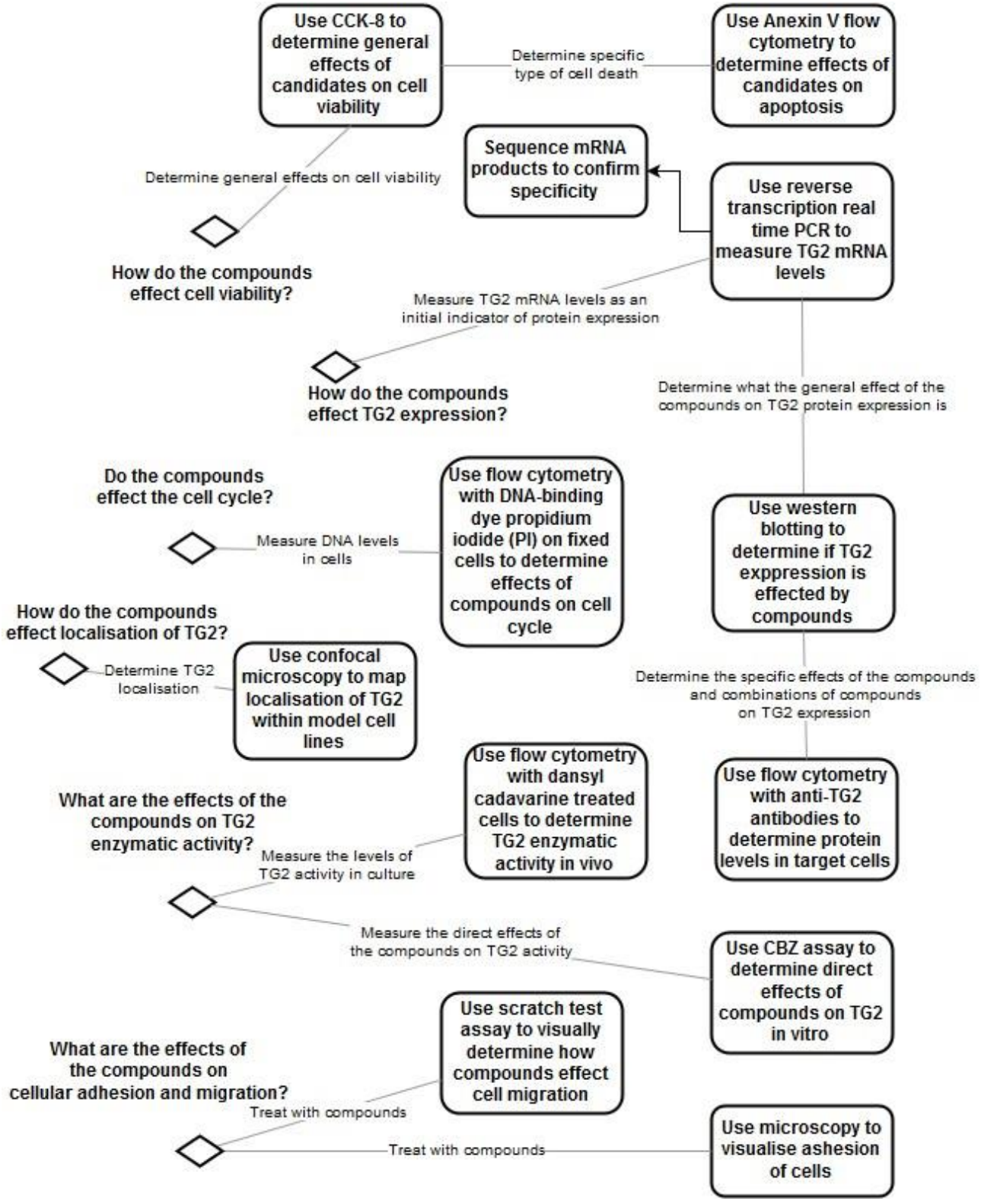


Figure 2.1. A decision tree showing the selection criteria for the methods used in this project

2.3 Methods

2.3.1 Cell maintenance

The human renal carcinoma cell line Caki-2 was maintained in McCoys 5A medium, supplemented with 10% foetal bovine serum, 1% l-glutamine, and 1% penicillin streptomycin (Sigma). Human hepatocarcinoma HepG2 and human astrocytoma 1321N1 were maintained in DMEM medium, also supplemented with 10% foetal bovine serum, 1% l-glutamine, and 1% penicillin streptomycin (Sigma). All cells were incubated at 37°C in a 95% air, 5% CO2 humidi fied incubator and were fed 2/3 times per week. All media and compounds were warmed in a water bath to 37°C before transferal to the flasks and/or well plates.

2.3.2 Cell thawing

Cryovials were removed from the liquid nitrogen dewer and agitated in a water bath at 37^0C until thawed. Cells were then centrifuged in 10 ml of pre-warmed cell culture medium for 4 mins at 1000 rpm to pellet out the cells. The media was discarded together with any dissolved cryoprotectant (DMSO). The cells were then re-suspended in growth media and transferred to cell culture flasks for incubation.

2.3.3 Cell freezing

At 70-80% con fluence, cell cultures were harvested and centrifuged (4 mins at 1000 rpm). Supernatants were removed and cells were re-suspended in chilled media containing 10% DMSO to prevent the formation of ice crystals in the cells during the freezing process. 1 ml aliquots were then transferred to cryovials and left overnight in a -80^0C freezer to slow freeze. Cryovials were subsequently transferred to liquid nitrogen dewers for long term storage.

2.3.4 Analysis of cell viability and apoptosis

2.3.4.1 Trypan blue assay

Trypan blue (TB) analyses of cell viability was performed in triplicate each time the cells were passaged to determine the general health of the populations. A 0.2% (w/v) TB solution was prepared by solubilising 1 g of TB and 3.38 g of NaCl in 500ml of distilled water. A 1:1 mixture of cells in culture medium and the TB solution was then incubated for 3 minutes in microcentrifuge tubes and cell viability was assessed under a microscope at 400X using a haemocytometer, based on

the appearance of the cells. Clear cells that failed to take up the TB were considered healthy, whilst cells that took up the TB dye had lost membrane integrity, allowing the dye to enter the cytoplasm and were therefore considered non-viable (see Figure 2.2).

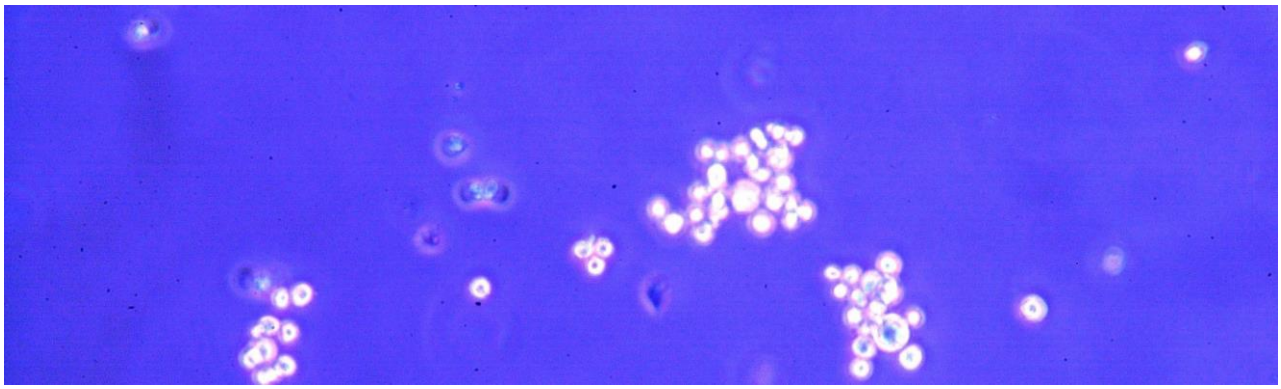


Figure 2.2. Trypan blue analyses of HepG2 cells

The brighter, clear cells have not taken up TB (Healthy). The large blue cells (necrotic) and the smaller, blue cells (apoptotic) have taken up TB.

2.3.4.2 CCK-8 assay

Initial determination of cell viability was measured using the CCK-8 kit (Dojindo Laboratories, Tokyo, Japan). The CCK-8 assay uses WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which in the presence of an electron carrier, 1-Methoxy PMS, produces a water-soluble formazan dye. Once added to cell cultures, WST-8 is bioreduced by cellular dehydrogenases to a soluble orange formazan product. The degree of formazan production is directly proportional to the number of living cells. In this application of the protocol, cells were seeded into 96-well plates for 24 hours, followed by a triplicate treatment of wells with resveratrol, berberine or both. After 24 hours incubation, all media was removed and the wells were washed 3 times with 100 ul of PBS; new media was next added to the wells (100 ul) and 10ul of CCK-8 solution was also added, the plates were then incubated for 4 hours at 37°C. Absorbence was measured at 490 nm using a micro-plate reader (Tecan).

2.3.4.3 Annexin V/Propidium iodide flow cytometric analysis

Based on CCK-8 results, the IC50 of cultured cells was determined and triplicates of T-25 flasks were treated with resveratrol, beberine or a combination of both. Levels of apoptosis and necrosis were determined using an Accuri C6 flow cytometer (BD, Biosciences) by staining with Anexin V and propidium iodide (BD Pharmingen) to determine phosphatidylserine (PS) cell surface levels and free DNA levels respectively.

Fluorescently labelled Annexin V-FITC binds to phosphatidylserine (PS), which translocates from

the inner to the outer membrane of cells during apoptosis and can be detected at 525 nm by excitation at 488 nm in a flow cytometer (Chemometec, 2013). PI binds to DNA and has an excitation/emission maxima of 493/636 nm, is not a membrane-permeable dye and is only able to pass into the cell and bind to DNA when the cell membranes become disrupted; which occurs in late apoptosis and more strongly in necrosis. By plotting Anexin V fluorescence against PI fluorescence, the percentage of healthy, apoptotic and necrotic cells can be measured (R&D Systems, 2013). The exact protocol used was as according to the manufacturer's instructions (BD Pharminogen, 2013) and the software used to analyse the results was FlowJo (LLC). See Figure 2.3 for an example of a flow cytometric analyses of a population of HepG2 cancer cells.

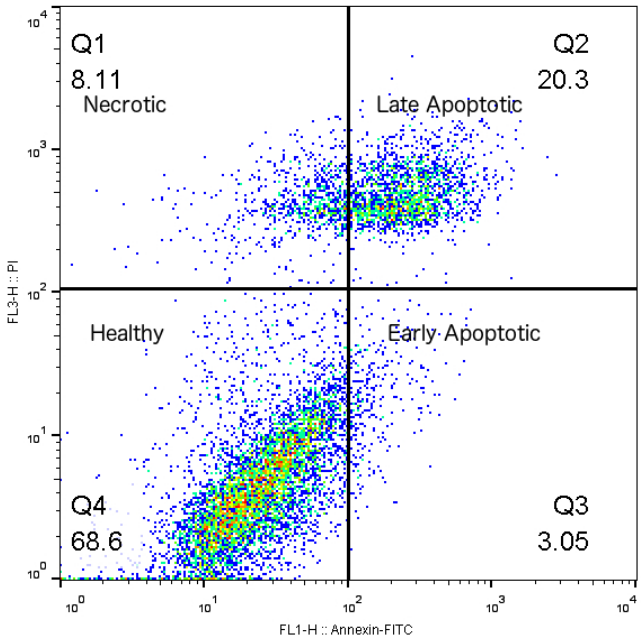


Figure 2.3. Flow cytometry analyses of Anexin V/PI treated HepG2 cells.
A graph generated by the BD accuri C3 software showing PI against anexin V. Low PI and annexin V indicates healthy cells (bottom left). Low PI and high annexin V (bottom right) shows that PS has switched from the inner to the outer cell membrane and indicates a rise in apoptosis. High PI and high anexin V indicates late apoptosis/necrosis as DNA is now leaking from the cell and can bind to PI, with PS binding to the cell membrane (top).

2.3.5 RNA extraction

Following trypsinisation, cells were lysed with RNeasy Lysis Buffer (RLT) (Qiagen) and total RNA extracted using the Qiagen RNeasy Mini Kit (50) (cat.number 74104) in accordance with the manufacturer's instructions (Qiagen, 2013). Prior to extraction, all surfaces, pipettes and glassware were cleaned with RNAseZap to neutralise any RNase activity of contaminating compounds. All steps of the protocol were performed on ice, with each centrifugation step performed at 4°C to slow down the degradation speed of RNA. Once extracted, RNA was suspended in RNase-free water and stored in microcentrifuge tubes at -80°C until required.

2.3.6 Total RNA Quanti fication

The Nanodrop ND1000 (ThermoScienti fic) spectrophotometer was used to measure total RNA concentration and purity according to the manufacturer's instructions, using 1 ul of total RNA in RNase free water for each measurement.

2.3.7 Determination of TG2 mRNA expression using reverse transcription real-time PCR

2.3.7.1 Determining the annealing temperature for the primers

Primers were designed using primer BLAST (NCBI) and purchased from Euro fins Genomics. The ideal annealing temperature to use in PCR applications for each primer set was determined by testing a series of temperatures around the temperature suggested in the guide document provided by the manufacturer. A TC-512 temperature gradient PCR machine (Techne) was used to test a range of temperatures in 1°C intervals around the mid-point. Agarose gel electrophoresis was performed on all samples and the predicted band length compared with a DNA ladder of known length. The strongest bands of the predicted length were isolated, puri fied and sequenced externally using Sanger sequencing (Cambridge University Department of Biochemistry). The returned sequences were run through NCBI BLAST to con firm speci ficity of the primers for the target sequences.

2.3.7.2 Relative measurement of mRNA levels

Real-time relative PCR was performed in a light cycler 2.0 (Roche) using the Syber Green protocol (BioRad). Total RNA was isolated from cultured cells following incubation with single or combined treatments of berberine/resveratrol (24 hours). Primers were designed for the Long TG2 isoform (TG2-L) and the shorter TG2 isoform (TG2-S) using the NCBI primer Blast tool. TG2-L forward primer was 5'-ATTCCCTCTCCTGCCCAGAT-3' and reverse was 5'- AGGGAGCTGGATTCCCTGAT-3'. For TG-S, the forward primer was 5'- CCCTGTGTTCCTGGAGCATT-3' and the reverse primer was 5'-CCAGAGTCAGCGCCATGTAA- 3'. For relative quanti fication the delta/delta method (Bustin, 1997) was used with beta actin being employed to normalise the results. The beta actin primers used were forward 5'- GCCTCGCCTTTGCCGA-3' and reverse was 5'-GTTGAAGGTCTCAAACATGATCTGG-3'.

To calculate the fold change of mRNA expression, firstly the CT values (PCR cycle threshold – the cycle number when the PCR product begins to amplify exponentially) for all samples were recorded and standard deviation determined.

Next, for each gene the C T was recorded, with controls standardised against actin to determine the difference between the gene expression induced by each treatment and the control. For controls, C T was calculated using the formula:

C T = CT – Actin Value

The C T for the controls was 0, – C T for the controls gave a value of 1.
For treated cells, the C T was calculated as above.

The C T was calculated using the formula below:

C T =C T - the average of the C T for the controls for the same gene

$2^{-\text{Delta CT}}$ as then calculated for each sample.

The $2^{-\text{Delta CT}}$ of each triplicate was averaged and fold changes were graphically illustrated

2.3.8 Gel electrophoresis

2% (w/v) agarose gels were made by dissolving 2 g of agarose powder in 100 ml of 1X TB Buffer. 1.5 l of ethidium bromide were then added to the solution and mixed. The solution was poured into a sealed electrophoresis tray, and a 16 well gel comb was slotted into the gel and left until the gel was set (20 mins dependant on ambient room temperature). The DNA or RNA was prepared for loading by mixing 4ul of loading dye and 5ul of RNA/DNA on a sheet of para film. Once the gel had set, the comb and masking tape were removed. 8 ul of sample was loaded into each well, the last well was loaded with Hyperladder 1 (Bioline). The gel was placed into an electrophoresis tank, filled with 1X TB buffer and run at 85 Volts for 1hour 30 minutes. The gel was then visualized under a UV transilluminator. See Figure 2.4 for an example of an agarose gel that was prepared using PCR products that had been amplified using the same Beta actin primers (see section 2.3.7) on a temperature gradient PCR machine.

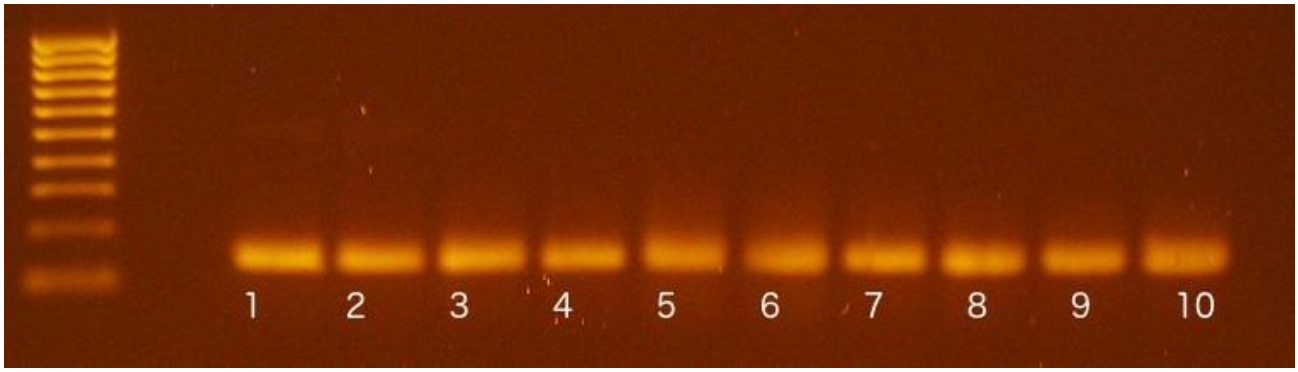


Figure 2.4. An agarose gel showing the speci ficity of the Beta actin primers over a range of temperatures 51°C-60°C

2.3.9 *In Situ* TG2 Enzymatic activity

Intracellular TG2 enzyme activity was measured using flow cytometry following incubation for 24 hours of cells with resveratrol, berberine or both in the presence of a fixed concentration (60uM) of the auto-fluorescent TG2 substrate dansyl cadavarine (Sigma). 10,000 events were recorded and substrate accumulation was detected at 533nm. In each flow cytomertry run, samples were included that had not been incubated with dansyl cadaverine to act as negative controls. Dansyl cadaverine was first used to monitor transglutaminase activity by Lorand, *et al* (1979).

2.3.10 Cell migration and adhesion assay

To determine if the candidate compounds affected cellular adhesion and migration, cells were grown to con fluence in 6 well plates and a horizontal scratch was made along the centre of the wells using a 200ul pipette tip. This created a path through the centre of the wells that was clear of cells. Images were then acquired of the cells under a phase contrast microscope (Nikon) and berberine, resveratrol or a combination of both were added to the media of the wells. Cells were then visualised under the microscope at 24 hours to ascertain the degree of migration across the empty area of the plates. As a final step, the cells were trypsinised and the time taken before 90% cellular detachment could be observed was measured to determine the strength of adhesion.

2.3.11 Stock solutions

The resveratrol was purchased from Sigma and has a molecular weight of 228.24. 1 mM stock solutions of resveratrol were prepared by dissolving 0.009 g of resveratrol powder in 30 ml of warm water in a sonicator for 10 mins. The berberine was also purchased from Sigma and has a molecular weight of 371.81. A 1 mM stock solutions were prepared dissolving 0.015 g of berberine powder in 40 ml of water whilst vortexing for 20 mins. The cystamine was purchased from Sigma and has a molecular weight of 225.20. 50 mM stock solutions were prepared dissolving 0.45 g of cystamine powder in 40 ml of water for 5 mins.

2.3.12 Statistical analyses

All the data from this study were analysed for statistical significance using the Graphpad Prism Statistics software (Version 7). The methods used were t-test to determine if any relation existed between concentration of phytochemicals and activity, the level of apoptosis and the cell cycle. One-way ANOVA was then used as a more robust method for con firming statistical signi ficance of all results. All data was presented with standard deviation were $P < 0.05$ was considered significant.

Chapter Three

The results of comparing the effects of berberine and resveratrol on cell viability and cell proliferation in different cancer cell lines

3.1. Introduction

As was discussed at length in the introduction (Section 1.1), apoptosis is a process that allows a cell to initiate a series of biochemical and molecular mechanisms including nuclear disruption, apoptotic body formation and membrane blebbing that result in organised cell death (Kerr *et al.*, 1972). This process displays a characteristic morphology and results in the safe removal of damaged cells from tissues (Lockshin, 2004). Necrosis in contrast is an uncontrolled form of cell death that results in the swelling and bursting of damaged cells and the release of powerful hydrolytic enzymes into surrounding tissues, which can cause damage to surrounding previously viable cells (Elmore, 2007). Failure to induce apoptosis means that damaged cells remain in tissues where over time an increasing number of genetic and/or epigenetic alterations eventually results in either a loss of some function within the tissue or in a gain of function. One such loss of function mutation/alteration might be a reduction in sensitivity to extrinsic pro-apoptotic signals, whilst a gain of function mutation/alteration might be self-sufficiency in pro-mitotic growth signalling (Bertram, 2000).

Carcinogenesis is characterised by the loss of control over the cell cycle and failure of cells to respond to either intrinsic or extrinsic apoptotic signals so any increase in such behaviour in damaged cells has the potential to lead to cancer. With this in mind, a common design strategy for scientists working to develop anti-cancer drugs, is to select for compounds that at certain doses will both selectively target cancer cells over healthy cells and also induce a combination of apoptosis and necrosis in the target cells. This combination of apoptosis and necrosis both clears away cellular debris following death of the cells (apoptosis) and attracts the immune cells of the patient to the site of the tumour (necrosis) resulting in the patients own immune system attacking the neoplastic cells.

To determine which cancer cell line was most responsiveto the candidate phytochemicals and for modelling the effects of the candidate phytochemicals on apoptosis and the cell cycle, toxicity curves were generated for resveratrol and berberine (isolated and combined treatments) using the CCK-8 cell viability assay (see materials and methods section 2.3.4.2). This initial ‘general’ cell viability assay was performed on the 1321N1 (astrocytoma), Caki-2 (renal carcinoma) and the HepG2 (hepatocarcinoma) cell lines.

Based on these results (outlined below in section 3.2), further analyses of the specific effects of

the phytochemicals on apoptosis and the cell cycle were performed using flow cytometry (section 3.3).

The following sections help to fulfill questions 1 and 2 of the reseach aims (section 1.5.1) – To determine if a novel phytochemical therapy can be developed that kills neoplastic cells and if so, if the doses needed are at clinically achievable levels.

3.2. Initial determination of cell viability using the CCK-8 assay

The CCK-8 assay determines the relative levels of healthy (viable) cells that are present in a cell culture well plate (in this case 96 well plates) using a water-soluble formazan dye that produces an orange colour in viable cells upon bioreduction. Although this assay does not differentiate between whether a particular treatment has caused cell death or merely inhibited the cell cycle, it is a good initial assay to evaluate if a particular treatment affects cell population size/viability. Also, as the assay can be performed in 96 well plates and is measured sprectrophotometrically, a large range of concentrations of a treatment together with a large number of replicates of data can be quickly and efficiently generated. This assay was, therefore, chosen to begin the selection process for a model cell line. Statistical analysis of the results was performed using a one-way ANOVA and Tukey for post hoc analysis was also performed on all treatment groups. A p value of .05 or less was regarded as significant.

3.2.1 Results of berberine treatment on cell viability

Following seeding of 96 well plates with either 321N1, Caki-2 or HepG2 cells and 24 hour incubation of all at cells at 37^0C in appropriate culture media (see Materials and Methods section 2.3.1) and the preparation of a stock 1 mM solution of berberine (see Materials and Methods section 2.3.11); triplicate wells were inoculated with berberine at a range of 0-75 uM in 5 uM increments. The well plates were then allowed to incubate for a further 24 hours at 37^0C before washing of the wells with PBS and the addition of 10 ul of CCK-8 in 100 ul of media to each well. Following 4 hours of further incubation, the absorbances of the wells were measured at 450 nm, the results of which are shown in Figure 3.1. The results show that berberine strongly effects the viability of both the 1321N1 astroytoma cell line at concentrations as low as 5 uM and the HepG2 cell line at concentrations of around 25-30 uM. Berberine does not seem to effect the viability of the Caki-2 cell line.

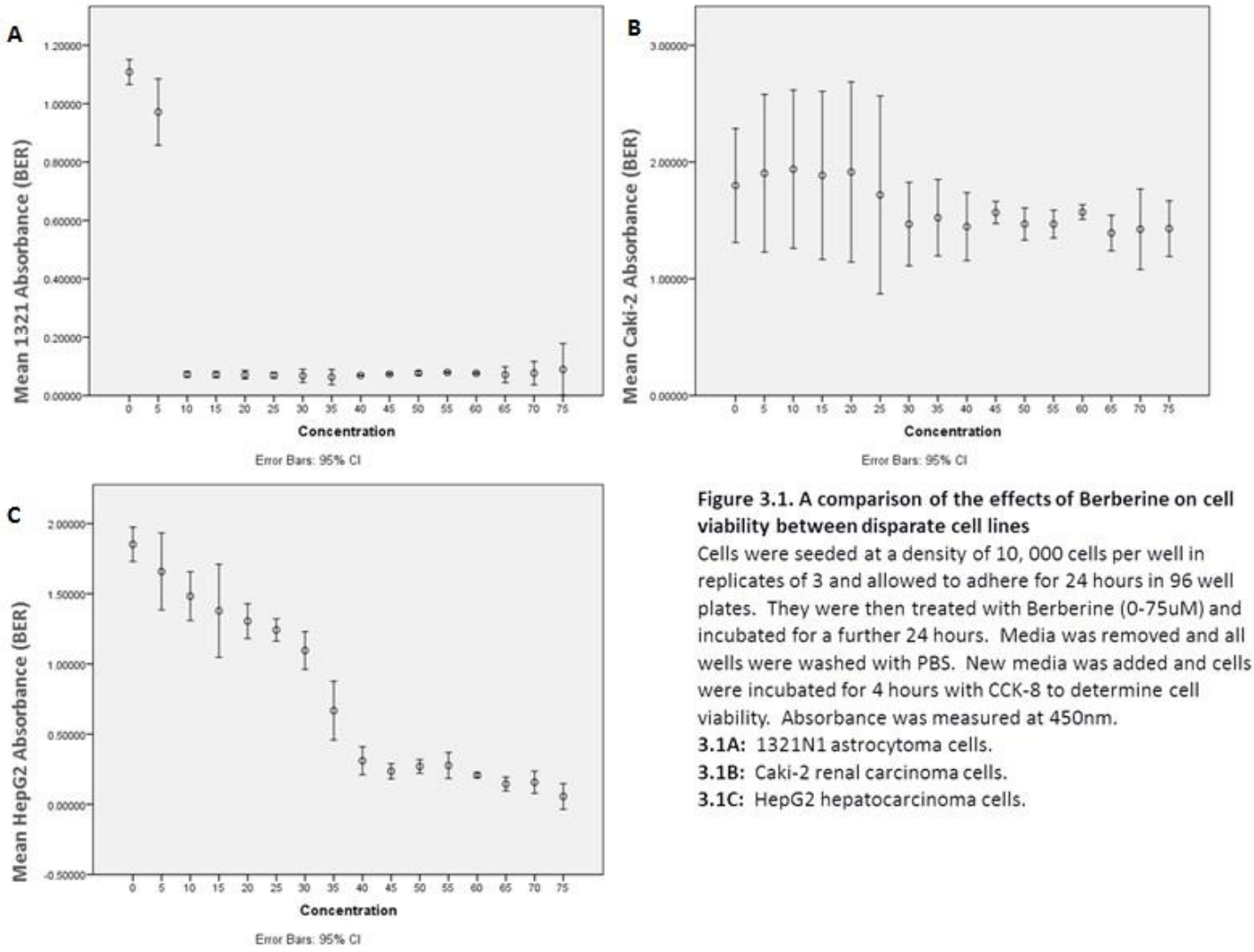


Figure 3.1. A comparison of the effects of Berberine on cell viability between disparate cell lines

Cells were seeded at a density of 10, 000 cells per well in replicates of 3 and allowed to adhere for 24 hours in 96 well plates. They were then treated with Berberine (0-75uM) and incubated for a further 24 hours. Media was removed and all wells were washed with PBS. New media was added and cells were incubated for 4 hours with CCK-8 to determine cell viability. Absorbance was measured at 450nm.

3.1A: 1321N1 astrocytoma cells.

3.1B: Caki-2 renal carcinoma cells.

3.1C: HepG2 hepatocarcinoma cells.

3.2.1.1 Discussion and interpretation of the results of investigating the effects of berberine on general cell viability

When comparing the effects of different concentrations of berberine (0-75 uM) on cell viability for the 1321N1 cell line, there was a statistically significant difference observed between groups as determined by one-way ANOVA p = < 0.000). A Tukey post hoc test to further analyse the difference in the effects of the treatments between 'specific' groups revealed that the cell viability was statistically significant when comparing the untreated group with groups treated with any of the other concentrations of berberine (5-75 uM) (p = < 0.000). There was also a statistically significant difference (p = < 0.000) between the group treated with 5uM of berberine and all other groups (10-75 uM). No statistically significant difference between any of the other treatment groups was observed. This result suggests that berberine treatment has a strong effect on either the viability or rate of growth of this cell line at concentrations as lowas 5uM. As the effects on the viability of this cell line were so strong in response to berberine at such a low dose and in line with the decision tree (Figure

1.8) and questions 1 and 2 of the research aims (section 1.5.1) it was therefore decided to investigate in more detail the effects of this phytochemical on the viability of 1321N1's using flow cytometry (see section 3.3 and Figure 3.6). This would gain a better insight into the type of cell death induced in this cell line by this treatment.

Statistical analysis of the effects of berberine (0-75 uM) on cell viability for the Caki-2 cell line, also showed a significant difference between groups as determined by one-way ANOVA ($p = < 0.002$). The Tukey post hoc test revealed that there was however no statistically significant difference in cell viability when comparing the untreated samples with samples treated at any specific concentration (5-75 uM) of berberine. The closest near significant value was $p = < 0.076$ when comparing the 10 uM treatment group with the 65 uM group, but as this p value falls above the accepted value of .005 this effect was deemed insignificant for this group comparison. This result suggests that berberine treatment has only a weak effect on cell viability/proliferation at concentrations up to 75 uM. This analysis does not however determine that this cell line will not be effected by higher concentrations of berberine. The effects of berberine on Caki-2 cell viability were much smaller than for 1321N1 (see Figure 3.1), but some reduction in cell viability was observed. Therefore, in line with the decision tree (Figure 1.8) and questions 1 and 2 of the research aims (section 1.5.1) it was decided to investigate in more detail the effects of berberine treatment on this cell line. It might also be useful as a control against cell lines that are less tolerant of this phytochemical.

Finally, for the HepG2 cell line, when comparing the effects of berberine (0-75 uM) on cell viability, there was observed a statistically significant difference between groups as determined by one-way ANOVA ($p = < 0.000$). A Tukey post hoc test revealed that the cell viability was statistically significant when comparing the untreated group with the 10uM group and when comparing the untreated group with all other groups ($p = < 0.000$). Post hoc analysis also showed a significant difference between most treatment groups. The result of this experiment suggests that berberine treatment has a strong effect on either the viability or rate of growth of this cell line at concentrations as low as 10 uM. As HepG2 is a cell line model of hepatocarcinoma and liver cells are a first point of contact for any orally ingested berberine, 10 uM is a concentration that is achievable clinically in the liver, thus answering for this cell line questions 1 and 2 of the research aims (section 1.5.1); berberine can be used at clinically achievable doses to reduce the viability of HepG2 cells. The results suggest that more detailed investigation into the exact effects of berberine on this cell line is warranted.

* See Appendix 1 for a full output of the one way ANOVA and Tukey tests for the above experimental data set.

3.2.2 Results of resveratrol treatment on cell viability

Following seeding of 96 well plates with either 1321N1, Caki-2 or HepG2 and 24hr incubation of all

at cells at 37^{0}C in appropriate culture media (see materials and methods section 2.3.1) and the preparation of a stock 1 mM solution of resveratrol (see materials and methods section 2.3.11); triplicate wells were inoculated with resveratrol at a range of 0-75 uM in 5 uM increments. The well plates were then allowed to incubate for 24 hours before washing of the wells with PBS and the addition of 10ul of CCK-8 in 100 ul of media to each well. Following 4 hours of further incubation, the absorbance of the wells was measured at 450 nm, the results of which are shown in Figure 3.2.

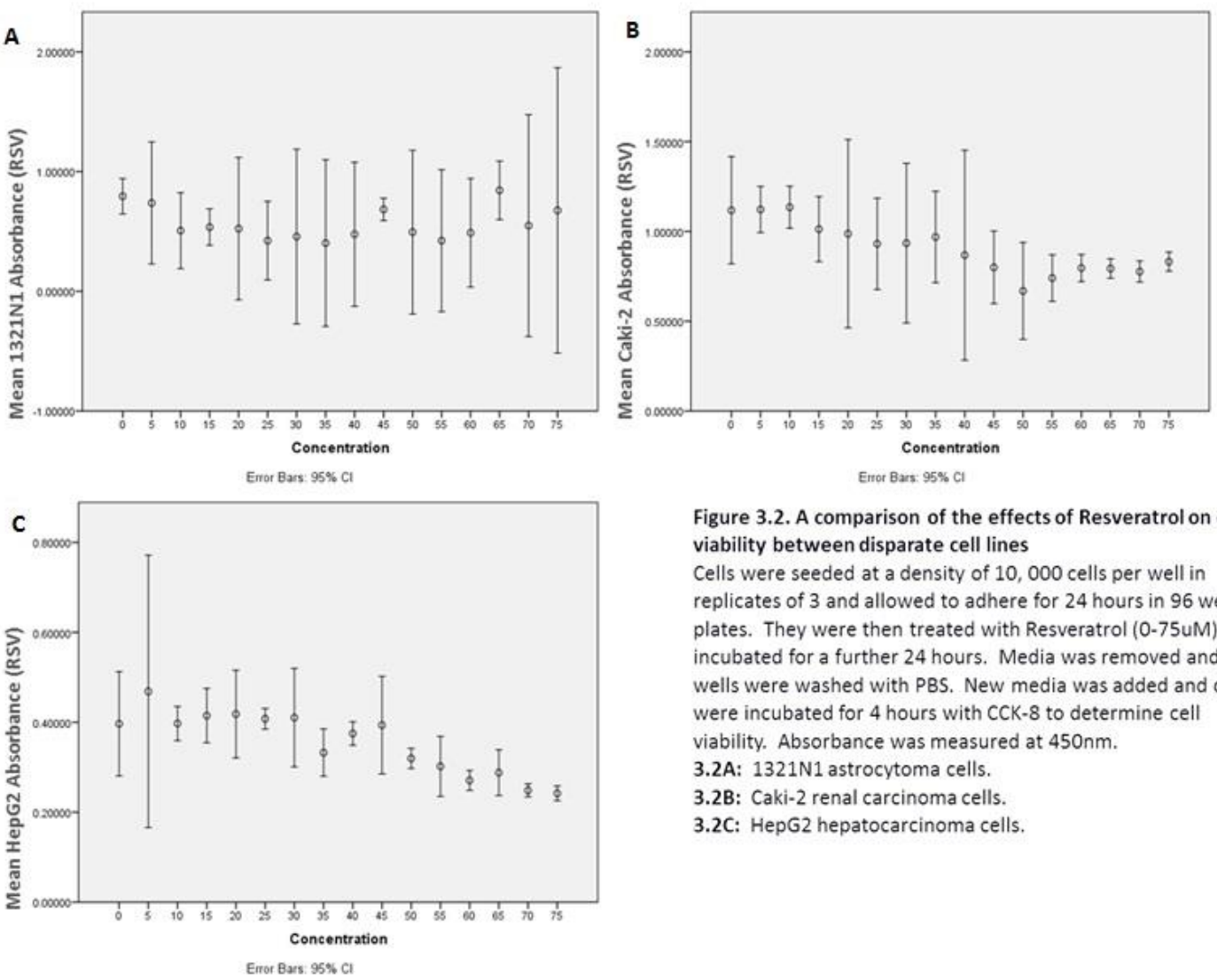


Figure 3.2. A comparison of the effects of Resveratrol on cell viability between disparate cell lines
Cells were seeded at a density of 10, 000 cells per well in replicates of 3 and allowed to adhere for 24 hours in 96 well plates. They were then treated with Resveratrol (0-75uM) and incubated for a further 24 hours. Media was removed and all wells were washed with PBS. New media was added and cells were incubated for 4 hours with CCK-8 to determine cell viability. Absorbance was measured at 450nm.
3.2A: 1321N1 astrocytoma cells.
3.2B: Caki-2 renal carcinoma cells.
3.2C: HepG2 hepatocarcinoma cells.

3.2.2.1 Discussion and interpretation of the results of investigating the effects of resveratrol on general cell viability

When comparing the effects of different concentrations of resveratrol (0-75 uM) on cell viability for the 1321N1 cell line, no statistically significant difference was observed between groups as determined by one- way ANOVA (p = < 0.464). A Tukey post hoc test also showed no statistically significant difference when comparing the untreated samples with samples treated at any of the other concentrations of resveratrol (5-75 uM). The most significant result was p = < 0.651 when comparing the 35 uM group with the 65 uM group, however as this p value is higher than 0.005 and other

samples in that data set did not show a similar pattern, this was not deemed significant. This result suggests that resveratrol treatment has only a weak effect on either the viability or rate of growth of this cell line at the concentration range used in this experiment. Referring back to questions 1 and 2 of the research aims (section 1.5.1) resveratrol does not seem to reduce cell viability in this astrocytoma cell line at the treatment range used.

Statistical analysis of the effects of resveratrol (0-75 uM) on cell viability for the Caki-2 cell line, showed a significant difference between groups as determined by one-way ANOVA ($p = < 0.000$).

The Tukey post hoc test revealed that there was a significant difference between the 5uM group and the 50 uM group ($p = .002$) and between the 10 uM group and the 50 uM group ($p = < 0.001$). Other group comparisons in the tukey test showed no significance. These results are inconclusive as there does not seem to be a clear relationship between the concentration of resveratrol and cell viability. Further analysis is therefore required to determine the exact effects of resveratrol (if any) on cell proliferation and viability and to answer questions 1 and 2 of the research aims.

The effects of resveratrol (0-75 uM) on cell viability in the HepG2 cell line revealed a statistically significant difference between groups as determined by one-way ANOVA ($p = < 0.000$). The Tukey post hoc test showed that cell viability was statistically significant when comparing the untreated group with the 75 uM group ($p = < 0.003$), although significance was almost met when comparing the untreated group with the 70 uM group. Tukey analysis also showed a significant effect between the 5 uM, 10 uM, 15 uM, 20 uM, 25 uM and 30 uM groups when compared with the 70 uM and 75 uM groups ($p = < 0.005$). This result suggests that resveratrol treatment does have a significant effect on either the viability or rate of growth of this cell line. Specifically, the results suggest that more detailed investigation into the exact effects of resveratrol on this cell line is warranted. This will be addressed further in sections 5.2-5.5 and in sections 6.5-6.9.

* See Appendix 1 for a full output of the one way ANOVA and tukey tests for the above experimental data set.

3.2.3 Results of combined berberine/resveratrol treatment on cell viability

To more thoroughly address questions 1 and 2 of the research aims and to build on the prevous sections, the phytochemicals berberine and resveratrol were tested in combination to determine if they had a greater ability to reduce cell viability when combined, than when used in isolation.

Following seeding of 96 well plates with either 1321N1, Caki-2 or HepG2 and 24hr incubation of all at cells at 370C in appropriate culture media (see materials and methods section 2.3.1) and the preparation of 1mM stock solutions of resveratrol and berberine (see materials and methods section

2.2.11); triplicate wells were inoculated with berberine at ranges of 0-35 uM in 5 uM increments together with 30 uM of resveratrol. The well plates were then allowed to incubate for 24 hours before washing of the wells with PBS and the addition of 10ul of CCK-8 in 100 ul of media to each well. Following 4 hours of further incubation, the absorbance of the wells was measured, the results of which are shown in Figure 3.3.

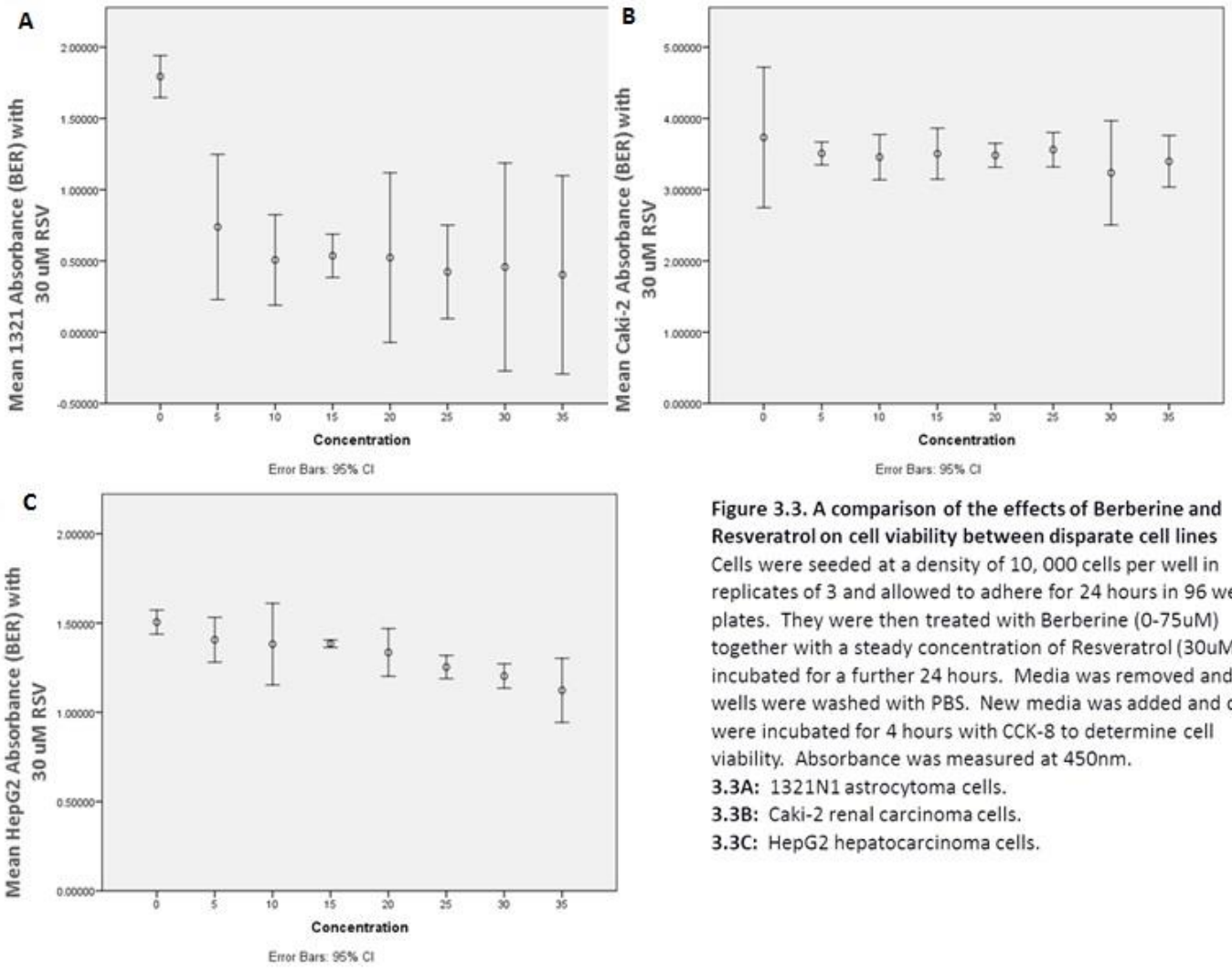


Figure 3.3. A comparison of the effects of Berberine and Resveratrol on cell viability between disparate cell lines
Cells were seeded at a density of 10, 000 cells per well in replicates of 3 and allowed to adhere for 24 hours in 96 well plates. They were then treated with Berberine (0-75uM) together with a steady concentration of Resveratrol (30uM) and incubated for a further 24 hours. Media was removed and all wells were washed with PBS. New media was added and cells were incubated for 4 hours with CCK-8 to determine cell viability. Absorbance was measured at 450nm.
3.3A: 1321N1 astrocytoma cells.
3.3B: Caki-2 renal carcinoma cells.
3.3C: HepG2 hepatocarcinoma cells.

3.2.3.1 Discussion and interpretation of the results of investigating the effects of a combined berberine/resveratrol treatment on general cell viability

When comparing the effects of different concentrations of berberine (0-35 uM) with a steady 30 uM concentration of resveratrol on cell viability for the 1321N1 cell line, there was a statistically significant difference observed between groups as determined by one-way ANOVA (p = < 0 .000). Specifically, a tukey post hoc test showed statistically significant differences between untreated groups and all other treatment groups treated (p = < 0.000). Tukey analyses between the remaining treatment groups showed no significance. This result suggests that the combined treatment has a strong effect on either the viability

or rate of growth of this cell line at concentrations as low as 5 uM. More detailed analysis is required to elucidate the exact effects of this treatment combination on this cell line. This will be performed using a more detailed cell viability assay involving flow cytometry (section 3.3).

Statistical analysis of the effects of berberine (0-35 uM) combined with 30 uM of resveratrol on cell viability for the Caki-2 cell line, showed no significant difference between groups as determined by one-way ANOVA ($p = < 0.242$). The Tukey post hoc test also showed no significant difference between any of the treatment groups. This result suggests that the combined treatment is no more effective than a single treatment on cell viability/proliferation for this cell line.

When comparing the effects of different concentrations of berberine (0-75 uM) with a steady 30 uM concentration of resveratrol on cell viability for the HepG2 cell line, there was a statistically significant difference between groups as determined by one-way ANOVA ($p = < 0.000$). The Tukey post hoc test revealed that cell viability was statistically significant when comparing the untreated group with either the 25 uM, 30 uM or 35 uM groups ($p = < 0.000$). The 35 uM treatment group when analysed for any significant difference in viability compared with the 0 uM, 5 uM, 10 uM, 15 uM and 20 uM groups also showed significance ($p = < 0.000$). This result suggests that the combined treatment has less effect on general cell viability than the berberine treatment, but is more effective than resveratrol alone.

However as the CCK-8 assay is non-specific, further analysis using is required to validate these results.

* See Appendix 1 for a full output of the one way anova and tukey tests for the above experimental data set.

3.2.4 Summary of CCK-8 data

In summary, CCK-8 analysis of 24 hour treatment with berberine at a range of 0-75uM shows a significant effect on general cell viability, shows a statistically significant effect on 1321N1, Caki-2 and the HepG2 cell lines, with the 1321N1 line showing the greatest sensitivity to berberine, followed by HepG2, with Caki-2 showing the lowest sensitivity to the compound. This differing sensitivity may be a result of the differing levels of TG2 activity in these cell lines or in an inherent differential ability to effectivily absorb/metabolise the phytochemicals. Absorbtion and metabolism of the compounds was not analysed during this project, however TG2 activity was analysed (section 4.2-4.3) in order to answer question 3 of the research aims (section 1.5.1). Resveratrol (0 uM-75 uM) had the most significant effect on viability in the Caki-2 cell line, followed by the HepG2 line, with no significant effect being observed in the 1321N1 cell line. When the subject cell lines were exposed to a combined resveratrol (30 uM) and berberine (0-35 uM) treatment for 24 hours, no

significant effect on viability was observed in Caki-2, however 1321N1 cells were highly sensitive to the combination as were HepG2 cells to a lesser extent (5 uM and 35 uM of berberine for 1321N1 and the HepG2 lines respectively).

3.3. Determination of the effects of berberine and resveratrol on apoptosis and necrosis

Following CCK-8 analyses of the initial effects of berberine and resveratrol on general cell viability, the specific effects of the compounds on apoptosis and necrosis were measured using the Annexin V/Propidium Iodide apoptosis assay (see Materials and Methods section 2.3.4). This assay was used to help to ascertain whether cells were responding to the treatments by halting the cell cycle. This work will be built on further when cell cycle analysis is specifically performed using methanol-fixed cells stained with Propidium Iodide (section 5.4) or by inducing either controlled cell death (apoptosis) or uncontrolled cell death (necrosis). The assay involves treating cells with Annexin V, a compound that attaches to the membrane-bound molecule Phosphotidyl serine and is conjugated with the green fluorescent molecule FITC. This binding to Phosphatidyl serine only occurs when PS is transferred from the inner, to the outer cell membrane as cells undergo apoptosis; at the same time as treating cells with Propidium Iodide, a red fluorescent compound that binds to DNA when the cells become permeable during late apoptosis and Necrosis. Annexin V and Propidium Iodide florescence were measured on a BDAccuri C6 flow cytometer (BD Bioscience) using the FL1 and FL3 channels(533/30 and 585/40 nm respectively).

3.3.1 Ensuring that cell populations were correctly identi fied and gated appropriately

In order to accurately measure the levels of apoptotic and necrotic cells in a given population using flow cytometry and the Annexin V/Propidium Iodide (PI) method, the cells of interest were first identified, separated from debris, and any doublets removed from measured populations. This process requires a number of steps involving gating of populations, followed by further gating of sub-populations. Without proper gating, error has the possibility of not only being included in the final results, but it can also become amplified at each subsequent step of the analysis. Gating can be considered an 'all or nothing' process of data reduction whereby cells that are included in an initial gate are analysed further in the next step, which often includes further gating. As ungated cells are not included in further data analyses steps, they therefore must be categorised correctly in early

steps to avoid inaccurate results.

In the proceeding section, the apoptosis gating strategy used in this series of experiments will be outlined using a representative data sample of HepG2 cells as an example. It should be noted however that prior to any sample being run through the flow cytometer, cells were all processed using the same preliminary steps as even small differences in harvesting techniques, centrifugation speeds/times and treatment concentrations such as incubation time with Annexin V/PI, can result in a differing in cell size or shape Any one of these variables can effect adversely the observed percentages of viable, apoptotic and necrotic cells identified. For example, if the concentration of Annexin V that cells are exposed to is increased, then so will the signal strength detected on the FL1 filter, which will move viable cells closer and in some cases into the early apoptotic gate, providing false positives.

3.3.1.1 Gating strategy for the Annexin V/PI assay

All cells in suspension with Annexin binding buffer were run through an Accuri C6 flow cytometer at a medium flow rate of 35 µL/min, with a 16 µm core and a count of 10,000 gated events was recorded for each sample. In some samples the sensitivity of the cells to berberine meant that it was not possible to record 10,000 events. In those cases, an attempt to record 5,000 events was made. This was due to a high proportion of cells bursting at the very highest concentrations of berberine. In all cases, it will be stated if the flow cytometer was unable to record 10,000 events. Once counts had been made, 3 gating steps were performed to determine the percentage of viable, early apoptotic, late apoptotic and necrotic cells in each sample (see below). Note that the initial choice of the gating parameters to use for each cell line was based upon untreated control cells that had been shown to be viable by staining with Trypan blue (See Figure 3.4).

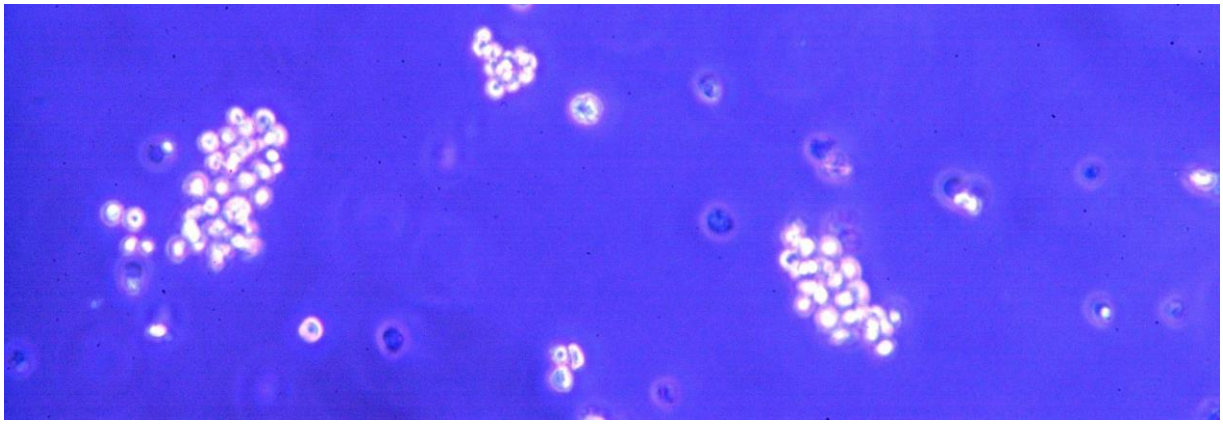


Figure 3.4. Trypan blue validation of viable cell population.

To gain a general overview of the viability of control (untreated) HepG2 cells, they were first trypsinised, then incubated with a 1:1 ratio of trypan blue buffer (see materials and methods 2.2.4.1) and DMEM cell culture media for 3

minutes at room temperature. Cells that failed to take up the dye and therefore had an intact membrane were considered viable (the white cells), whilst those that did take up the dye were considered to have lost membrane cohesion and were considered non-viable (the blue cells).

Step One. Removal of cell debris.

Once cells had been run through the flow cytometer and events had been collected, a graph was generated including all events using the FlowJo software (BD BioSciences) with FSC-A (forward scatter area) on the x axis and SSC-A (side scatter area) on the y axis. Forward scatter detectors in the flow cytometer collect light at acute angles relative to the incident beam (light scattered in the forward direction), which allows for the accurate measurement of cell size. The side scatter detectors however, measure light that interacts with cells and is scattered at right angles to the beam. The side scatter detector therefore allows cells and cellular debris to be distinguished from one another based on heterogeneity of the sample. Generating a graph of FSC-A against SSC-A is an effective strategy for separating the genuine population of cells from the cell debris (BD BioSciences). The debris can then be removed by placing a gate around the events with a larger FSC-A/SSC-A signal and using that gated population for further analysis (see Figure 3.5A).

Step Two. Deletion of Doublets and clumped cells.

Depending on the cell line being observed, cells can pass through the flow cytometer laser in doublets or even small clumps. These clumps will be misinterpreted by the forward scatter detector as single events with a large area (FSC-A) due to the fact they will take longer to pass through the laser than singlets. In the case of the Annexin V assay this can result in a stronger apoptotic or necrotic fluorescent signal being recorded than would otherwise be detected. To account for this issue, gates were generated with FSC-A on the y axis and FSC-H (forward scatter height) on the x axis.

Singlets are observed as a dense population clustered around a diagonal region between the two axis representing the singlets and discounting any cell debris. Gating of this population allows for the false signal generated by the doublets to be removed so that these events do not interfere with further analysis (see Figure 3.5B). If any doublets were present, then they would appear outide of this narrow FSC-A range due to the longer time that it takes for doublets to pass through the detector.

Step Three. Measurement of cell viability.

The final gating step is to isolate events based on their Annexin V-FITC and PI florescence. This allows for a relatively accurate measurement of the percentage of viable, early apoptotic, late

apoptotic and necrotic cells (BD BioSciences). On the y axis, FL3-A is used to detect PI and FL1-A on the x axis is used to detect Annexin staining (see Figure 3.5C).

Note: For the purposes of displaying data in a clear and concise manner, the raw flow cytometry outputs for the various treatment regimes in the remainder of this chapter and exemplars of flow cytometry outputs have been included in Appendix 2

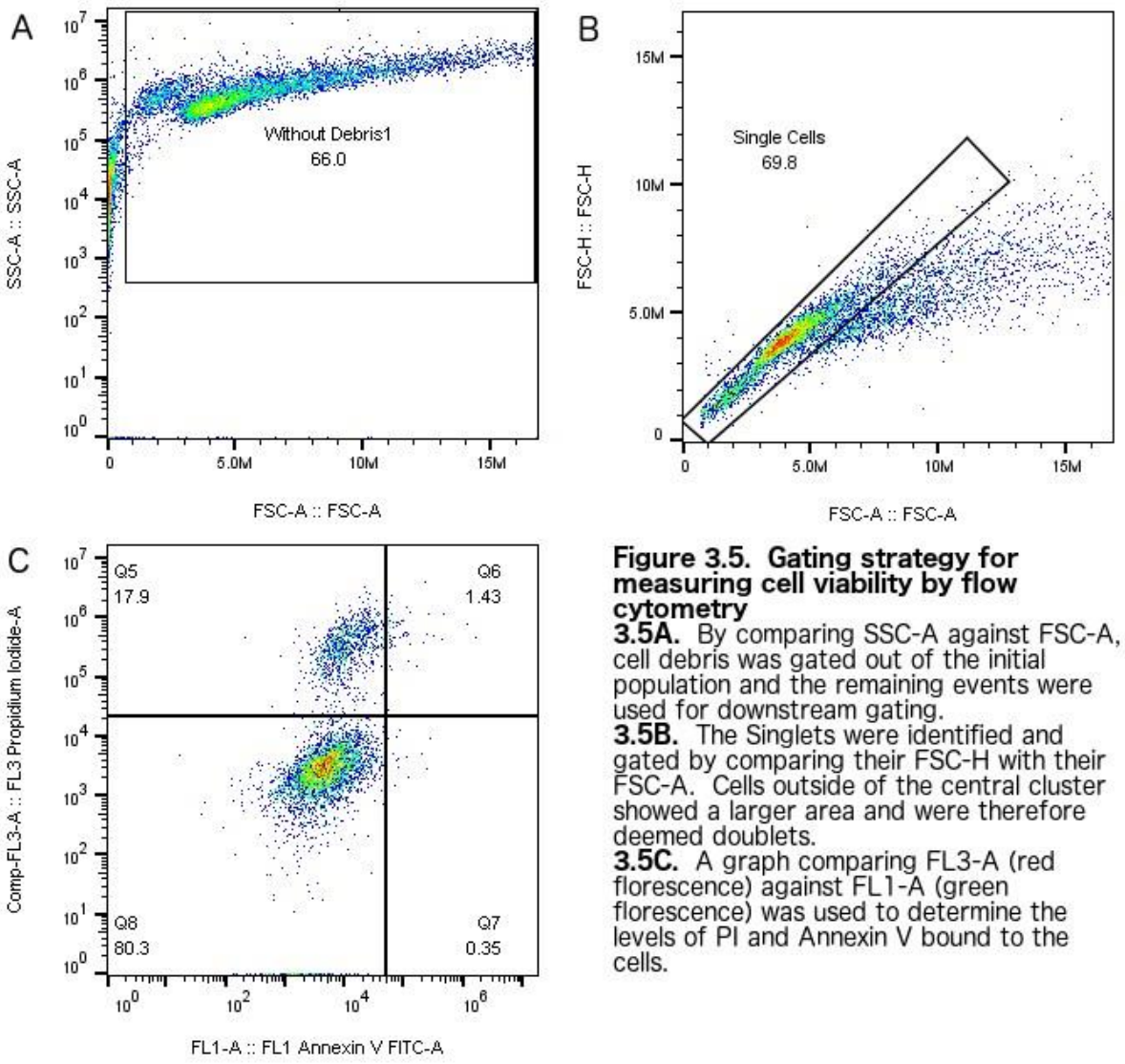


Figure 3.5. Gating strategy for measuring cell viability by flow cytometry
3.5A. By comparing SSC-A against FSC-A, cell debris was gated out of the initial population and the remaining events were used for downstream gating.
3.5B. The Singlets were identified and gated by comparing their FSC-H with their FSC-A. Cells outside of the central cluster showed a larger area and were therefore deemed doublets.
3.5C. A graph comparing FL3-A (red florescence) against FL1-A (green florescence) was used to determine the levels of PI and Annexin V bound to the cells.

3.3.2 Analyses of the effects of berberine treatment on apoptosis and necrosis in the 1321N1, Caki-2 and HepG2 cell lines

Following seeding of 1321N1, Caki-2 and HepG2 cells in T-25 flasks and the preparation of a stock 1mM solution of berberine (see materials and methods section 2.3.1.1); triplicate flasks were

inoculated for 24 hours with berberine at a range of 0-120 uM**.** The cells in the flasks were then trypsinised, washed in PBS and resuspended in Annexin binding buffer with 5ul of Annexin V-FITC and PI. Following 15 minutes incubation in a dark cupboard at room temperature, samples were run through a BD Accuri C6 flow cytometer. The results were then gated to remove any cell debris and doublets and the remaining populations were analysed for viability (Figures 3.6-3.8). Statistical analysis of the results was performed using an unpaired t-test with a p value of <0.05 or less regarded as significant.

As the 1321N1 cell line was previously found to be sensitive to berberine (see section 3.2.1 and Figures 3.1-3.3) at much lower concentrations than either Caki-2 or HepG2 cells, it was decided that a single treatment of 10 uM of berberine for 24 hours would be used to measure viability. An independent-samples t-test was conducted to compare the percentage of viable cells in an untreated control group of 1321N1 cells with cells treated with 10 uM berberine for 24 hours.

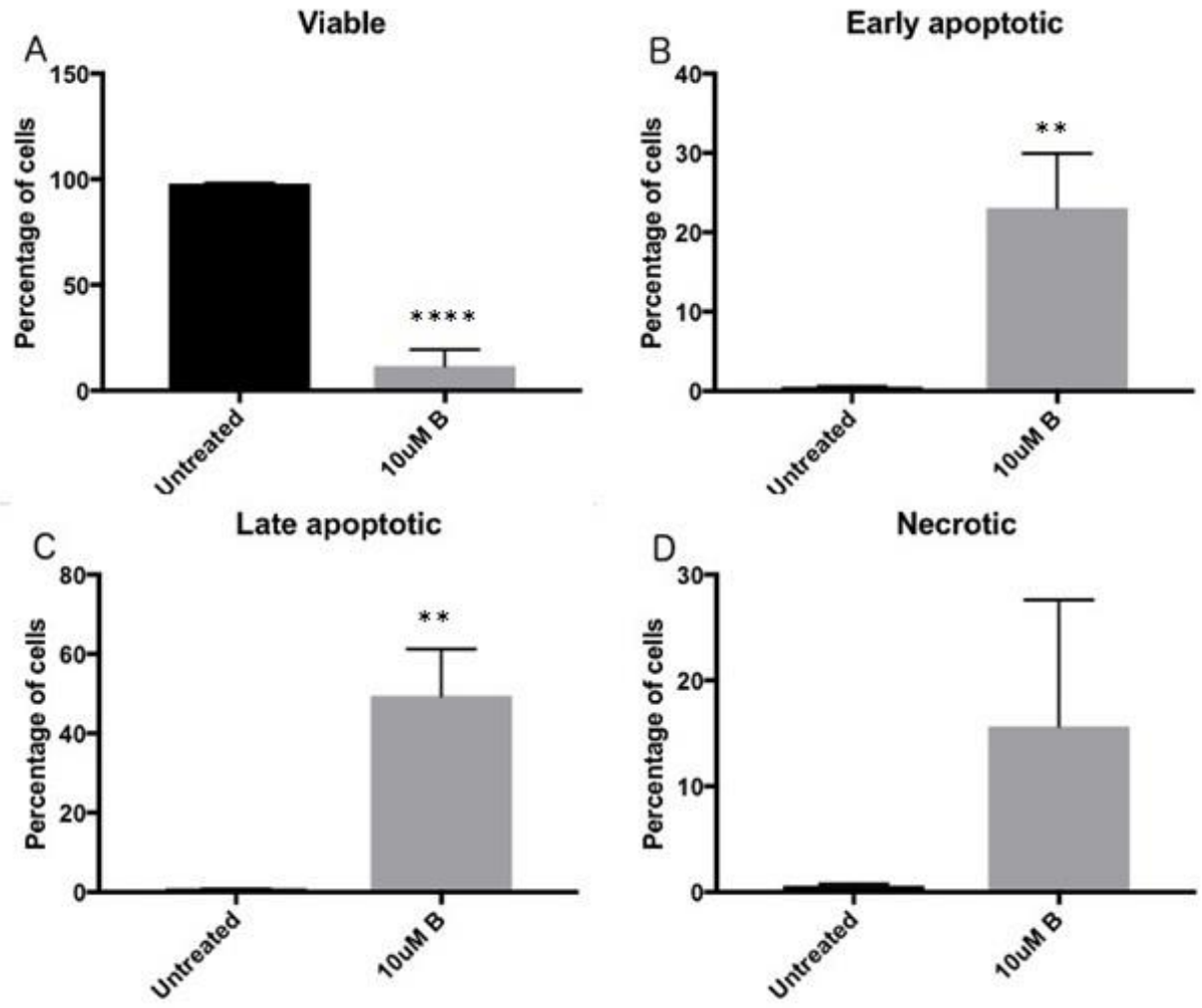


Figure 3.6. Effect of Berberine concentration on 1321N1 cell viability

This Figure shows the percentages of viable, apoptotic and necrotic 1321N1 astrocytoma cells present in triplicate T-25 flasks following 24 hours treatment with 10 uM of berberine**.**

3.6A: This graph compares the percentage of viable cells present in an untreated control group with that of a 10uM berberine-treated group.

3.6B: This graph compares the percentage of early apoptotic cells present in an untreated control group with that of a 10uM berberine-treated group.

3.6C: This graph compares the percentage of late apoptotic cells present in an untreated control group with that of a 10uM berberine-treated group.

3.6D: This graph compares the percentage of necrotic cells present in an untreated control group with that of a 10uM berberine-treated group.

Analyses of the data shown in Figure 3.6 showed that there was a significant difference in the scores for the untreated group (M=98.17, SD=0.1764) and the berberine treatment group (M=11.69, SD=4.372) conditions; t (19.77)=4, p = < 0.0001. For early apoptotic cells, t-test also revealed a significant difference between the untreated group (M=0.5267, SD=0.02728) and the berberine treatment group (M=23.1, SD=3.98) conditions; t (5.671)=4, p = < 0.0048. t-test analysis of late apoptotic cells showed significant difference in scores between the untreated group (M=0.7267,

SD=0.09135) and the berberine treatment group (M=49.5, SD=6.856) conditions; t (7.113)=4, p = < 0.0021. No significant difference was observed however between the untreated group (M=0.58, SD=0.105) and the treated group (M=15.7 SD=6.879) conditions; t (2.198)=4, p = < 0.0929 when comparing the percentage of necrotic cells. This result suggests that berberine treatment does have a highly significant effect on the viability of this cell line at a low concentration. Specifically, the results suggest that berberine induces a high level of apoptosis in 1321N1 at this dose over a 24 hour period. This answers question 1 of the research aims for 1321N1 in regards to berberine. This compound effectively reduces the viability of this astrocytoma cancer model. It also goes some way to answering question 2 of the research aims. 10 uM of berberine is an achievable dose in a clinical setting.

The Caki-2 cell line had previously been found to be highly resistant to berberine treatment (see section 3.2.1 and Figure 3.1) so the concentration range used for Flow cytometric analyses was 0-120 uM (see Figure 3.7). This is a dose range that would be difficult to reach clinically, but would help determine if berberine does have a potential at higher doses to induce cell death in this renal carcinoma model, thus helping to answer research question 2 (see section 1.5.1).

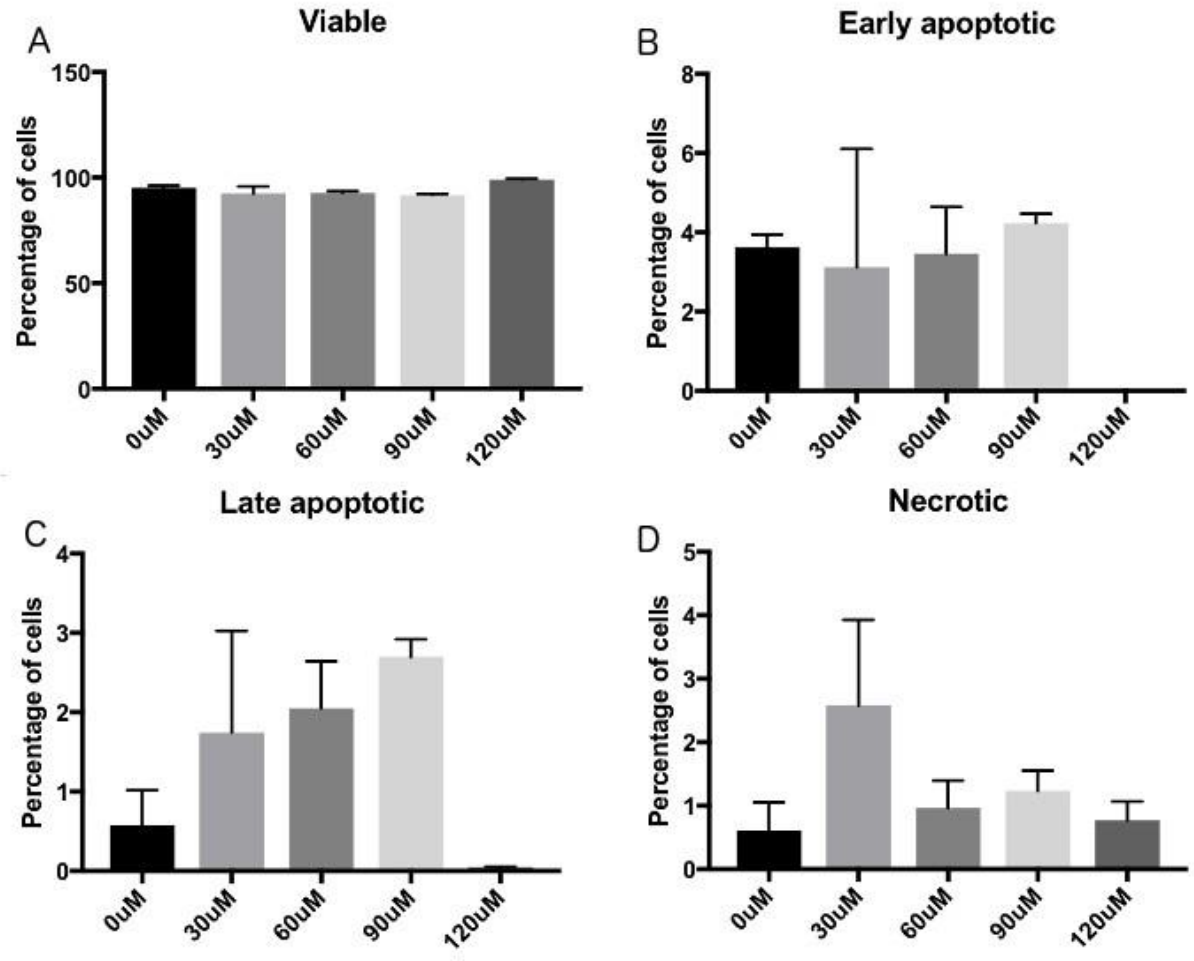


Figure 3.7. Effect of Berberine concentration on Caki-2 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic Caki-2 renal carcinoma cells present in triplicate T-25 flasks following 24 hours treatment with berberine (0 uM-120 uM).

3.7A: This graph compares the percentage of viable cells observed when treated with a concentration range of berberine.

3.7B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of berberine.

3.7C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of berberine.

3.7D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of berberine.

When comparing the effects of 24 hours treatment of berberine (0-120 uM) on the percentage of viable Caki- 2 cells, a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0018$). A Tukey post hoc test determined that significance was however only observed when comparing the 0 uM, 30 uM, 60 uM and 90 uM treatments with the

120uM treatment (p = < 0. 001). Comparisons between all other treatments showed no significance in the percentage of viable cells.

For early apoptotic percentages a statistically significant difference between the treatment groups was noted (p = < 0.0345), but this difference only became significant when comparing the 90 uM group, with the 120 uM group. The difference in the percentage of late apoptotic cells between the treatment groups was also significant (p = .0037), with Tukey showing significance when comparing the 0 uM group with the the 90 uM group and when comparing the 60 uM and 90 uM groups with the 120 uM group, but not between any other groups. When looking at the difference in the percentage of necrotic cells over the treatment range, significance was observed (p = < 0.0366). Tukey post hoc analysis identified that this significance was only present when comparing the 0 uM group with the 30 uM group. This result suggests that berberine treatment does have a significant effect on the viability of this cell line.

Specifically, the results indicate that berberine induces a fairly low level of apoptosis (up to 7%) in Caki-2 cells over a 24 hour period at a range of treatments between 0-120 uM and a small degree of necrosis (up to 3%). In response to research questions 1 and 2, berberine can reduce the viability of this renal carcinoma model, but it is indeterminate whether the doses required are clinically achievable to have a large effect on tumourgenesis.

As the HepG2 cell line had previously been observed to be more sensitive to berberine than the Caki-2 line, but has a greater sensitivity than 1321N1 (see section 3.2.1 and Figure 3.1), a smaller treatment range of 0-90 uM was used (see Figure 3.8) to determine the effects on viability of berberine treatment on this hepatocarcinoma cancer model.

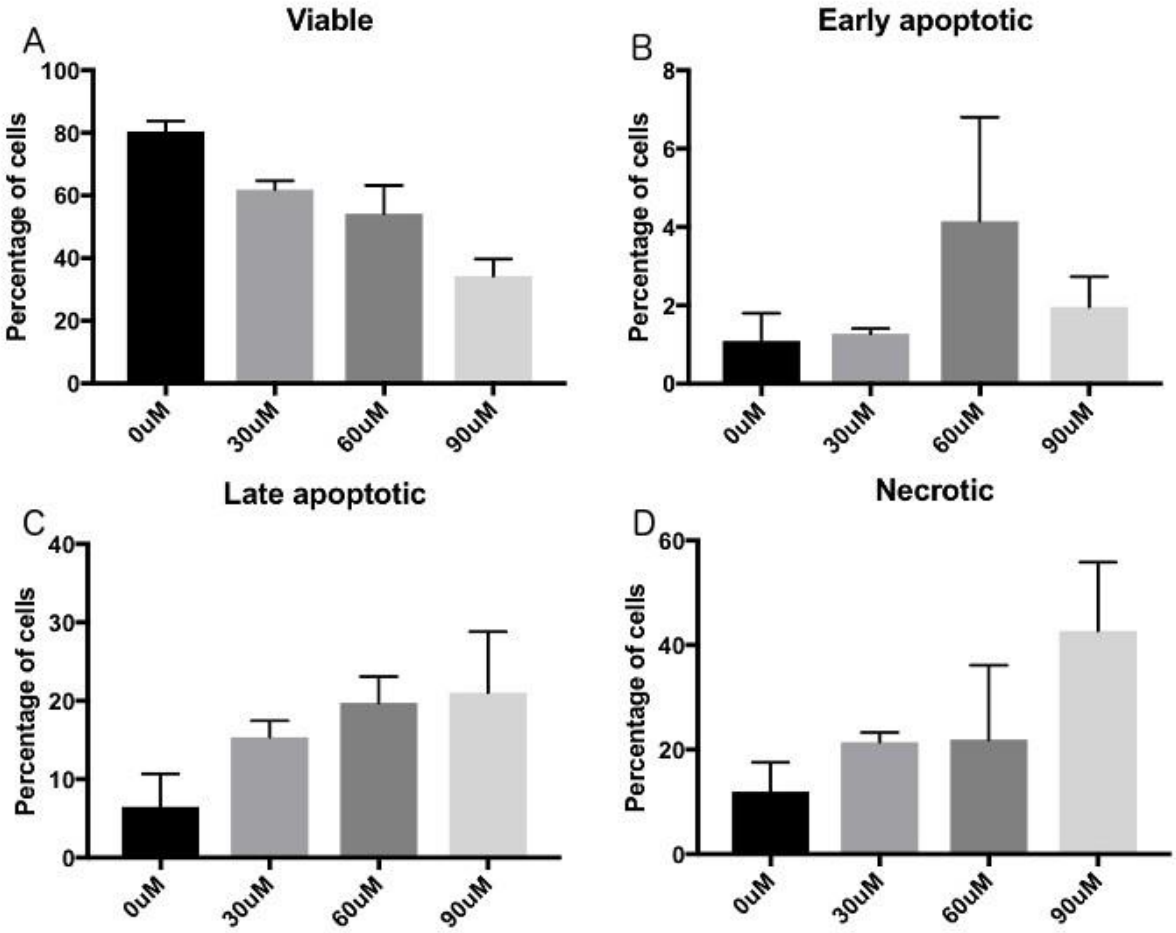


Figure 3.8. Effect of Berberine concentration on HepG2 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic HepG2 hepatocarcinoma cells present in triplicate T-25 flasks following 24 hours treatment with berberine (0 uM-90 uM).

3.8A: This graph compares the percentage of viable cells observed when treated with a concentration range of berberine.

3.8B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of berberine.

3.8C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of berberine.

3.8D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of berberine.

When comparing the effects of 24 hours treatment of berberine (0-90 uM) on the percentage of viable HepG2 cells, a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0001$). A Tukey post hoc test revealed that there was a significant difference between all groups ($p = < .001$) except when comparing the 30 uM group with the 60uM treatment group. For early apoptotic cells, ANOVA showed no significant difference between the groups ($p = < 0.1016$) and Tukey also showed no significant difference between groups. The difference in the percentage of late apoptotic cells between the treatment groups was

significant (p = < 0.0236), with Tukey post hoc showing significance when comparing the 0 uM group with the 60 uM and 90 uM groups (p = < 0.0405 and < 0.0257 respectively), but revealed no significance between other groups. When comparing the necrotic cells over the treatment range, significance was also revealed (p = < 0.0314). Tukey post hoc analysis however identified that this significance was only present when comparing the 0 uM group with the 90 uM group.

These results suggest that berberine treatment does have a significant effect on the viability of this cell line. Specifically, the results suggest that berberine induces apoptosis and secondary necrosis at concentrations of 60 uM and 90 uM over a 24 hour period and some general loss of cell viability at a dose of 30 uM. This backs up the CCK-8 results (section 3.2.1) clearly showing that berberine is effective at killing this hepatocarcinoma model at clinically-achievable doses (answering questions 1 and 2 of the research aims – 1.5.1).

3.3.3 Effects of resveratrol on apoptosis and necrosis

Following seeding of 1321N1, Caki-2 and HepG2 in T-25 flasks and the preparation of a stock 1 mM solution of resveratrol (see materials and methods section 2.3.11); triplicate flasks were inoculated for 24 hours with resveratrol at a range of 0-150 uM**.** The cells in the flasks were then trypsinised, washed in PBS and resuspended in Annexin binding buffer with 5ul of Annexin V-FITC and PI. Following 15 minutes incubation, samples were run through a BD Accuri C6 flow cytometer. The results were then gated to remove any doublets and the remaining population was analysed for viability (see Figures 3.9-3.11).

1321N1 cells had previously been shown to be highly tolerant of resveratrol (see section 3.2.2.1 and Figure 3.2A) as a result of which it was decided to treat this line with a higher concentration range of the compound than would be used on the other cell lines (maximum 150 uM).

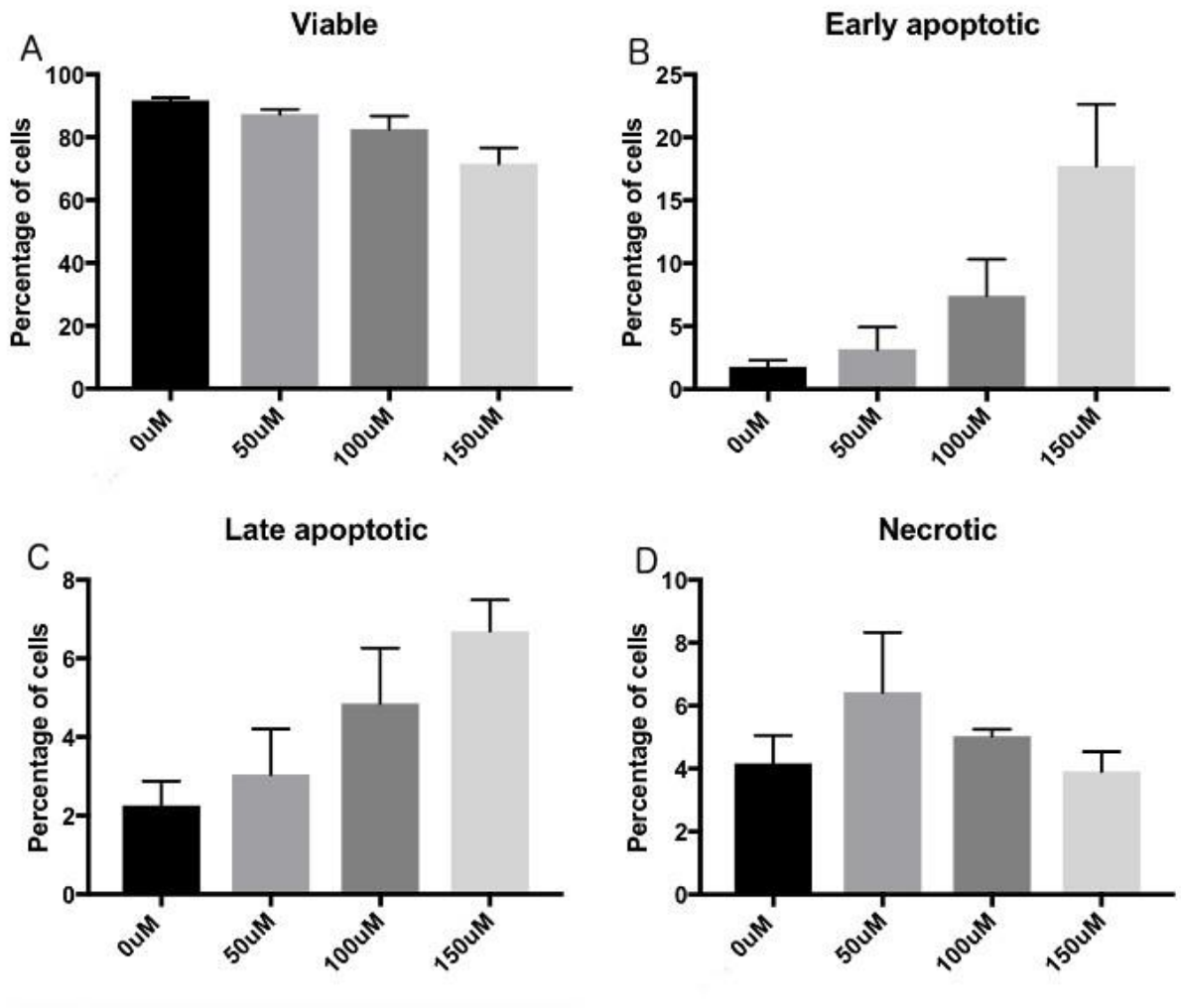


Figure 3.9. Effect of Resveratrol concentration on 1321N1 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic 1321N1 astrocytoma cells present in triplicate T-25 flasks following 24 hours treatment with resveratrol (0 uM-150 uM). Note that the Y axis percentage scale is smaller in the apoptotic and necrotic graphs in order to show the patterns more clearly.

3.9A: This graph compares the percentage of viable cells observed when treated with a concentration range of resveratrol.

3.9B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of resveratrol.

3.9C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of resveratrol.

3.9D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of resveratrol.

Statistical analysis of the effects of 24 hours treatment of resveratrol (0-150 uM) on the percentage of viable 1321N1 cells using ANOVA revealed a significant difference between groups ($p = < 0.0001$). Tukey post hoc analysis showed that there was a significant difference between mostgroups ($p = < 0.01$) except when comparing the 0 uM group with the 50 uM treatment group

and the 50 uM with the 100 uM groups. Analysis of early apoptotic cells, also showed a significant difference between the groups ($p = < 0.0001$) and Tukey post hoc revealed a significant difference between all groups ($p = < 0.05$) except when comparing the 0 uM group with the 50uM treatment group and the 50 uM with the 100 uM groups. ANOVA showed a significant difference in the percentage of late apoptotic cells between the treatment groups ($p = < 0.0001$), with Tukey revealing a significant difference when comparing all groups with each other, apart from when comparing the 0 uM group with 50 uM group, the 50 uM group with the 100 uM group and the 100 uM group with the 150 uM group. Finally, when comparing these secondary necrotic cells over the treatment range, significance was observed ($p = < 0.0099$), however Tukey only revealed significant differences between individual groups when comparing the 0 uM group with the 50 uM group and the 50 uM with the 150 uM group. These results indicate that resveratrol treatment does have a significant effect on the viability of this cell line. Specifically, the results suggest that resveratrol induces a small, but significant level of apoptosis and necrosis in 1321N1 cells over a 24 hour period of incubation at the highest concentration (120 uM).

This answers question 1 of the research aims for resveratrol-treated 1321N1's; resveratrol does induce cell death in this line. Question 2 is also answered; as the high doses of resveratrol required to induce a significant degree of cell death in this cell line would be difficult to achieve clinically, added to which only a small percentage of resveratrol (as little as 1%) is able to cross the blood/brain barrier, this phytochemical shows low clinical promise at this stage as an astrocytoma therapy.

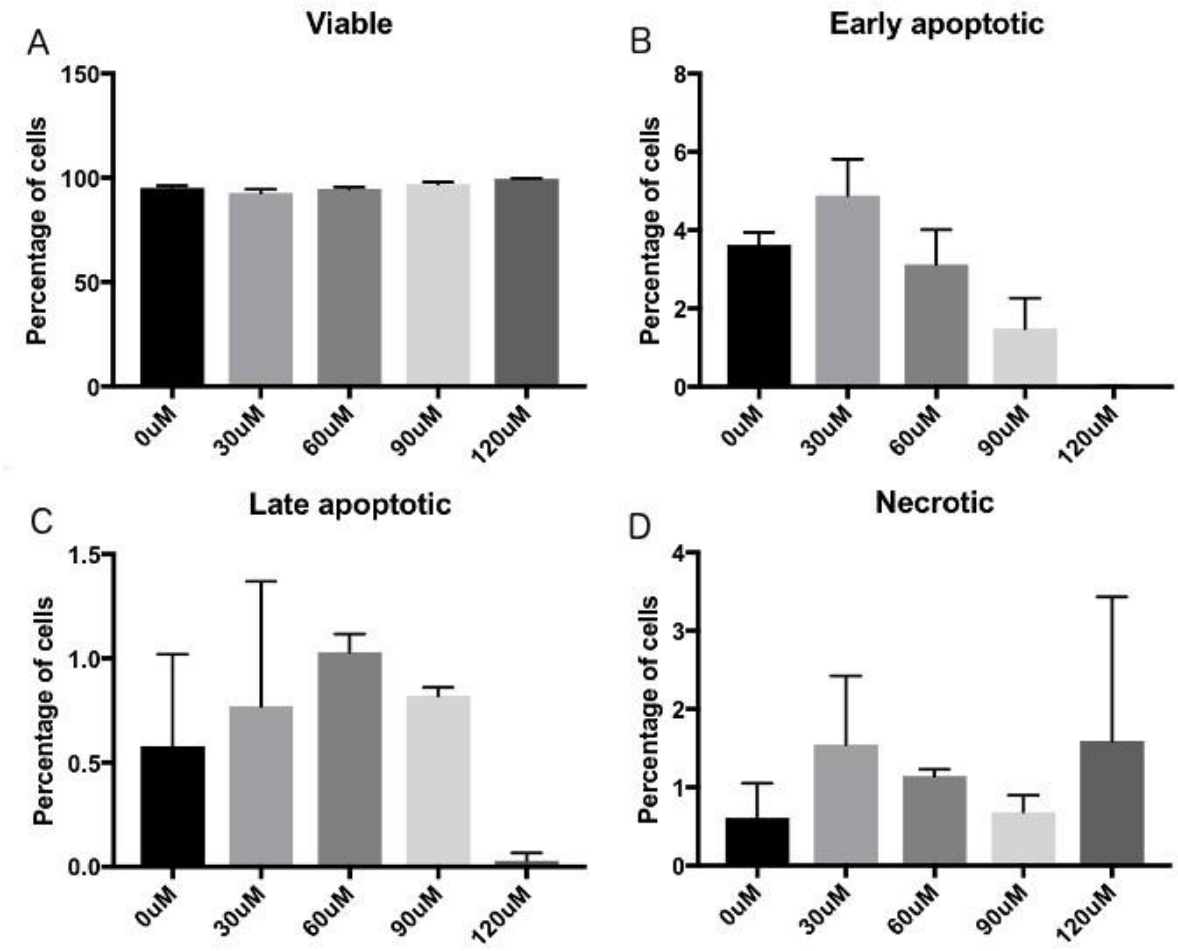


Figure 3.10. Effect of Resveratrol concentration on Caki-2 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic Caki-2 renal carcinoma cells present in triplicate T-25 flasks following 24 hours treatment with resveratrol (0 uM-120 uM).

3.10A: This graph compares the percentage of viable cells observed when treated with a concentration range of resveratrol.

3.10B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of resveratrol.

3.10C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of resveratrol.

3.10D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of resveratrol.

When comparing the effects of 24 hours treatment of resveratrol (0-120 uM) on the percentage of viable Caki-2 cells, a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0005$). A Tukey post hoc test identified specific significance when comparing the 0 uM with the 120 uM treatment group ($p = < 0.007$). Tukey post hoc also revealed significant differences between the 30 uM group compared to the 90 uM and 120 uM groups ($p = < 0.05$). A final difference in the percentage of viable cells was also noted when comparing the 60 uM group with the 120 uM group ($p = < 0.0035$). Analysis of early

apoptotic percentages revealed significant difference between the treatment groups ($p = < 0.0001$), with significant differences of $p = < 0.05$ observed when comparing the 0 uM group with the 90 uM and 120 uM groups, when comparing the 30 uM group with the 60 uM, 90 uM and 120 uM groups and when comparing the 60 uM group with the 120 uM group. The difference in the percentage of late apoptotic cells between the treatment groups was not significant ($p = <0.0590$), with Tukey post hoc only revealing a slight significance when comparing the 60 uM with the 120 uM treatment group ($p = < 0.0430$). When looking at the difference in the percentage of necrotic cells over the treatment range, no significance was observed ($p = < 0.6684$). Tukey post hoc analysis also showed no significance when directly comparing the effects of resveratrol on the percentage of necrotic cells between any of the treatment groups. Analysis of these results suggest that resveratrol treatment does have a significant (although small) effect on the viability of this cell line. Specifically, the results suggest that resveratrol induces a 'very' low level of apoptosis in Caki-2 cells over a 24 hour period.

Note: For such a close result (although significant) it can be observed that resveratrol has little effect on viability, which goes some way towards answering questions 1 and 2 of the research aims, showing that this phytochemical does not have a strong toxic effect on Caki-2 (and therefore possibly renal carcinoma's generally).

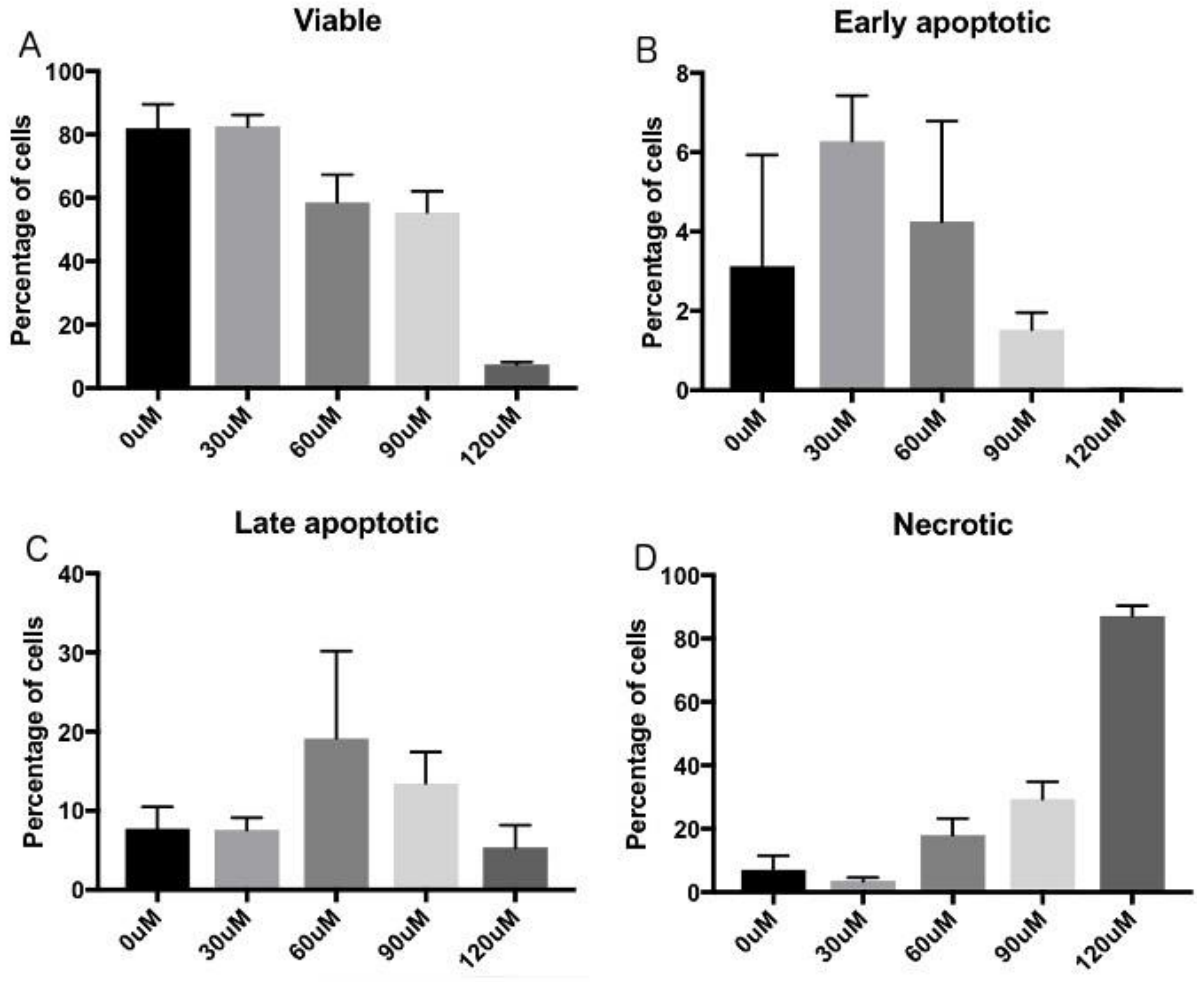


Figure 3.11. Effect of Resveratrol concentration on HepG2 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic HepG2 hepatocarcinoma cells present in triplicate T-25 flasks following 24 hours treatment with resveratrol (0 uM-120 uM).

3.11A: This graph compares the percentage of viable cells observed when treated with a concentration range of resveratrol.

3.11B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of resveratrol.

3.11C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of resveratrol.

3.11D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of resveratrol.

When comparing the effects of 24 hours treatment of resveratrol (0-120 uM) on the percentage of viable HepG2 cells, a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0001$). Tukey post hoc analysis revealed that there was a significant difference between all groups ($p = < 0.006$) except when comparing the 0 uM group with the 30 uM treatment group and between the 60 uM and 90 uM groups. For early apoptotic cells,

ANOVA showed significant difference between the groups ($p = < 0.0127$), with Tukey revealing however that a significant difference only existed when comparing the 30 uM treatment group with the 120 uM group ($p = < 0.0103$). No significant difference was observed in the percentage of late apoptotic cells between the treatment groups ($p = < 0.0700$). When checking for significance in the percentage of necrotic cells between groups, significance was observed ($p = < 0.0001$) and Tukey revealed that this significance was present when comparing any treatment group with any other group. Analysis of these results suggest that resveratrol treatment does have a significant effect on the viability of this cell line. Specifically, the results show that resveratrol induces both apoptosis and necrosis in HepG2 cells over a 24 hour period.

This answers question 1 of the research aims for resveratrol-treated HepG2's; resveratrol does induce cell death in this line. Question 2 however is less clearly answered at this stage as the high doses of resveratrol required to induce a significant degree of cell death in this cell line would be difficult to achieve clinically.

3.3.4 Effects of resveratrol and berberine on apoptosis and necrosis

Based on the results obtained in the previous 2 sections and to follow up on the CCK-8 results (section 3.2), it was decided to treat Caki-2 and HepG2 cells with a combined resveratrol/berberine treatment.

*** Note.** Based on the results illustrated in Figures 3.1-3.3 and Figures 3.6 and 3.9 it was decided not to examine the effects of a combined berberine/resveratrol treatment on the 1321N1 cell line. Further reasoning behind this decision included consideration of the fact that whilst resveratrol does have a limited capacity to pass through the blood/brain barrier, with brain tissue being previously observed one hour following iv administration into the blood of resveratrol to contain 6% of that found in blood plasma (Wang *et al.,* 2002), this cell lines low sensitivity to resveratrol and the fairly low bioavailability in the brain meant that it would potentially prove more difficult to use this drug to treat astrocytoma clinically than either renal or hepatocarcinoma. As a result of these observations, further experiments did not include the 1321N1 cell line in this project.

Following seeding of Caki-2 and HepG2 in T-25 flasks and the preparation of stock 1 mM solutions of resveratrol and berberine (see materials and methods section 2.3.11); triplicate flasks were inoculated for 24 hours with 30 uM of resveratrol and either 30 uM, 60 uM, 90 uM or 120 uM of berberine. The cells in the flasks were then trypsinised, washed in PBS and resuspended in Annexin binding buffer with 5ul of Annexin V and PI. Following 15 minutes incubation, samples were run through a BD Accuri C6 flow cytometer. The results were then gated to remove any doublets and the remaining population was analysed for viability (see Figures 3.12-3.13).

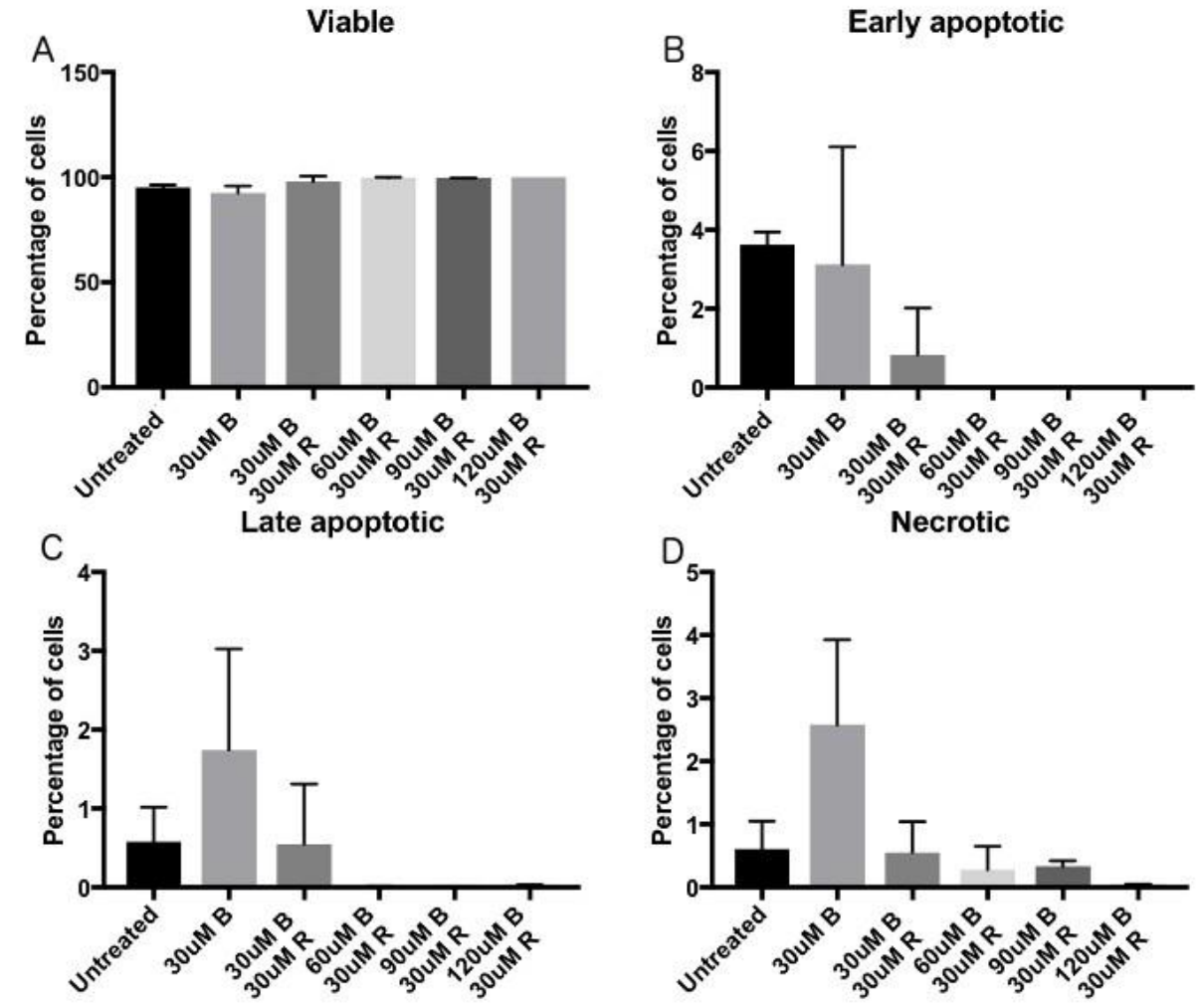


Figure 3.12. Effect of Berberine/Resveratrol combined treatment on Caki-2 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic Caki-2 renal carcinoma cells present in triplicate T-25 flasks following 24 hours treatment with 30 uM resveratrol (30 uM R) and berberine (0 uM-120 uM).

3.12A: This graph compares the percentage of viable cells observed when treated with a concentration range of berberine in the presence or resveratrol.

3.12B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of berberine in the presence or resveratrol.

3.12C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of berberine in the presence or resveratrol.

3.12D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of berberine in the presence or resveratrol.

One-way ANOVA analysis comparing the effects of 24 hours treatment of 30 uM of resveratrol in combination with berberine (0-120 uM) on the percentage of viable Caki-2 cells revealed a statistically significant difference between groups ($p = < 0.0026$). Tukey post hoc analysis showed

that a significant difference was only observed however when comparing the 30 uM berberine (30 uM B) treatment group with the 60 uM B/30 uM R (resveratrol) group and when comparing the 30 uM B group with the 90 uM B/30 uM R and the 120 uM B/30 uM R groups ($p = < 0.0059$). For early apoptotic cells, ANOVA revealed a significant difference in the data set ($p = < 0.0266$), Tukey analysis failed however to show a specific significant difference between any individual groups. When comparing the late apoptotic percentages, a comparison of the groups showed a significant difference ($p = < 0.0607$), with Tukey post hoc analysis once again failing to reveal any significant difference when comparing individual treatment groups. When checking for significant difference,the percentage of necrotic cells, significance was observed $p = < 0.0114$) with Tukey in this case revealing significance when comparing the untreated group with the 30 uM B group ($p = < 0.0429$) and when comparing the 30uM B group with the 60uM B/30uMR, 90uM B./0uM R and 120uM B/30uM R groups ($p = < 0.0203$). These results clearly show that the combined therapy has only a small effect on apoptosis/necrosis in Caki-2 cells (up to approximately 4%), thus backing up the data collected using the CCK-8 assay (see section 3.2.3).

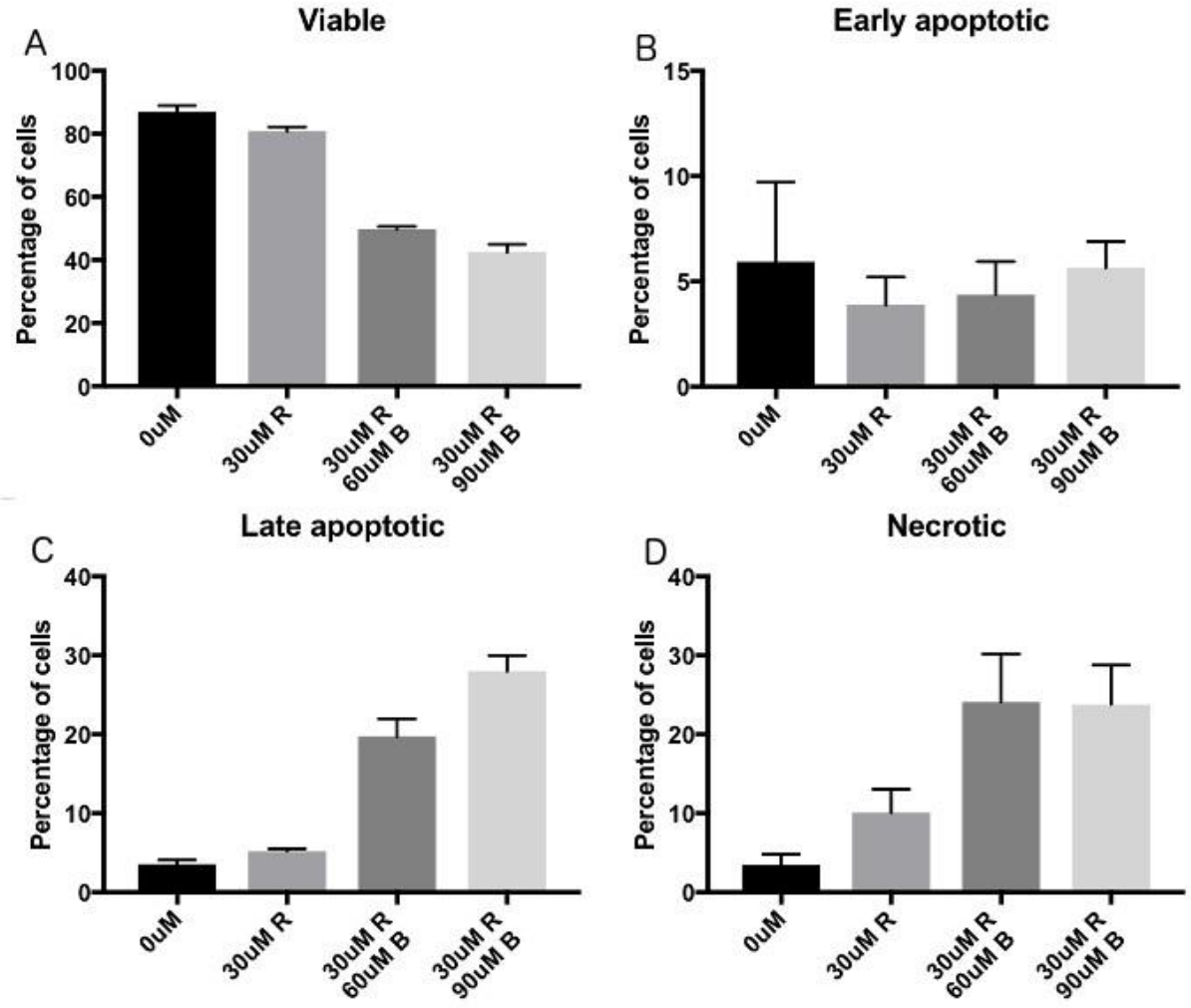


Figure 3.13. Effect of Berberine/Resveratrol combined treatment on HepG2 cell viability

Flow cytometry analysis of the percentages of viable, apoptotic and necrotic HepG2 hepatocarcinoma cells present in triplicate T-25 flasks following 24 hours treatment with 30 uM resveratrol (30 uM R) and berberine (0 uM-90 uM).

3.13A: This graph compares the percentage of viable cells observed when treated with a concentration range of berberine in the presence or resveratrol.

3.13B: This graph compares the percentage of early apoptotic cells observed when treated with a concentration range of berberine in the presence or resveratrol.

3.13C: This graph compares the percentage of late apoptotic cells observed when treated with a concentration range of berberine in the presence or resveratrol.

3.13D: This graph compares the percentage of necrotic cells observed when treated with a concentration range of berberine in the presence or resveratrol.

When comparing the effects of 24 hours treatment of 30 uM of resveratrol in combination with berberine (0- 90 uM) on the percentage of viable HepG2 cells (Figure 3.13), a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0001$). Tukey post hoc analysis revealed that there was a significant difference between all

groups (p = < 0.03). For early apoptotic cells, anova revealed no significant difference between the groups (p = 0.7184), with tukey also showing no significant difference between groups. For late apoptosis, a comparison of the groups showed a significant difference (p = <0 .0001), with tukey showing a significant difference when comparing all treatment groups, except when comparing the 0uM control group with the 30 uM resveratrol group (p = < 0.0020). When checking for significance in the percentage of necrotic cells between groups, significance was observed (p = 0.0017) and tukey revealed that this significance was present when comparing all treatment groups, except when comparing the 0 uM group with the 30 uM resveratrol group and when comparing the 30 uM resveratrol group with the 30 uM resveratrol/90 uM berberine group. This result suggests that the combined resveratrol/berberine treatment does have a significant effect on the viability of the HepG2 cell line.

3.3.4.1. T-test analyses of combined resveratrol/berberine treatment on apoptosis and necrosis

To help to elucidate the exact effects of the combined resveratrol/berberine ttreatment on apoptosis and necrosis, flow cytometry analysis using the Annexin V/PI protocol was conducted using the Caki-2 and HepG2 cell lines following 24 hours incubation with 30uM of resveratrol in combination with 30uM of berberine (see Figures 3.14-3.15). The results were further analyses using independent sample t-tests.

The decision to use t-tests for these analysis is due to the fact that only 2 cocentrations were compared, and it was believed that this test would be a more robust statistical test to use under these conditions.

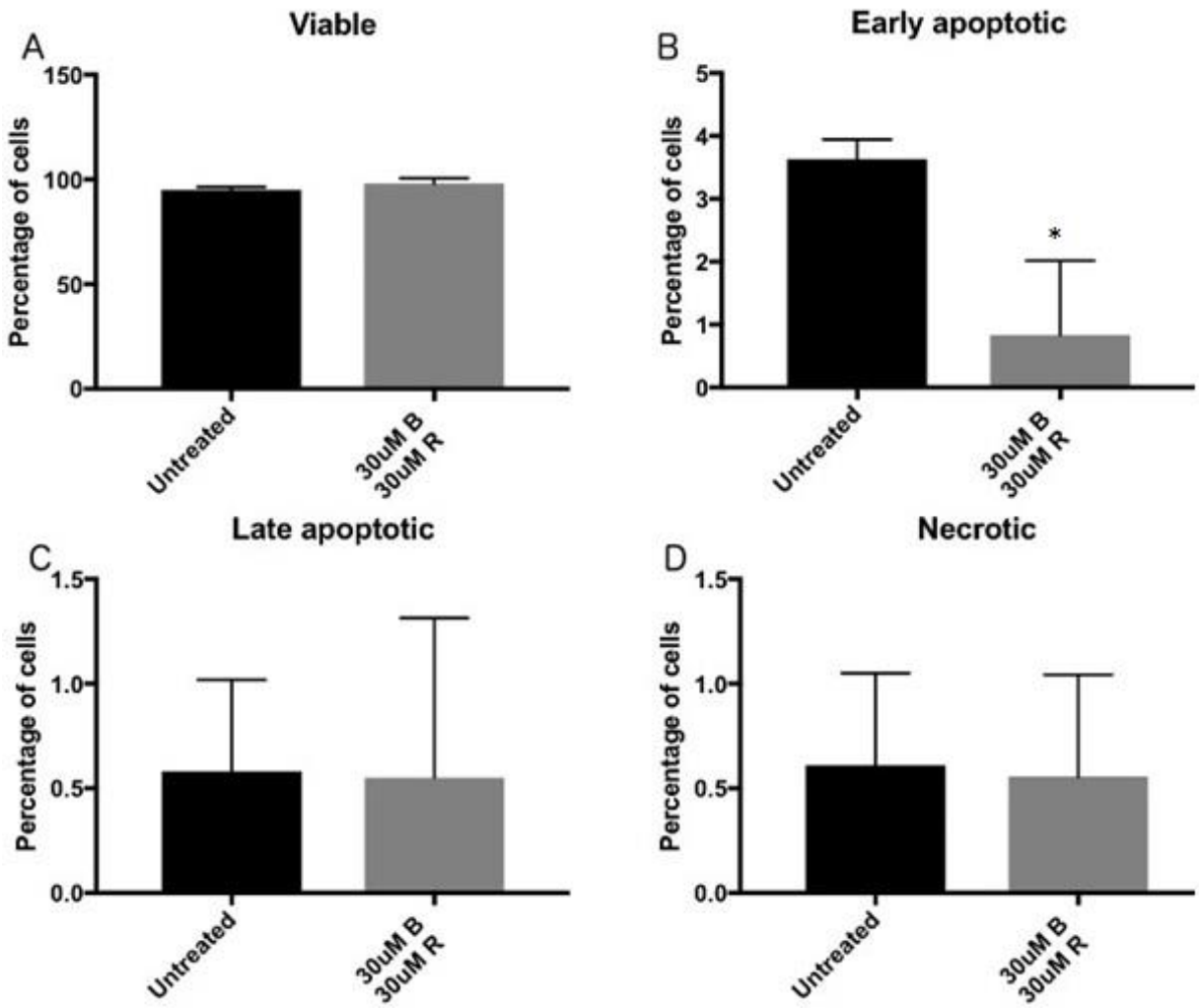


Figure 3.14. A single-dose direct comparison of the effects of Berberine/Resveratrol combined treatment on Caki-2 cell viability

A comparison of the effects of 30uM berberine in combination with 30 uM of resveratrol on apoptosis and necrosis in the Caki-2 cell line.

3.14A: This graph compares the percentage of viable cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

3.14B: This graph compares the percentage of early apoptotic cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

3.14C: This graph compares the percentage of late apoptotic cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

3.14D: This graph compares the percentage of necrotic cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

An independent-samples t-test was conducted to compare the percentage of viable cells in an untreated control group of Caki-2 cells with cells treated with 30 uM berberine and 30 uM resveratrol for 24 hours. There was no significant difference in the scores for the untreated group (M=95.2, SD=0.6429) and the berberine/resveratrol treatment group (M=98.05, SD=1.75)

conditions; t (1.843)=3, p = .1625. For early apoptotic cells, t-test revealed a significant difference between the untreated group (M=3.63, SD=0.1833) and the berberine/resveratrol treatment group (M=0.835, SD=0.835) conditions; t (4.198)=3, p = < 0.0247. t-test analysis of late apoptotic cells revealed no significant difference in scores between the untreated group (M=0.581, SD=0.2532) and the berberine/resveratrol treatment group (M=0.5515, SD=0.5385) conditions; t (0.05699)=3, p = < 0.9581. Finally, for necrotic cells, t-test revealed no significant difference in scores between the untreated group (M=0.61, SD=0.2542) and the berberine/resveratrol treatment group (M=0.555, SD=0.345) conditions; t (0.1319=3, p = < 0.9034. Although a significant difference was observed when comparing the levels of early apoptotic cells between the untreated group and the treated group, the levels of cell death are low, which is in agreement with the results observed when testing this treatment combination using the CCK-8 assay; and indicate a low susceptibility to apoptosis/necrosis in Caki-2 cells for this treatment regime (see section 3.2 and Figures 3.1-3.3).

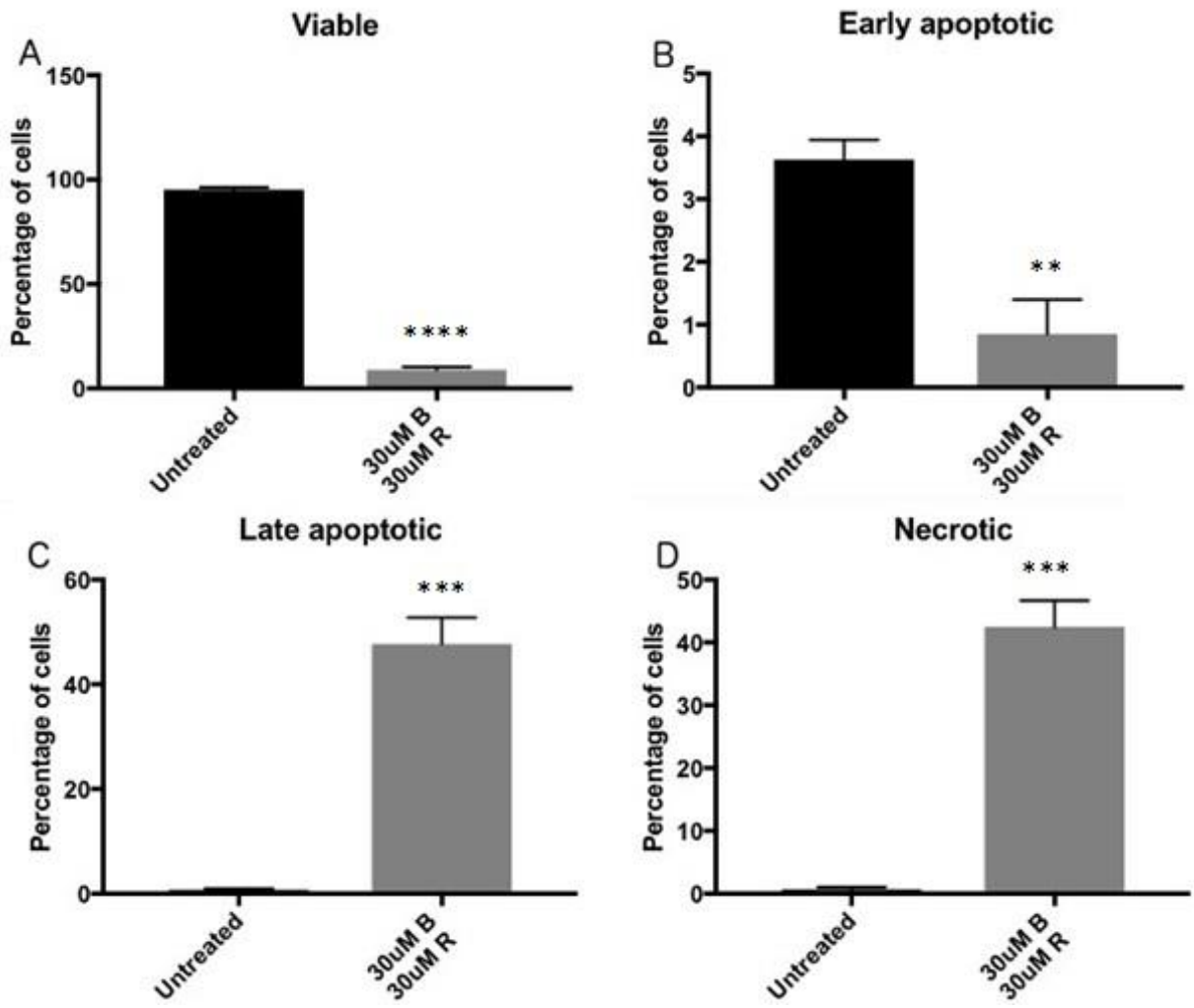


Figure 3.15. A single-dose direct comparison of the effects of Berberine/Resveratrol combined treatment on HepG2 cell viability

A comparison of the effects of 30 uM berberine in combination with 30 uM of resveratrol on apoptosis and necrosis in the HepG2 cell line.

3.15A: This graph compares the percentage of viable cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

3.15B: This graph compares the percentage of early apoptotic cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

3.15C: This graph compares the percentage of late apoptotic cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

3.15D: This graph compares the percentage of necrotic cells observed when treated with a combination of 30 uM of berberine and 30 uM of resveratrol.

An independent-samples t-test was conducted to compare the percentage of viable cells in an untreated control group of HepG2 cells with cells treated with 30uM berberine and 30 uM resveratrol for 24 hours. There was a significant difference in the scores between the untreated

group (M=95.2, SD=0.6429) and the berberine/resveratrol treatment group (M=8.9, SD=0.96) conditions; t (78)=3, p = < 0.0001. For early apoptotic cells, t-test also revealed a significant difference between the untreated group (M=3.63, SD=0.1833) and the berberine/resveratrol treatment group (M=0.855, SD=0.385) conditions; t (7.461)=3, p = < 0.0050. t-test analysis of late apoptotic cells showed significant difference in scores between the untreated group (M=0.581, SD=0.2532) and the berberine/resveratrol treatment group (M=47.75, SD=3.55) conditions; t (17.69)=3, p = < 0.0004. Finally, there was revealed a significant difference between the untreated group M=0.61, SD=0.2542) and treated group (M=42.5 SD=3) conditions; t (18.54)=3, p = < 0.0003 when comparing the percentage of necrotic cells. These results suggest that a combined berberine/resveratrol (30uMB/30uMR) treatment has a significant and strong effect on both apoptosis and necrosis and that this treatment regime is a more effective inducer of cell death than either resveratrol or berberine alone.

3.4. Summary

Both resveratrol and berberine have differing biovalabilities and pharmakokinetics, with resveratrol in particular having a short halve life *in vivo* of usually no more than an hour or so (Zhang, 2013). The final metabolism of both compounds takes place in the liver, resulting in an accumulation of the phytochemicals in hepatocytes *in vivo*, an observation that increases the potential of using the HepG2 cell line as a model system. Conversely, the relatively poor availability of the compounds elsewhere in the body (particularly in tissues protected by the blood/brain barrier) and the lack of sensitivity of the 1321N1 cell line to resveratrol indicates that 1321N1 has not proven to be a good model system for further analysis. The Caki-2 cell line shows some response to the compounds and as a potential clinical model is not blocked from the phytochemicals by the blood-brain barrier, so further analysis of this model would provide a useful comparitor for results generated with the HepG2 line.

The results outlined in this chapter taken together indicate that berberine and resveratrol have very differing effects on cell viability and that these effects are cell line specific. This could be due to the levels of TG2 activity or sensitivity between these cell lines. This will be discussed further in sections 4.2 and 4.3. To develop the model system further, only the cell lines that had responded most effectively to the compounds at ‘clinically achievable’ dosses were taken forward for further analyses. Based on these results, the HepG2 cell line was chosen to analyse further in order to develop the model system; and to later evaluate any relationship between its sensitivity to apoptosis/necrosis and TG2 expression/activity (see chapter 4). It was also decided on balance to

investigate the Caki-2 cell line further, as although it had proven that only a low level of apoptosis/necrosis was induced in this line by resveratrol and berberine, further analysis would still be required to determine the effects of the phytochemicals on cell migration.

Chapter Four
The results of analysing the relationship between transglutaminase 2 (TG2) and cell viability

4.1. Introduction

During chapter 3, the 1321N1 cell line was eliminated as a potential model cell line for determining the relationship between apoptosis/necrosis and the expression/activity of transglutaminase 2 (TG2). This was due to the poor bioavailability of resveratrol in the CSF, combined with the lack of sensitivity of this astrocytoma cell line to resveratrol. This leaves, from the initially evaluated cell lines, Caki-2 and HepG2's as potential models. Both of these cell lines exhibited differing sensitivities to berberine and resveratrol, with HepG2 showing a much greater sensitivity than Caki-2 to berberine, and neither cell line proving sensitive to resveratrol at the concentration range used.

To explore the relationship between the phytochemicals and transglutaminase 2, TG2 enzyme activity was measured using flow cytometry followed by an enzyme inhibition assay (see section 2.3.9). The results would help to elucidate if modulation of TG2 was relevant in modulating the processes involved in apoptosis/necrosis in either the TG2 high-expressing HepG2 or the low-expressing Caki-2 cell lines.

Note: For the purposes of displaying data in a clear and concise manner, the raw flow cytometry outputs for the various treatment regimes in the remainder of this chapter and exemplars of flow cytometry outputs have been included in Appendix 3

4.2. Determination of TG2 enzyme activity

Monodansylcadaverine (MDC) is non-toxic competitive auto-fluorescent substrate for TG2, that can be detected at a wavelength of 533nm using the FL1 filter[533/30nm] on a BD Accuri C6 flow cytometer. Cells were subjected to treatment with either berberine, resveratrol or a combination of berberine and resveratrol in the presence of MDC (60uM or 10uM). Enzymatic activity was measured using FL1-H based on changes in the relative fluorescent units (RFU's).
Prior to analysis, cells were gated to remove debris and doublets (see Figure 4.1). Statistical analysis of the results was performed using one-way ANOVA, unpaired t-test and Tukey for post

hoc analysis. A p value of .05 or less was regarded as significant.

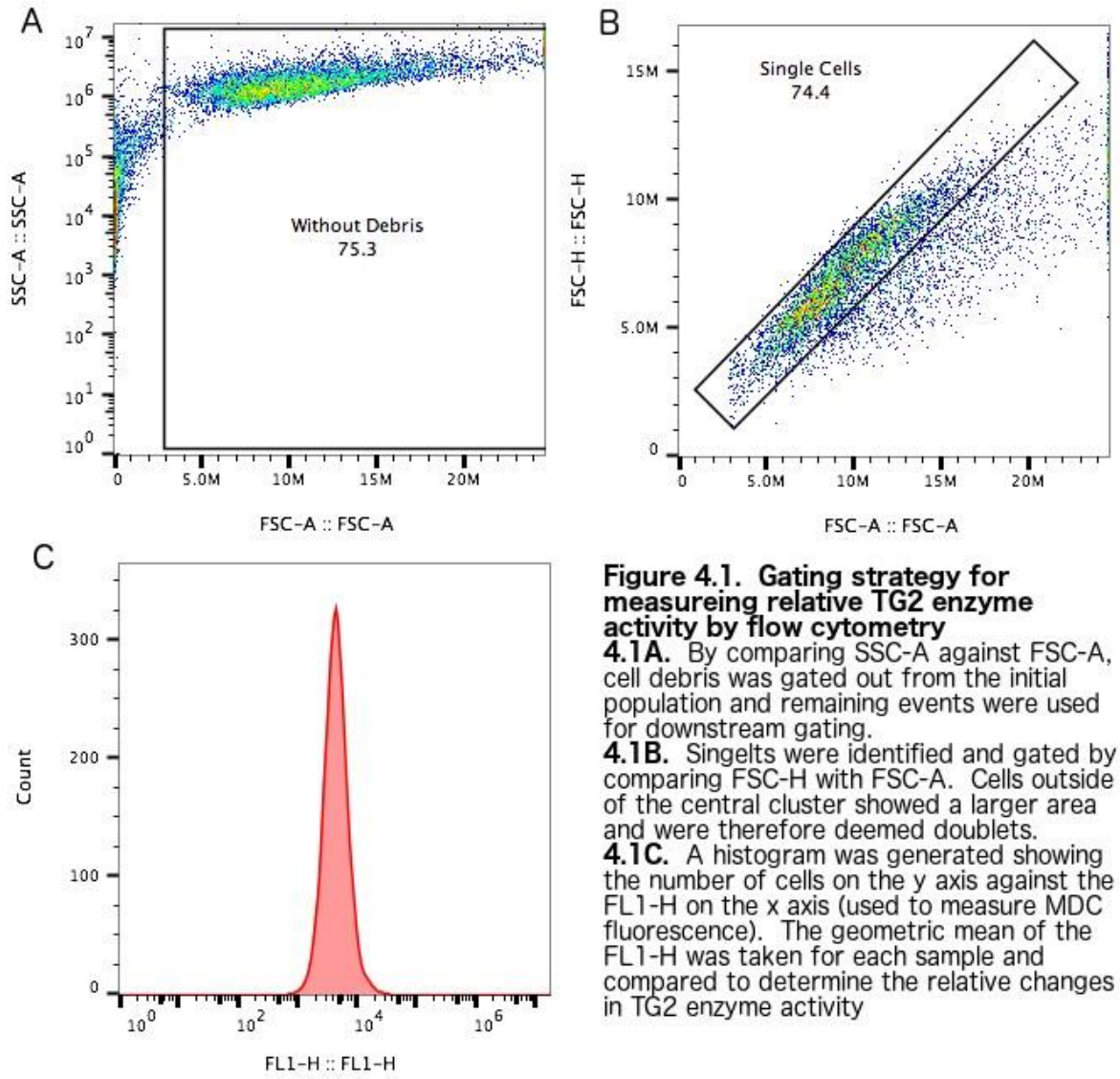


Figure 4.1. Gating strategy for measureing relative TG2 enzyme activity by flow cytometry
4.1A. By comparing SSC-A against FSC-A, cell debris was gated out from the initial population and remaining events were used for downstream gating.
4.1B. Singelts were identified and gated by comparing FSC-H with FSC-A. Cells outside of the central cluster showed a larger area and were therefore deemed doublets.
4.1C. A histogram was generated showing the number of cells on the y axis against the FL1-H on the x axis (used to measure MDC fluorescence). The geometric mean of the FL1-H was taken for each sample and compared to determine the relative changes in TG2 enzyme activity

4.2.1 Effects of berberine and resveratrol on TG2 activity in Caki-2 cells

Following seeding of Caki-2 cells in T-25 flasks and the preparation of 1mM stock solutions of berberine, resveratrol and monodansylcadaverine (MDC) (see materials and methods section 2.3.9); triplicate flasks were inoculated with either berberine (30 uM), resveratrol (30 uM), a combined 30 uM B/20 uM R or combined 20 uM B/30 uM R, together with 60 uM of MDC**.** After 24 hours of incubation, cells were trypsinised, washed in PBS and resuspended in 1ml of PBS. All samples were then run through a BD Accuri C6 flow cytometer at a medium flow rate of 35L /min, with a 16m core and a count of 10,000 singlet events was recorded for each sample. The results were then gated to remove any cell debris and doublets and the remaining populations were analysed for viability (Figure 4.2). The choice to use these concentrations of berberine and resveratrol was based on the results of the toxicity assays performed in Chapter 3 of this thesis.

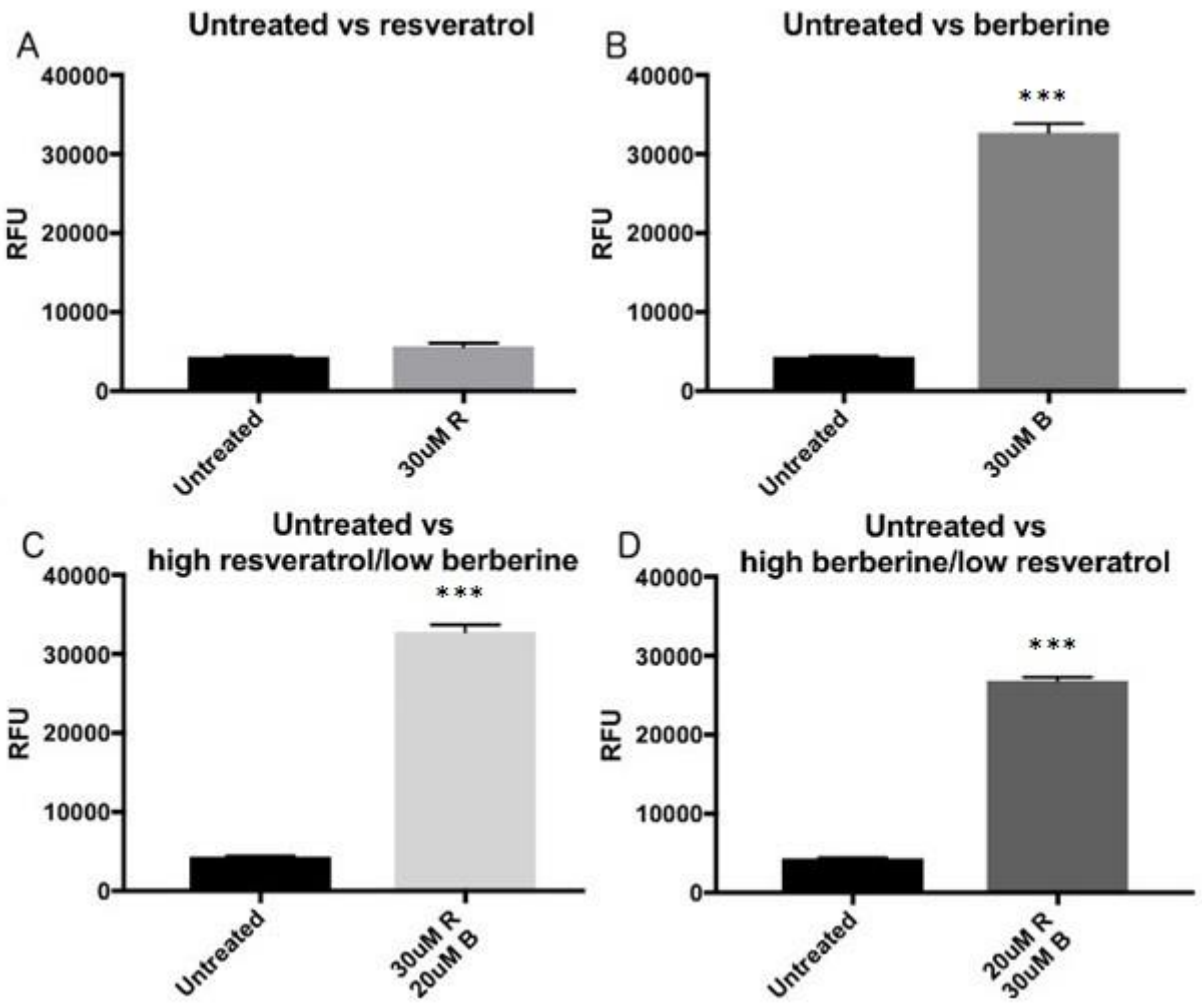


Figure 4.2. Effects of Berberine and Resveratrol on TG2 activity in Caki-2 cells

Flow cytometry analysis of the relative TG2 enzymatic activity of berberine and resveratrol treated Caki-2 cells**.** All cells were pre-treated with MDC (60 uM) and incubated for 24 hours with resveratrol, berberine or a combination of resveratrol and berberine. Flow cytometry was used to measure the relative fluorescent units (RFU) values as a measure of TG2 enzyme activity in Caki-2 cells following 24 hours of incubation

4.2A: This graph compares the relative TG2 activity of cells presented with 30 uM of resveratrol with untreated controls.

4.2B: This graph compares the relative TG2 activity of cells presented with 30 uM of berberine with untreated controls.

4.2C: This graph compares the relative TG2 activity of cells presented with 30 uM of resveratrol in and 20 uM of berberine with untreated controls.

4.2D: This graph compares the relative TG2 activity of cells presented with 30 uM of resveratrol in and 30 uM of berberine with untreated controls.

4.2.1.1. Statistical analyses of the effects of berberine and resveratrol on TG2 activity in Caki-2 cells

An independent-samples t-test was conducted to compare the relative TG2 enzyme activity in an untreated control group of Caki-2 cells with cells treated with 30 uM resveratrol for 24 hours. There was no signi ficant difference in the scores for the untreated group (M=4357, SD=31) and the berberine treatment group (M=5635, SD=321) conditions; t (3.963) = 2 p = < 0.0582. When comparing untreated cells with cells challenged with 30 uM of berberine, t-test revealed a significant difference between untreated and the berberine group (M=32701, SD=823 conditions; t (34) = 2, p = < 0.0008. A t-test analysis of a combined resveratrol/berberine treatment (30 uM R/20 uM B) showed signi ficant difference in scores between the untreated group and the 30 uM R/20 uM B treatment group (M=32772, SD=646) conditions; t (43.94)=2, p = < 0.0005. Finally, a signi ficant difference was also observed in TG2 activity when comparing the untreated group and the 20 uM R/30 uM B treatment group (M=26852 SD=322) conditions; t (69.54) = 2, p = < 0.0002.

These results suggest that berberine treatment does have a signi ficant effect on TG2 activity in this cell line. Speci fically, the results suggest that berberine induces a slight, but signi ficant increase in TG2 activity in Caki-2 cells over a 24 hour period, whereas resveratrol does not induce a signi ficant increase in activity. As the combined treatments induce a greater level of TG2 activity when the berberine concentration is higher (30 uM as averse to 20 uM) than the resveratrol concentration and as resveratrol treatment alone does not show a significant effect; resveratrol can be concluded to have only a minimal effect on TG2 enzyme activity in these experimental conditions, thus answering question 3 of the research aims for this phytochemical/cell line combination. Berberine does increase TG2 activity, whilst resveratrol does not under these experimental conditions.

4.2.2. Effects of berberine and resveratrol on TG2 activity in HepG2 cells

Following seeding of HepG2 cells in T-25 flasks and the preparation of 1mM stock solutions of berberine, resveratrol and monodansylcadaverine (MDC) (see materials and methods section 2.3.9); triplicate flasks were inoculated with either berberine (30 uM), resveratrol (30 uM), a combined 30 uM B/20 uM R or combined 20 uM B/30 uM R, together with 60uM of MDC**.** After 24 hours of incubation, cells were trypsinised, washed in PBS and resuspended in 1ml of PBS. All samples were then run through a BD Accuri C6 flow cytometer at a medium flow rate of 35L /min, with a 16m core and a count of 10,000 singlet events was recorded for each sample. The results were then gated to remove any cell debris and doublets and the remaining populations were analysed for viability (Figure 4.3).

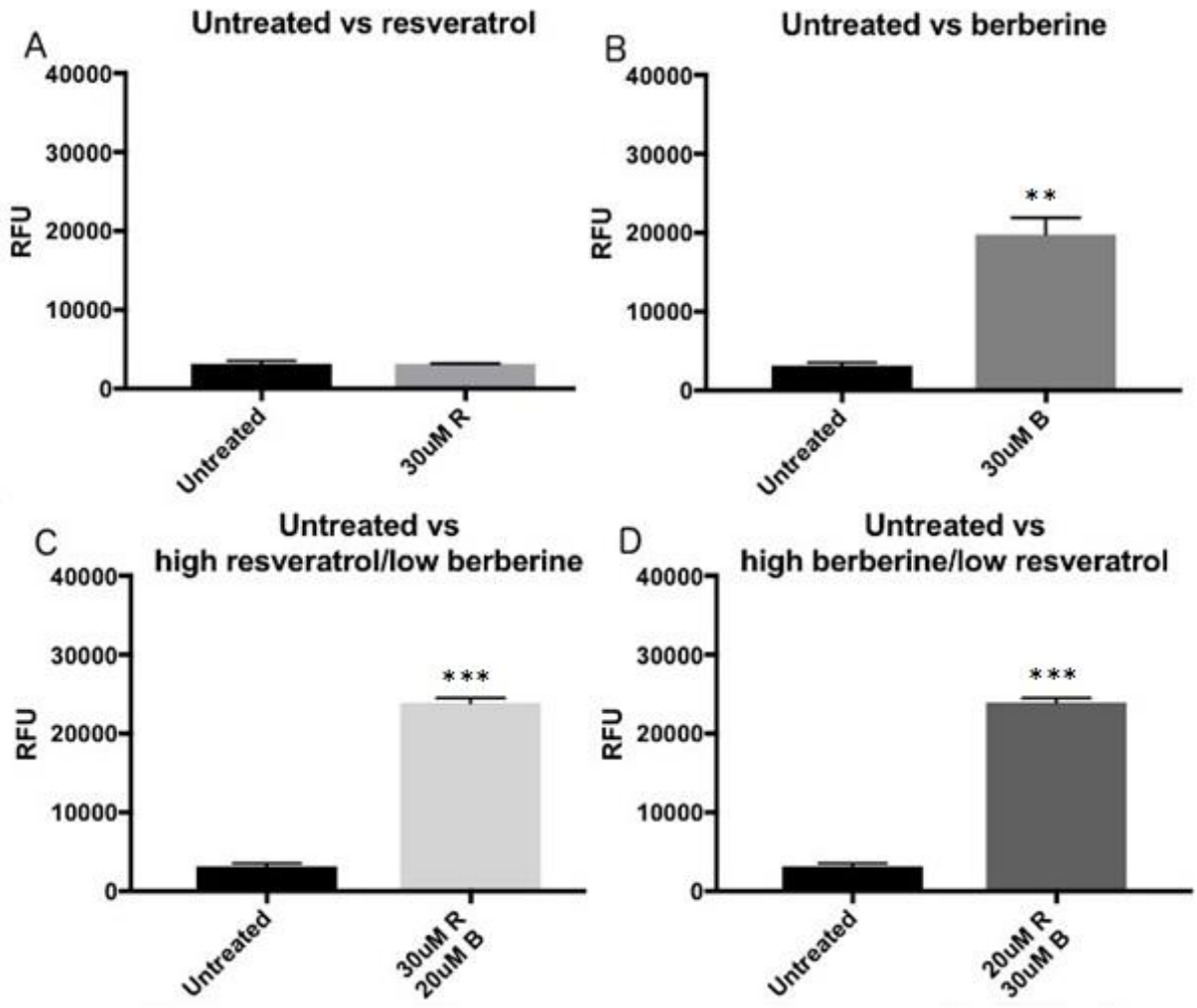


Figure 4.3. Effects of Berberine and Resveratrol on TG2 activity in HepG2 cells

Flow cytometry analysis of the relative TG2 enzymatic activity of berberine and resveratrol treated HepG2 cells. All cells were pre-treated with MDC (60uM) and incubated for 24 hours with resveratrol, berberine or a combination of resveratrol and berberine. Flow cytometry was used to measure the relative fluorescent units (RFU) values as a measure of TG2 enzyme activity in HepG2 cells following 24 hours of incubation

4.3A: This graph compares the relative TG2 activity of cells presented with 30 uM of resveratrol with untreated controls.

4.3B: This graph compares the relative TG2 activity of cells presented with 30 uM of berberine with untreated controls.

4.3C: This graph compares the relative TG2 activity of cells presented with 30 uM of resveratrol in and 20 uM of berberine with untreated controls.

4.3D: This graph compares the relative TG2 activity of cells presented with 32uM of resveratrol in and 30uM of berberine with untreated controls.

4.2.2.1 Statistical analyses of the effects of berberine and resveratrol and on TG2 activity in HepG2 cells

An independent-samples t-test was conducted to compare the relative TG2 enzyme activity in an untreated control group of HepG2 cells with cells treated with 30 uM resveratrol for 24 hours. There was no signi ficant difference in the scores for the untreated group (M=3165, SD=258) and the berberine treatment group (M=3164, SD=4.5) conditions; t (0,0058)=2 p = < 0.9959. When comparing untreated cells with cells challenged with 30uM of berberine, t-test revealed a significant difference between untreated and the berberine group (M=19770, SD=1550 conditions; t (10.57)=2, p = < 0.0088. A t-test analysis of a combined resveratrol/berberine treatment (30 uM R/20 uM B) also showed significant difference in scores between the untreated group and the 30 uM R/20 uM B treatment group (M=23891, SD=428) conditions; t (41.47)=2, p = < 0.0005. When analysing the 20 uM R/30 uM B group, a significant difference was observed in TG2 activity compared with the 20 uM R/30 uM B treatment group (M=23961 SD=359) conditions; t (47)=2, p = < 0.0005.

These results indicate that berberine treatment does have a significant effect on TG2 activity in this cell line. Specifically, the results suggest that berberine alone induces large and signi ficant increase in TG2 activity in HepG2 cells over a 24 hour period, whereas resveratrol alone does not induce a significant increase in activity. Unlike with Caki-2 cells (see section 4.2.1) HepG2 cells show a larger increase in TG2 activity when a combined resveratrol/berberine treatment is presented to the cells, than with berberine alone.

4.2.2.2 Investigation of the effects of concentration ranges of berberine and resveratrol on TG2 activity in HepG2 cells

Following on from the previous section where individual concentrations of berberine, resveratrol, or both were used to treat cells and the TG2 enzyme activity measured, in this section a range of concentrations of the phytochemicals was used to determine what effects differing concentrations of the phytochemicals have on TG2 activity. This will help to answer question 3 of the research aims (section 1.5.1); specifically - Do the phytochemical treatments tested exert their effects on cancer by modulating TG2 expression and/or activity.

Following seeding of HepG2 cells in triplicate T-25 flasks and the preparation of 1mM stock solutions of berberine, resveratrol and monodansylcadaverine (MDC) (see materials and methods section 2.3.11); all flasks were inoculated with either a concentration range of berberine (0 uM-40 uM) or a concentration range of resveratrol (0 uM-250 uM) together with 10uM of MDC. A lower concentration of MDC was used here to reduce noise from the readings and to reduce any likelihood of MDC producing any toxic effects to the cells (even if only slight). A higher range of

concentrations of resveratrol was used as previous experiments had shown only a minimal effect o this compound on TG2 activity so a higher range was chosen to determine if a higher range would induce any modulation of enzyme activity**.** After 24 hours of incubation, cells were trypsinised, washed in PBS and resuspended in 1ml of PBS. All samples were then run through a BD Accuri C6 flow cytometer at a medium flow rate of 35L /min, with a 16m core and a count of 10,000 events was recorded for each sample. The results were then gated to remove any cell debris and doublets and the remaining populations were analysed for viability (Figure 4.4).

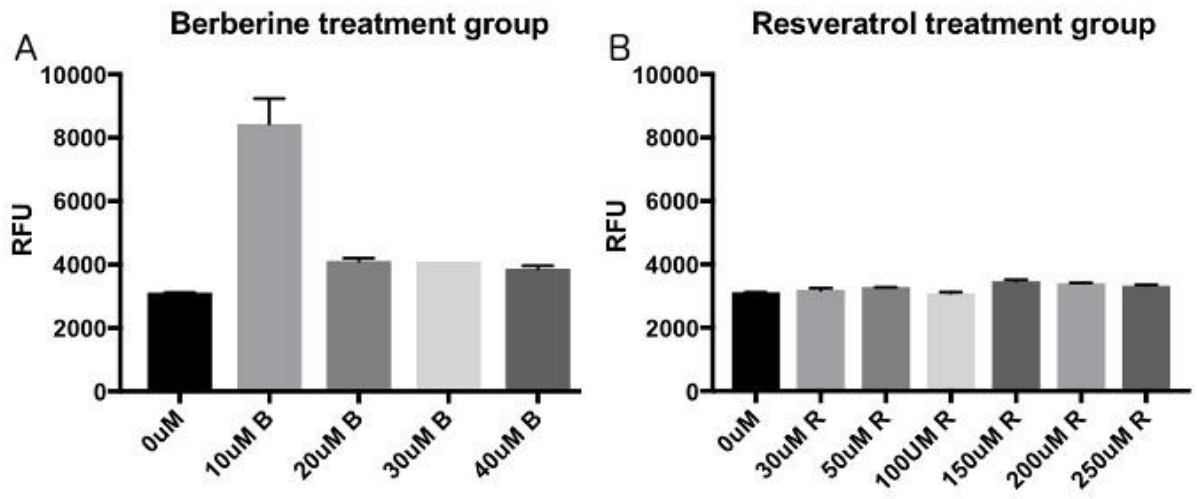


Figure 4.4. Effects of Resveratrol and berberine in isolation on TG2 activity in HepG2 cells

Flow cytometry analysis of the relative enzymatic activity of TG2 in resveratrol and berberine treated HepG2 cells. Flow cytometry was used to measure the relative fluorescent units (RFU) values as a measure of TG2 enzyme activity in HepG2 cells following 24 hours of incubation with either berberine or resveratrol and 10 uM of MDC.

4.4A: This graph compares the relative TG2 activity of cells presented with a range of concentrations of berberine (0-40 uM) for 24 hours.

4.4B: This graph compares the relative TG2 activity of cells presented with a range of concentrations of resveratrol (0-250 uM) for 24 hours.

4.2.2.2.1. Statistical analysis of the effects of concentration ranges of berberine and resveratrol on TG2 activity in HepG2 cells

When analysing the effects of 24 hours treatment of berberine (0-40 uM) on TG2 activity in HepG2 cells, a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0001$). A Tukey post hoc test revealed a significant difference when comparing the 0uM treatment group with the 10 uM group ($p = < 0.0001$). Significance was also observed when comparing the 10 uM berberine group with all other treatment groups ($p = < 0.0005$). This result suggests that berberine treatment does have a significant effect on the TG2 activity in this

cell line. Specifically, the results indicate that a 10 uM berberine treatment over 24 hours induces a large and significant increase in TG2 activity compared with the other treatments.

When analysing the effects of 24 hours treatment of resveratrol (0-250 uM) on TG2 activity in HepG2 cells, a statistically significant difference was observed between groups as determined by one-way ANOVA ($p = < 0.0001$). A Tukey post hoc test revealed a significant difference when comparing all treatment groups, except when comparing the 0 uM with the 30 uM and 100 uM group, when comparing the 30 uM with the 50 uM and 100 uM groups, the 50 uM with the 250 uM group, and when comparing the 150 uM group with the 200 uM and 250 uM groups. This result, although significant is small compared to the effects of berberine (at 10 uM ($p = < 0.0005$) and shows that berberine treatment has a far stronger effect on TG2 enzyme activity than does resveratrol.

* Based on the berberine results, it was decided to run an additional experiment comparing untreated HepG2 cells with 10 uM and 30 uM berberine treatments to ascertain if the effects of the 10 uM berberine treatment were consistent (see Figure 4.5). The specific reasoning for this was that the 10 uM spike of TG2 activity observed in Figure 4.4 was so striking that it was thought worth repeating further.

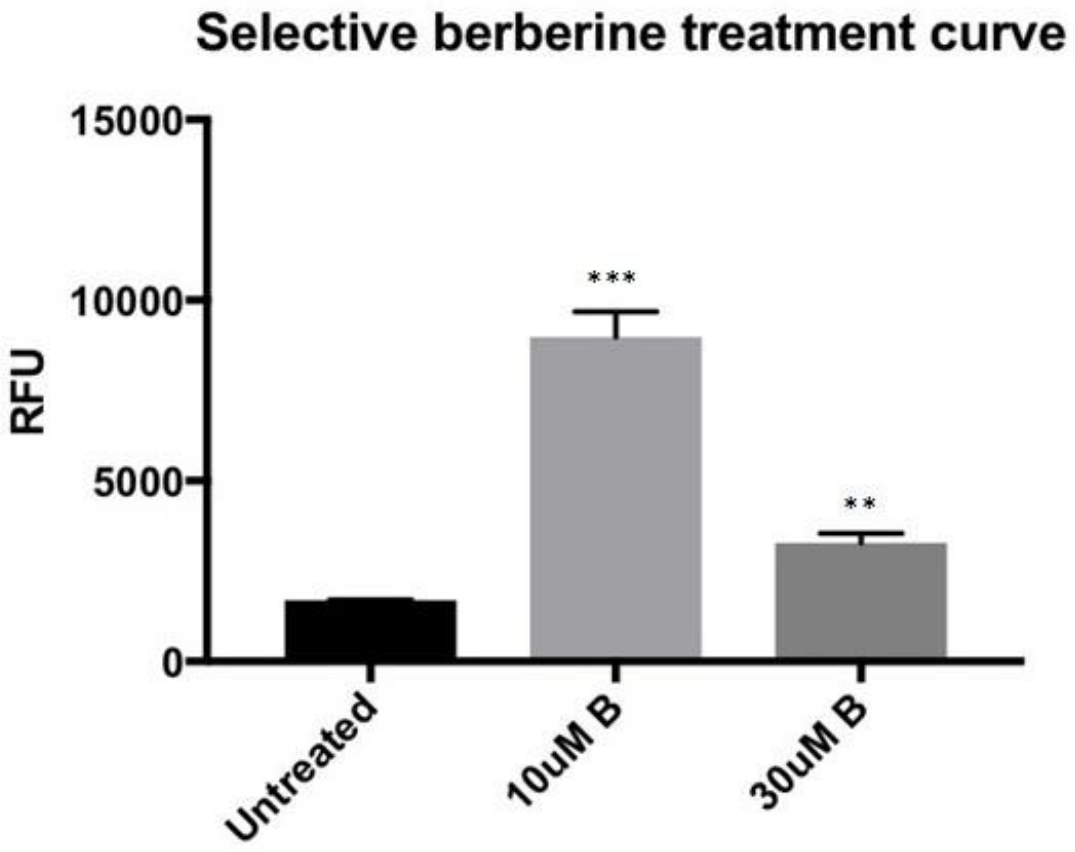


Figure 4.5. Effects of Berberine at 2 concentrations in isolation on TG2 activity in HepG2 cells

The effects of 24 hours dose-specific berberine treatments on TG2 enzyme activity in the HepG2 cell line. Cells were pre-treated with 10uM of MDC prior to incubation with berberine for 24 hours at concentrations of 0 uM, 10 uM or 30 uM. Flow cytometry was used to measure the relative fluorescent units (RFU) values as a measure of relative TG2 enzyme activity.

A comparison of the effects on TG2 enzyme activity in HepG2 cells following 24 hours treatment of berberine at 10 uM and 30 uM against untreated controls showed a statistically significant difference between groups as determined by one-way ANOVA ($p = < 0.0009$). A Tukey post hoc test revealed a significant difference when comparing the 0 uM treatment group with the 10 uM group ($p = < 0.0010$) and when comparing the 10 uM group with the 30 uM group ($p = < 0.0019$). No significant difference was observed however when comparing the 0 uM group with the 30 uM group, although this comparison almost reached significance ($p = < 0.0674$). This result backs up the result outlined in above and suggests that berberine treatment at a sub-lethal concentration of 10 uM over 24 hours induces a large and significant increase in TG2 activity compared with the lethal higher concentrations on berberine.

4.3 Effects of inhibiting TG2 on cell viability

Based on the decision tree (Figure 1.8) and in order to answer question 3 of the research aims – "Do the phytochemical treatments tested exert their effects on cancer by modulating TG2 expression and/or activity?" An inhibitor of TG2 activity was used to determine if this modulated the effectiveness of the phytochemicals and reduced cell viability. The inhibitor used was Cystamine.

Cystamine is a diamine (see Figure 4.6) that acts as a competitive amine inhibitor of TG2 (Jeitner *et al.*, 2005). It has also been shown to inhibit TG2 by other mechanisms, such as by inhibiting the thiol-dependant caspase 3 and thus increasing intracellular production of glutathione (Lesort et al., 2003) and perhaps by the formation of disul fide bonds between cystamine and the cysteine present in TG2's active site (Lorand, 1998). To determine the effects of inhibiting TG2 on the viability of the Caki-2 and HepG2 cell lines, triplicateT-25 flasks were treated with cystamine (2 mM) for 24 hours, with untreated flasks being used as negative controls (see materials and methods section 2.3.11). *** Note** that 2 mM was a dose found to inhibit TG2 effectively in Western blot silencing assays (Dipak Meshram, internal correspondence). After 24 hours incubation, cells were trypsinised, washed in PBS and resuspended in Annexin binding buffer with 5 ul of Annexin V-FITCand PI. Following 15 minutes incubation in a dark cupboard at room temperature, samples were run through a BD Accuri C6 flow cytometer. The results were then gated to remove any cell debris and doublets and the remaining populations were analysed for viability. Statistical analysis of the results was performed using an unpaired t-test with a p value of .05 or less being regarded as signi ficant. (see Figures 4.7 and 4.8).

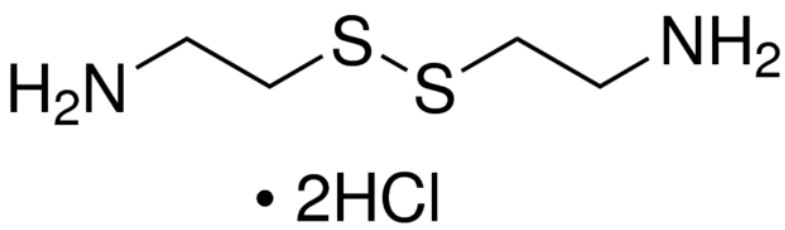


Figure 4.6. The molecular structure of cystamine hydrochloride (Chemspider, 2017).

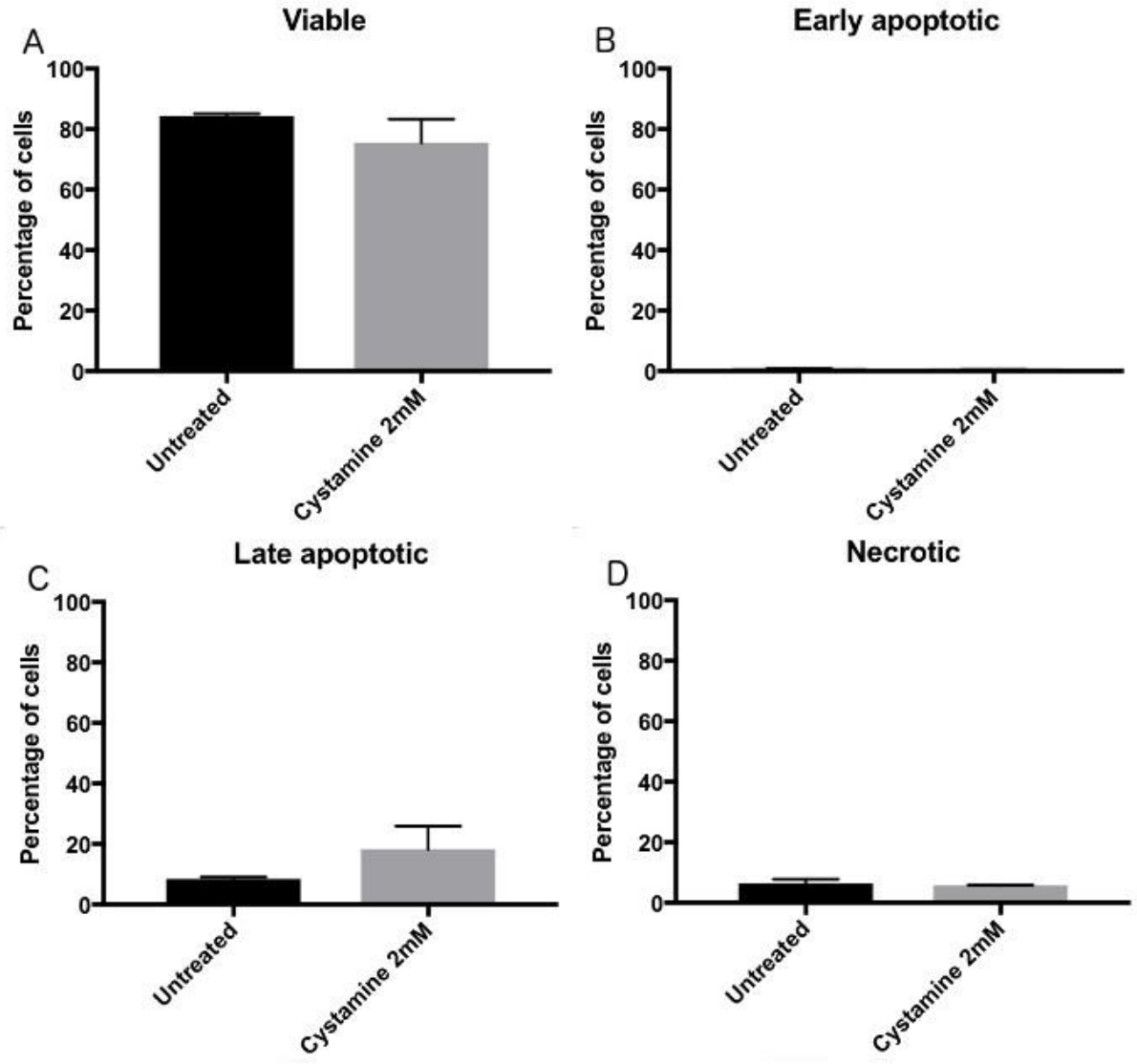


Figure 4.7. The effects of cystamine inhibition of TG2 activity on cell viability in the Caki-2 cell line.

Cells were treated for 24 hours with 2mM of cystamine to competatively inhibit TG2 activity. Flow cytometry was then performed using the Annexin V/PI protocol and the percentages of viable, apoptotic and necrotic cells present in triplicate T-25 flasks were measured.

4.7A: This graph compares the percentage of viable cells in untreated control group against the percentage of viable cells in a cystamine (2 mM) treatment group.

4.7B: This graph compares the percentage of viable cells in untreated control group against the percentage of early apoptotic cells in a cystamine (2 mM) treatment group.

4.7C: This graph compares the percentage of viable cells in un untreated control group against the percentage of late apoptotic cells in a cystamine (2 mM) treatment group.

4.7D: This graph compares the percentage of viable cells in un untreated control group against the percentage of necrotic cells in a cystamine (2 mM) treatment group.

4.3.1 Observations and analyses of the effects of cystamine inhibition of TG2 on cell viability in the Caki-2 cell line

Inhibition of TG2 activity in the Caki-2 cell line with cystamine did not effect viability, which shows that the Caki-2 renal carcinoma model does not seem to be dependent on TG2 for survival. This helps to answer question 3 of the research aims (section 5.1.1). Perhaps in this low-expressing TG2 cell line, the activity of the TG2 enzyme is not sufficient to perform a protective role. The details of the results of the analyses of the cystamine data now follow.

An independent-samples t-test was conducted to compare the percentage of viable cells in an untreated control group of Caki-2 cells with cells treated with 2 mM of cystamine for 24 hours. No significant difference in the scores between the untreated group (M=84.47, SD=0.3712) and the cystamine treatment group (M=75.5, SD=4.554) was observed, conditions; t (1.962)=4, p = < 0.1212. For early apoptotic cells, t-test also revealed no significant difference between the untreated group (M=0.6333, SD=0.1155) and the cystamine treatment group (M=0.4867, SD=0.09528) conditions; t (0.9795)=4, p = < 0.3828. A t-test analysis of late apoptotic cells showed no significant difference in scores between the untreated group (M=8.463, SD=0.3768) and the cystamine treatment group (M=18.3, SD=4.406) conditions; t (2.224)=4, p = < 0.0902. No significance was revealed between the untreated group (M=6.43, SD=0.7858) and treated group (M=5.68 SD=0.1127) conditions; t (0.9448)=4, p = < 0. 03983 when observing the percentage of necrotic cells. These results suggest that inhibition of TG2 in this cell line has a small, but insignificant effect on cell viability.

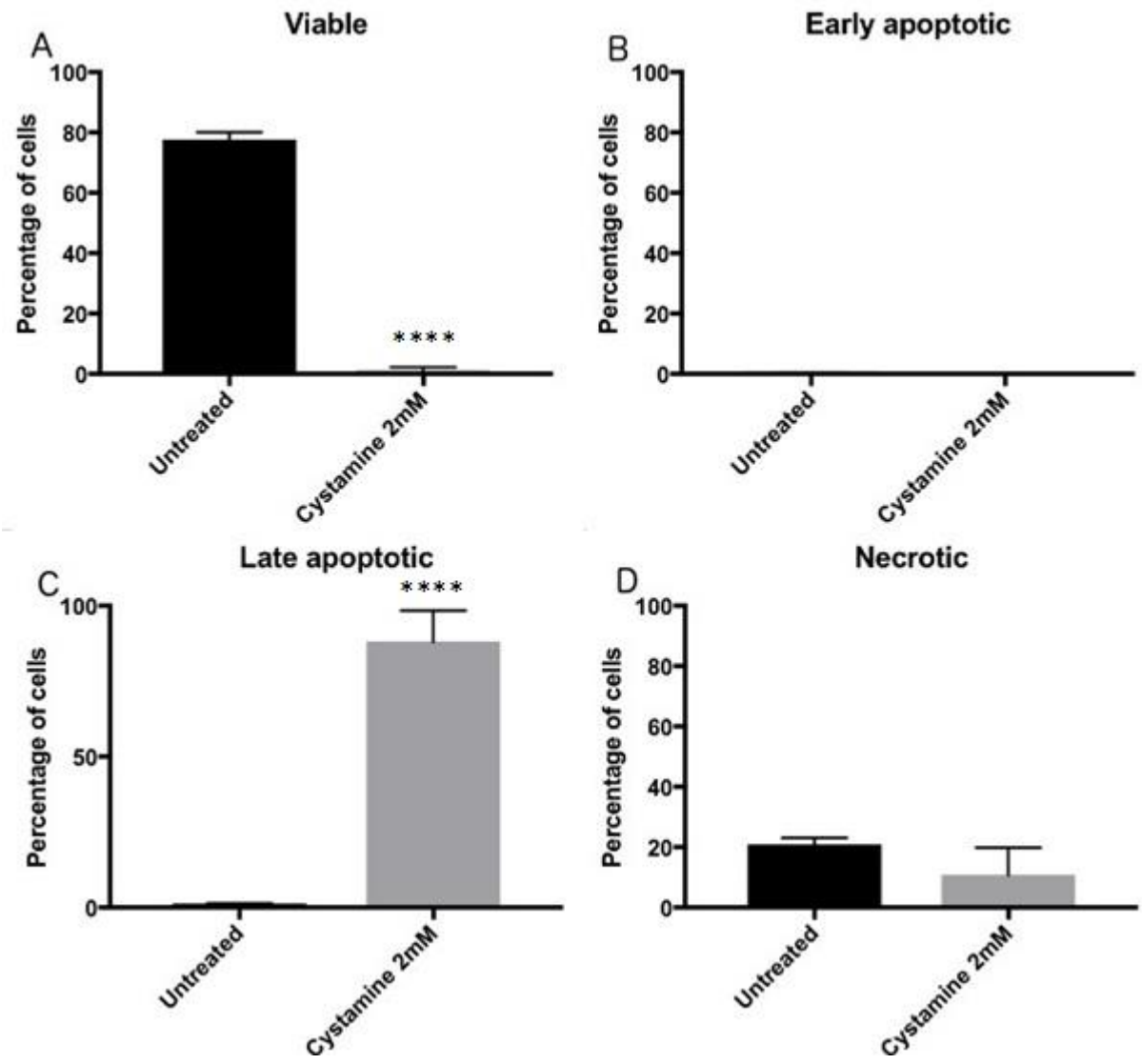


Figure 4.8. The effects of cystamine inhibition of TG2 activity on cell viability in the HepG2 cell line.

Cells were treated for 24 hours with 2 mM of cystamine to competatively inhibit TG2 activity. Flow cytometry was then performed using the Annexin V/PI protocol and the percentages of viable, apoptotic and necrotic cells present in triplicate T-25 flasks were measured.

4.7A: This graph compares the percentage of viable cells in untreated control group against the percentage of viable cells in a cystamine (2 mM) treatment group.

4.7B: This graph compares the percentage of viable cells in untreated control group against the percentage of early apoptotic cells in a cystamine (2 mM) treatment group.

4.7C: This graph compares the percentage of viable cells in untreated control group against the percentage of late apoptotic cells in a cystamine (2 mM) treatment group.

4.7D: This graph compares the percentage of viable cells in untreated control group against the percentage of necrotic cells in a cystamine (2 mM) treatment group.

4.3.2 Statistical analyses of the effects of cystamine inhibition of TG2 on cell viability in the HepG2 cell line

An independent-samples t-test was conducted to compare the percentage of viable cells in an untreated control group of HepG2 cells with cells treated with 2 mM of cystamine for 24 hours. There was a significant difference in the scores between the untreated group (M=77.67, SD=1.359) and the cystamine treatment group (M=1.02, SD=0.7543) conditions; t (49.3)=4, p = < 0.0001. For early apoptotic cells, t-test failed to reveal a significant difference between the untreated group (M=0.3033 SD=0.08413) and the cystamine treatment group (M=0.066, SD=0.0185 conditions; t (2.755)=4, p = < 0.00511. t- test analysis of late apoptotic cells showed significant difference in scores between the untreated group (M=0.97, SD=0.1808) and the cystamine treatment group (M=88.1, SD=5.979) conditions; t (14.57)=4, p = < 0.0001. Finally, no significant difference between the untreated group (M=21.03, SD=1.217) and treated group (M=10.8, SD=5.311) was revealed, conditions; t (1.878)=4, p = < 0.1336 when comparing the percentage of necrotic cells. These results revealed a significant effect on viable, late apoptotic and necrotic populations when cells were challenged with cystamine and the effect on early apoptotic percentages was not significant using a P value of < 0.005, but almost did reach significance with a P value of 0.0051 . Specifically, these results indicate that inhibition of TG2 in the HepG2 hepatocarcinoma model has a significant and strong effect on cell viability. The observed reduction in viability and increase in apoptosis/necrosis indicates that in the high-expressing TG2 cell line HepG2, TG2 is essential for the survival of the cells. Manipulation of its activity, whether increasing or decreasing its overall activity reduces significantly the viability of the cell line. This observation will be discussed further in section 6.5 and may have clinical implications for the treatment of hepatocarcinoma.

4.4. Identification of the TG2-S and TG2-L isoforms of transglutaminase 2

As stated in the introduction (section 1.3.4), it was observed as early as 1997 (Monsonego) that there were 2 isoforms of TG2 (TG2-L and TG2-S) and that these isoforms appeared to have opposing effects on apoptosis, with TG2-S being broadly observed to be pro-apoptotic and TG2-L being anti-apoptotic (Antonyak, 2006). In this project, it was not possible with the resources available to differentiate between the TG2-L and TG2-S isoforms at the protein level as antibodies for the isoforms were not then commercially available. However, ass several papers have been published relating to the isoforms and their relevance to drug resistance and cell viability , it was decided to attempt to measure the relative levels of the isoforms to develop the model system further (see decision tree – Figure 2.1).

To provide a guide to TG2 isoform expression, reverse transcription RT-PCR was used as described in the materials and methods chapter (section 2.3.7) to determine if the TG2 isoforms were present in the available cell lines and to quantify the relative expression of the isoforms at the mRNA level. At the time of writing, the TG2-L and TG2-S isoforms had not yet been identified in either Caki-2 or the HepG2 cell lines. It was during this project that these isoforms were identified for the first time in these model cell lines. This novel discovery may lead to further insights into the role of the isoforms in cancer and perhaps help to lead to future clinical interventions for cancer patients.

This measurement would also allow for a preliminary analysis of whether the expression of the isoforms of TG2 correlated with a susceptibility to apoptosis when exposed to the candidate compounds. Note that the decision to use mRNA expression as an initial model for determining relative TG2 isoform protein expression was taken as a correlation has previously been established between TG2 mRNA levels and TG2 protein expression (Tee, 2009) so mRNA analyses would act as a good first indicator of TG2 isoform levels in the model system.

Before performing large scale RT-PCR, traditional PCR was used to ascertain if the TG2 isoforms were present. Primers were designed that specifically bound to either the TG2-L or the TG2-S isoform. This specificity was achieved by designing the primers to exon jump sequences that were present on the sister isoform (see section 2.3.7.2 for the primer sequences). Primer specificity was determined via PCR amplification of a cDNA library that was generated using total mRNA extracted from the cell lines. Figure 4.9A confirms the successful isolation of RNA from cultured HepG2 cells. The distinctive bands of the gel indicate the presence of purified RNA. Figure 4.9B shows the spectrophotometric results of a HepG2 extract following creation of cDNA from the purified RNA, which confirms the generation of cDNA from the RNA extract.

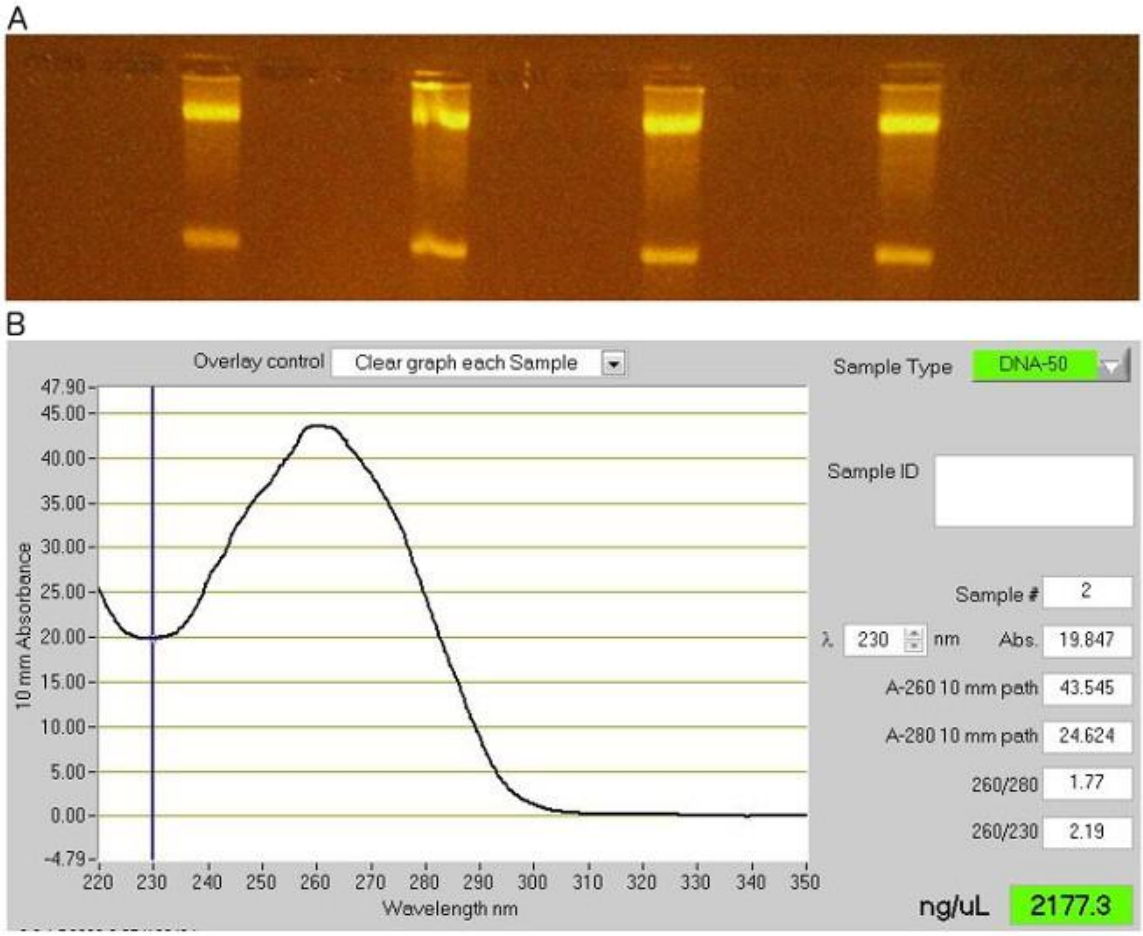


Figure 4.9. RNA extraction and creation of a cDNA library from cultured HepG2 cells

4.9A. Agarose gel of sample RNA extraction replicates from HepG2 cultures. The top band in the lanes is 28S rRNA and the bottom band is 18S rRNA.

4.9B. Spectrophotometric confirmation that RNA was converted to cDNA library's following the cDNA synthesis assay (see materials and methods).

4.4.1 Validation of primers and identification of the presence of TG2-L and TG2-S isoforms in HepG2 cultures

Following validation of the RNA extraction and cDNA library generation assays, the TG2-L and TG2-S primers were tested on the HepG2 cDNA libraries using PCR at a temperature range of 51- 59°C to find the ideal annealing temperatures. Following PCR, the amplified products were run on a 2% agarose gel at 85V for 2 hours (see Figure 4.10).

From the results shown in Figure 4.10, it can be seen that at most temperatures a single product was amplified, the QiaQuick gel extraction kit (Qiagen) was then used to extract the products from lane 3 (TG2-L) and lane 10 (TG2-S) as these appeared to be the strongest and most specific signals. The purified extracts were then sent for Sanger sequencing (Cambridge department of

Biochemistry) and the sequences were uploaded to the NCBI BLAST utility to confirm the identity of the products. It was confirmed that the amplified products were TG2-L and TG2-S. It was, therefore, confirmed that HepG2 expressed both isoforms of TG2 at the mRNA level, which had previously not been observed in either HepG2 cells specifically or in any other forms of liver cancer. It is, however, noteworthy that in a paper by Phatak *et al* (2013) TG2-L and TG2-S were found to be significantly higher (twofold and ninefold, respectively) in fetal liver cells compared to adult liver.

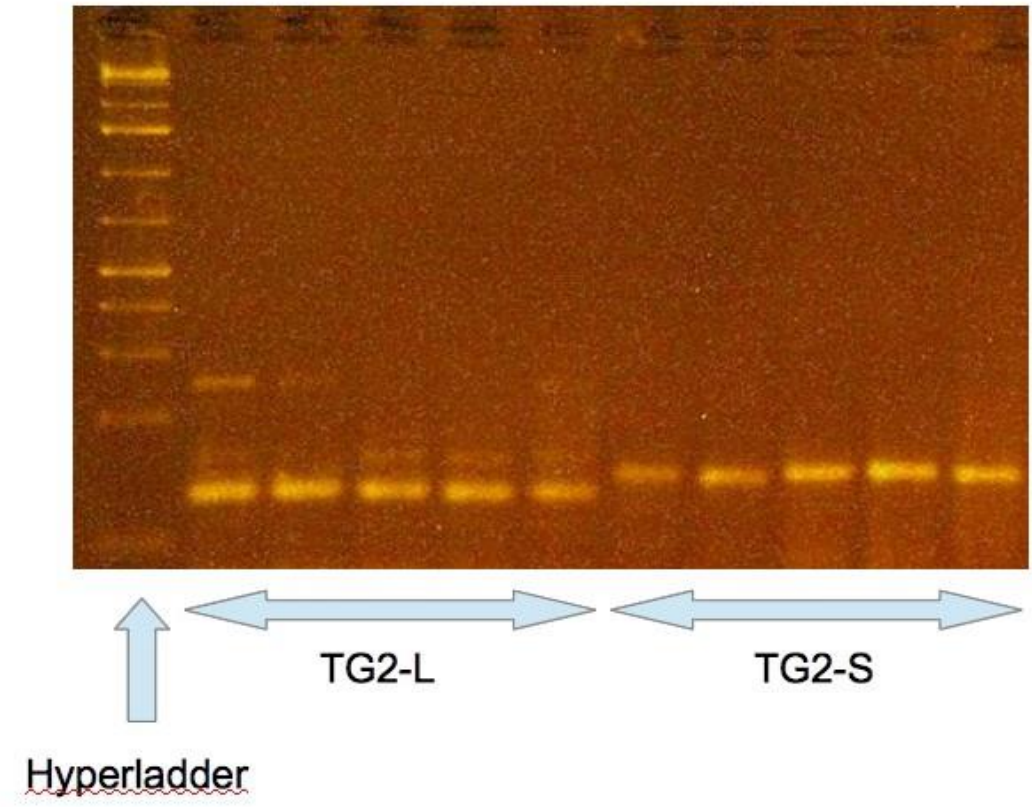


Figure 4.10. An agarose gel of amplified HepG2 TG2-L and TG2-S cDNA

From left to right, lane 1 contains a hyperladder (hyperladder 1, bioline), lanes 2-6 contain amplified TG2-L products amplified with annealing temperatures of 51°C, 53°C, 55°C, 57°C and 59°C. Lanes 7-11 contain amplified TG2- S products amplified with annealing temperatures of 51°C, 53°C, 55°C, 57°C and 59°C.

4.4.1.1 Determination of the presence of the TG2 isoforms in the HepG2 and Caki-2 cancer cell lines

Once the primer speci ficity had been con firmed and the isoforms identi fied in HepG2 cells, the Caki-2 cell line was also tested for the presence of the isoforms and both isoforms were detected (see Figure 4.11).

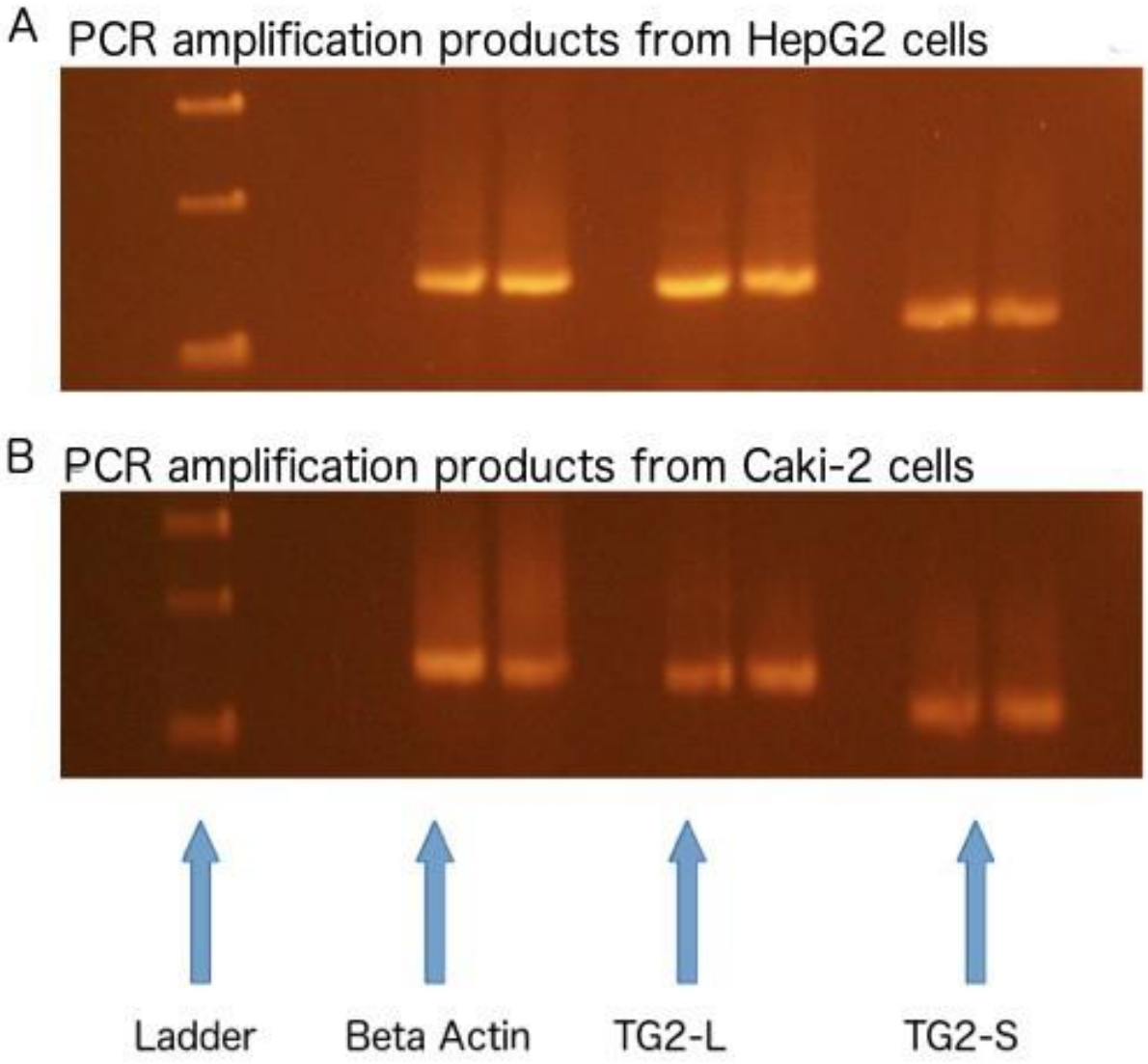


Figure 4.11. Agarose gels showing ampli fied Beta Actin and TG2-isoforms obtained from cDNA libraries of HepG2 and Caki-2 cells

4.11A shows from left to right, DNA hyperladder 1 (Bioline), Beta Actin (control), TG2-L and TG2-S ampli fied from a cDNA library created from HepG2 total RNA.

4.11B shows from left to right, DNA hyperladder 1 (Bioline), Beta Actin (control), TG2-L and TG2-S ampli fied from a cDNA library created from Caki-2 total RNA.

From Figure 4.11 it can be observed that the TG2-L and TG2-S isoforms are present in HepG2 and Caki-2 cells. Previous to this research, the TG2 isoforms had not been identified in either of these cancer models, an observation which may indicate that the isoforms are more prevalent than was previously understood.

4.4.2 Reverse transcription real time PCR amplification of the TG2 isoforms

Following the confirmation of the presence of the isoforms, the HepG2 and Caki-2 cell lines were treated with berberine (30 uM) or resveratrol (30 uM) for 24 hours and total RNA was then extracted from the cultures. Reverse transcription real time PCR was performed to determine how the compounds effected isoform expression, with untreated controls used to determine the baseline levels of the isoforms, all measurements being normalised against Beta Actin (see materials and methods). Figure 4.12 shows an example of a set of amplification and melting curves from this series of experiments.

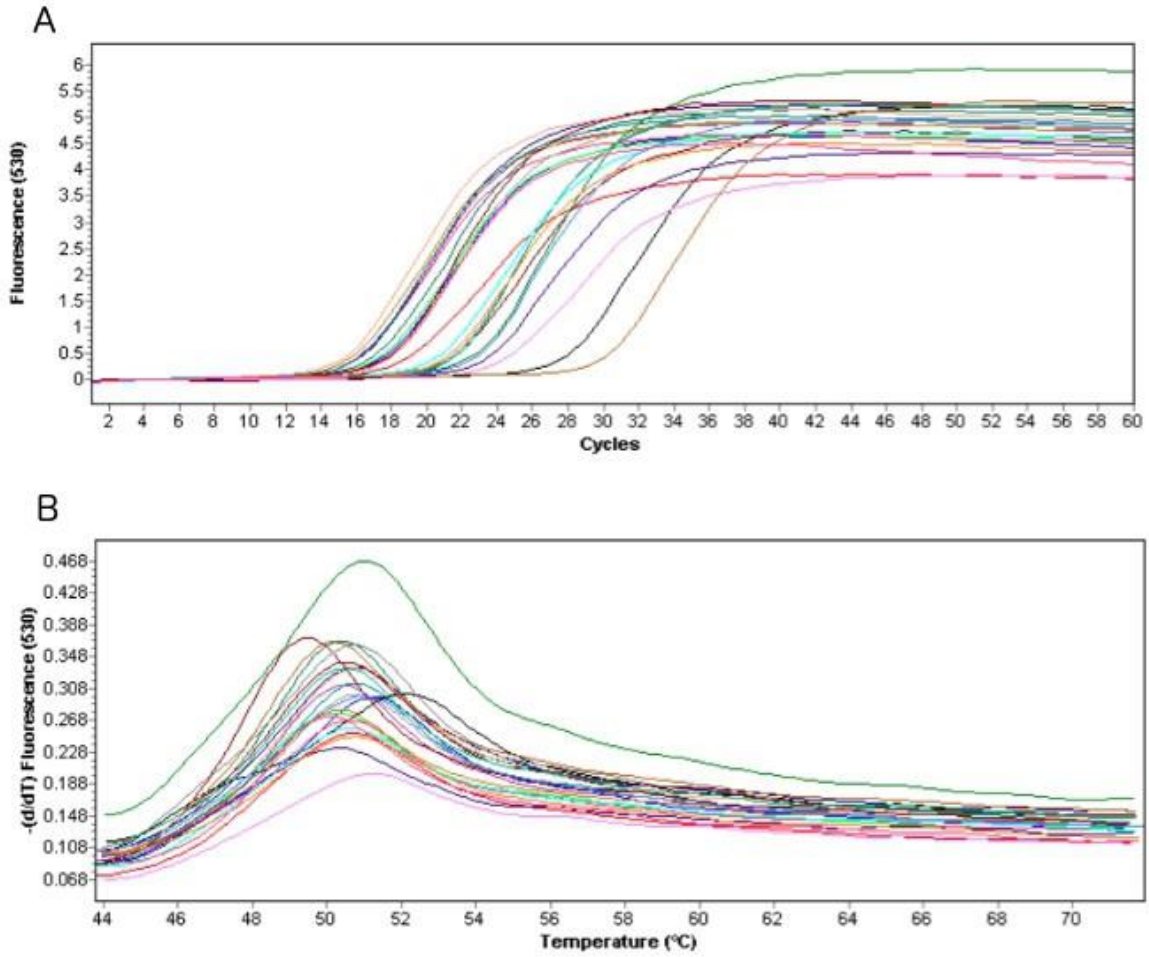


Figure 4.12. Example amplification and melting curves obtained following RT-PCR

Products were amplified from cDNA isolated from HepG2 cells

4.12A: This shows amplification curves generated from cDNA created from total RNA extracted from HepG2 and Caki-2 cells. The primers used were for Beta actin, TG2-S and TG2-L.

4.12B: This shows melting curves generated from cDNA created from total RNA extracted from HepG2 and Caki-2 cells. The primers used were for Beta actin, TG2-S and TG2-L.

Once amplification of the cDNA had been performed, relative quantification was performed using

the Delta-Delta CT method (Livak and Schmittgen, 2001) and the fold changes in gene expression as compared with untreated controls were plotted on graphs (see Figures 4.13 and 4.14). Note that using the Delta-Delta CT method the baseline level of the target gene is considered 1 or (100%) and change in expression is indicated as a fold change, for example a fold change value of 1.5 in a treatment group compared with an untreated control would indicate an increase in gene expression of 50% (see materials and methods 2.2.7.2).

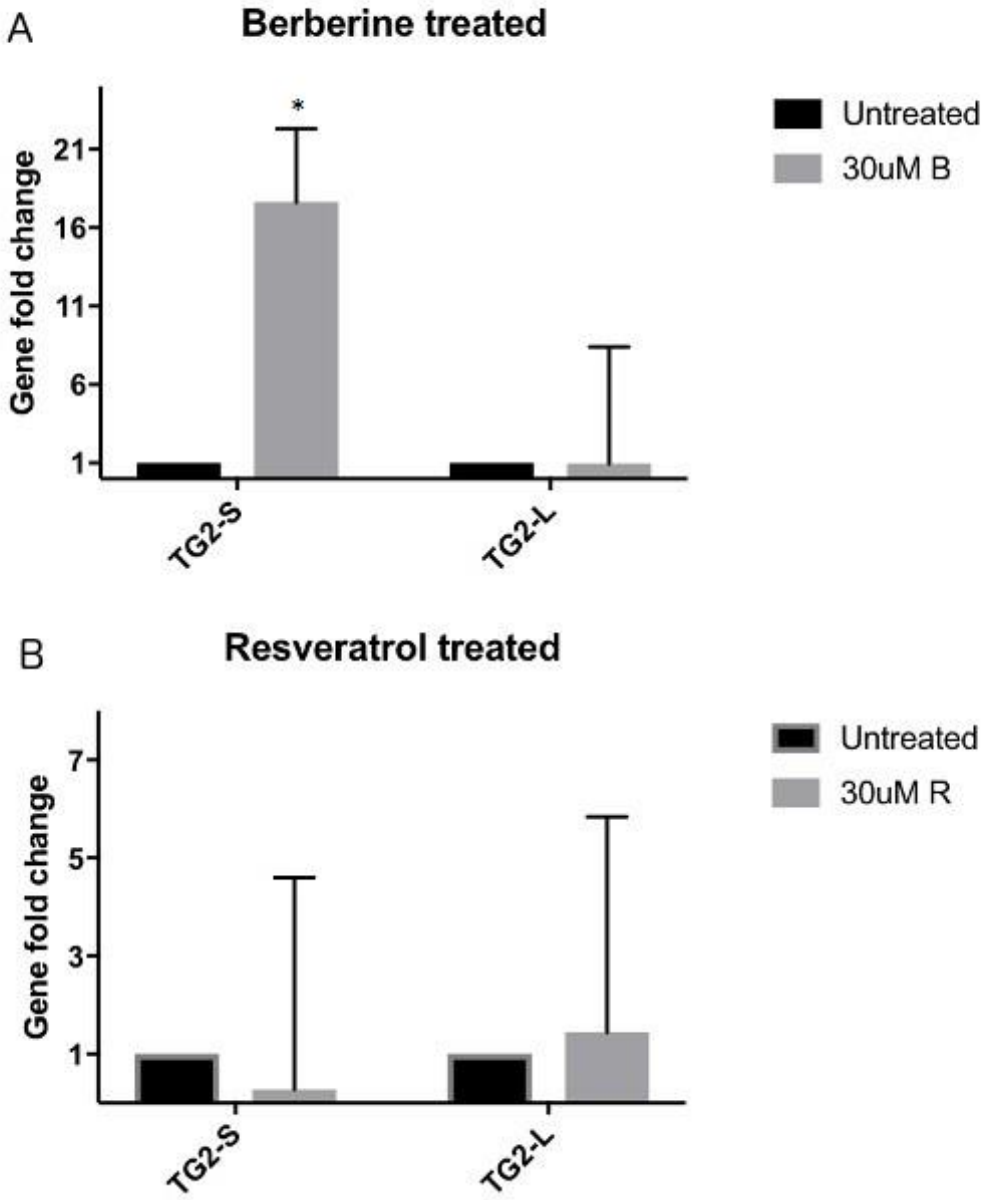


Figure 4.13. Relative gene expression of TG2-S, and TG2-L in HepG2 cells when challenged with berberine or resveratrol.

4.13A: This shows the fold change in gene expression relative to untreated controls of TG2-S and TG2-L in HepG2 cells when treated with 30 uM of berberine for 24 hours.

4.13B: This shows the fold change in gene expression relative to untreated controls of TG2-S and TG2-L in HepG2 cells when treated with 30 uM of resveratrol for 24 hours.

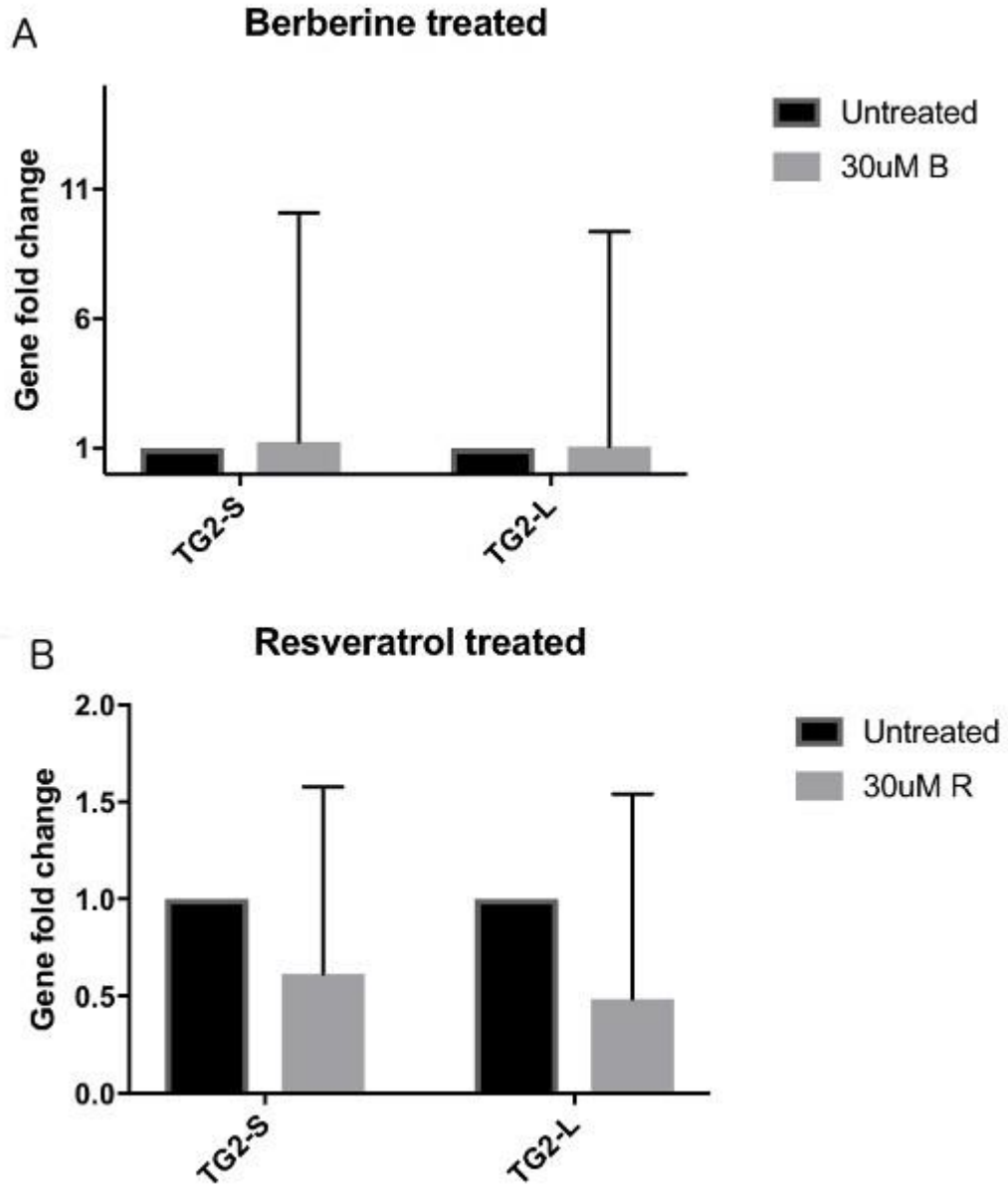


Figure 4.14. Relative gene expression of TG2-S and TG2-L in Caki-2 cells when challenged with berberine or resveratrol.

4.14A: This shows the fold change in gene expression relative to untreated controls of TG2-S and TG2-L in Caki-2 cells when treated with 30 uM of berberine for 24 hours.

4.14B: This shows the fold change in gene expression relative to untreated controls of TG2-S and TG2-L in Caki-2 cells when treated with 30 uM of resveratrol for 24 hours.

4.4.2.1. Analysis of TG2-S and TG2-L gene expression in HepG2 and Caki-2 cells treated with resveratrol or berberine.

Following real time PCR and calculation of fold changes, an independent-samples t-test was conducted to compare the fold change in gene expression of TG2-S and TG2-L (if any) induced by treatment with either berberine (30 uM) or resveratrol (30 uM) for 24 hours. There was a signi ficant difference in the scores between the untreated group (M=1, SD=0) and the berberine treatment group for TG2-S expression in the HepG2 cell line (M=31.03, SD=4.675) conditions; t (3)=4, p = < 0.0282. No signi ficant difference in gene expression was found in TG2-L expression under these conditions however. No significant difference in either TG2-S or TG2-L expression was found in HepG2 cells when treated with resveratrol. Finally, no signi ficant difference was observed in either TG2-S or TG2-L expression between untreated controls when treating Caki-2 cells with either berberine or with resveratrol. Taken together these results are inconclusive. One reason for this is that qPCR requires a large number of samples and replicates from which to draw any firm conclusions from, however as TG2-S has been previously implicated as an inducer of apoptosis (Antonyak, 2006) and this experiment shows a significant increase in TG2-S levels at a Berberine concentration shown in section 3.3 to induce apoptosis, this result is significant.
*Note that the error bars shown in these PCR results was in most cases quite large. This is probably due to the difficulty in extracting and the lack of stability of RNA. During this project a cold room or specific laboratory for extracting RNA was not available. It would perhaps be enlightening if this series of experiments was repeated using dedicated RNA extraction facilities.

4.5 Summary

In this chapter it has been shown that TG2 enzymatic activity is increased in both the HepG2 and Caki-2 cell lines when treated with berberine. TG2 activity was not however found to increase when treated with resveratrol in either cell line. Inhibition of TG2 activity with cystamine substantially decreased the percentage of viable HepG2 cells and there was also a small increase in cell death in the Caki-2 cell line. Of note is that TG2 activity was found to spike in HepG2 cells when treated with berberine at a sub-lethal concentration. This observation indicates that modulation of TG2 activity may have a role in inducing apoptosis or in protecting the cells from some pro-apoptotic compounds such as berberine. TG2 expression as measured by qPCR was inconclusive in most measurements, however the results may show a role in overexpression of the TG2-S isoform during apoptosis in the HepG2 hepatocarcinoma model. The results of this chapter have built on the results of chapter 3 and has added to the knowledge discussed in the literature

review (sections 1.3 and 1.4) regarding TG2's role in cell death and drug resistence.

Chapter Five

Determination of the affects of berberine and resveratrol on cellular adhesion, migration and the cell cycle

5.1. Introduction

It has been determined that HepG2 and Caki-2 cells have differing tolerances to berberine as an inducer of apoptosis and very similar tolerances to resveratrol. The levels of cell death induced by either phytochemical (in isolation or when combined) is minimal in the Caki-2 cell line, however berberine, either in isolation or in combination with resveratrol induces a high level of cell death at 30 uM (24 hours' exposure) in HepG2 cells. Cystamine inhibition of TG2 activity also reduced cell viability substantially in HepG2 cells (see section 4.3). The following series of experiments involve microscopic analysis of the cells when treated with berberine, resveratrol, a combination of both compounds or cystamine. This helps to ascertain any effects on morphology, cellular adhesion, migration and proliferation induced by these compounds.

5.2. Microscopic analysis of cellular adhesion

5.2.1 Affects of resveratrol and berberine on cellular adhesion in the Caki-2 cell line

Caki-2 cells were seeded in 6 well plates and 1 mM stock solutions of berberine and resveratrol and a 12 mM stock solution of cystmaine were prepared (see Materials and Methods 2.2.1.5). Wells were treated with either 30 uM resveratrol (30 uM R), 30 uM berberine (30 uM B), 20 uM R/30 uM B, 20 uM B/30 uM R or 2 mM of Cystamine. Cells were then incubated for 24 hours and images and observations were recorded (see Figure 5.1 below).

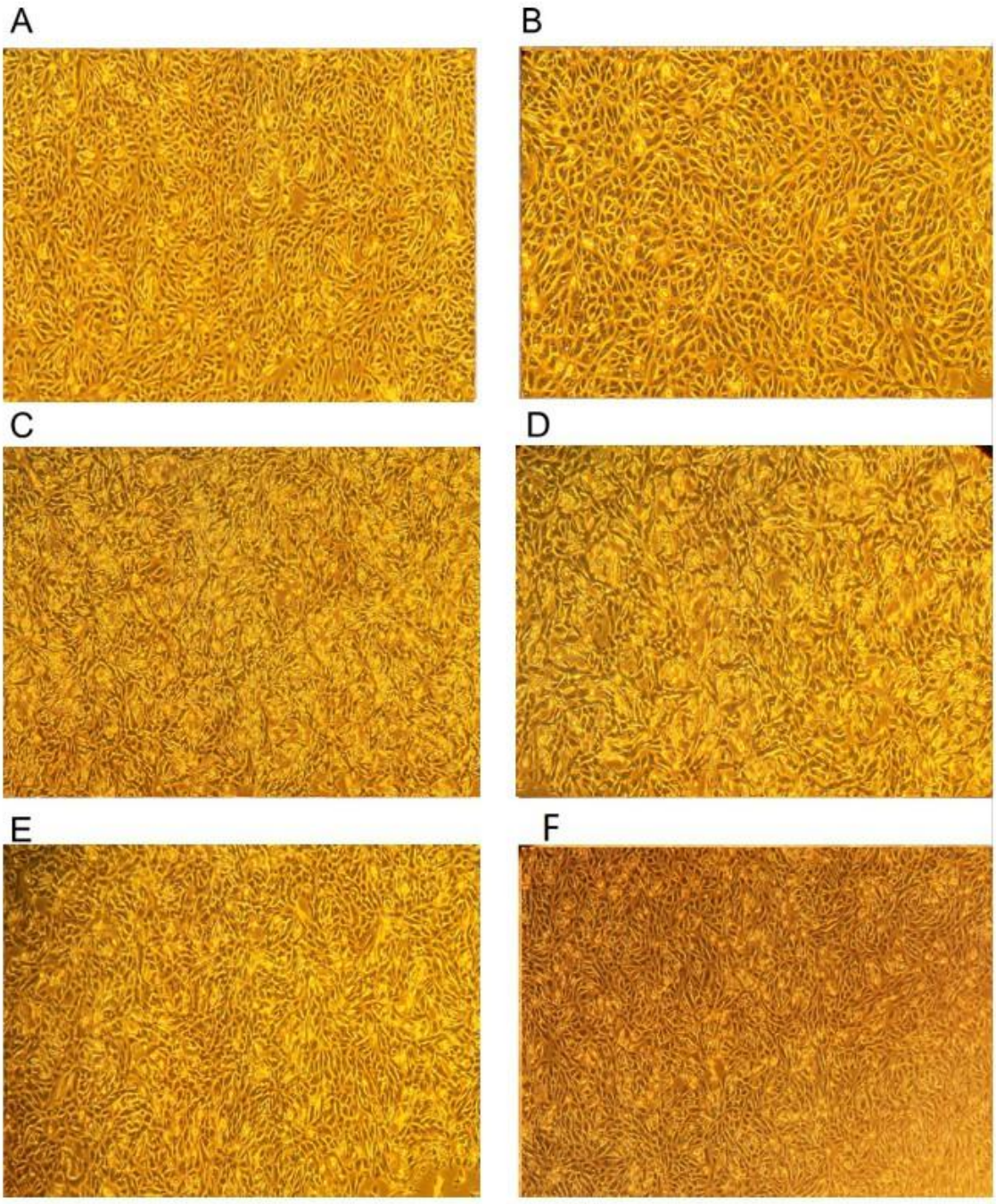


Figure 5.1. Microscopic images of Caki-2 cells treated with resveratrol, berberine, combined resveratrol/berberine or cystamine for 24 hours.

5.1 A: Untreated control. **5.1B:** Resveratrol treated (30 uM). **5.1C:** Berberine treated (30 uM). **5.1D:** Resveratrol/berberine combined treatment (20 uM R/30 uM B). **5.1E:** Berberine/resveratrol combined treatment (20 uM B/30 uM R). **5.1F:** Cystamine treatment (2 mM).

Treatment with resveratrol (5.1B) resulted in a change in morphology of the Caki-2 cells as compared with the controls, with the cells appearing larger, with a more distinctive nucleus. Berberine treatment alone (5.1C) resulted in a loss of shape of the cells as compared with the untreated controls, with cells appearing more hairlike in shape. A combined 20 uM R/30 uM B treatment (5.1D) induced a morphology that combined the larger size and clearer nucleus of the

resveratrol treatment with the more hairlike morphology of the berberine treatment, whilst a combined 20 uM B/30 uM R treatment (5.1E) produced a similar morphology, but with less of a hairlike appearance to the cells than was observed in 5.1D. Finally, treatment with the TG2 inhibitor cystamine (5.1F) resulted in cells that were similar in morphology to the untreated controls, but more densely con fluent. All treated conditions resulted in cells that were firmly adherent to the well plates. These results suggest that all compounds modulate cell morphology in some way, which may be a result of a modulation of the cell cycle in the case of resveratrol as the larger nuclei observed following this treatment may reflect an accumulation of DNA.

5.2.2 Effects of resveratrol and berberine on cellular adhesion in the HepG2 cell line

Following seeding of HepG2 cells in 6 well plates, and 1 mM stock solutions of berberine and resveratrol and a 12 mM stock solution of cystmaine were prepared (see Materials and Methods 2.2.1.5). Wells were treated with either 30 uM resveratrol (30 uM R), 30 uM berberine (30 uM B), 20 uM R/30 uM B, 20 uM B/30 uM R or 2 mM of Cystamine. Cells were then incubated for 24 hours and images and observations were recorded (see Figure 5.2).

Treatment with resveratrol (5.2B) resulted in less dense clusters of HepG2 cells as compared with the untreated controls. Berberine treatment alone (5.2C) resulted in a loss of shape of the cells and a change (lowering) of cell layers in clusters as compared with the controls or the resveratrol treated cells and the cells appeared non-viable and were observed to be only loosely attached to the wells. A combined 20 uM R/30 uM B treatment (5.2D) resulted in a loss of cellular adhesion, with cells floating in the media and appearing non-viable. A combined 20 uM B/30 uM R treatment (5.2E) was also observed to reduce viability and reduced cell density, but the cells remained adherent to the well plate. Finally, cystamine treatment (5.2F) appeared to kill all of the cells and they were observed to be granular in morphology and all had lost adhesion to the well plates.

Taking together, these results suggest that resveratrol inhibits the cell cycle in HepG2 cells, but increases cellular adhesion, whereas berberine (as was observed in chapter 3) induces cell death, but in combination with a higher concentration of resveratrol retains the adhesion of the non-viable cells to the well plates.

This helps to support the idea outlined in section 1.3 and 1.4 and that was clarified in the aims/objectives and research questions (section 1.5) that phytochemicals might be an effective method for modulating TG2 activity and that this modulation can result in the death of certain cancer cell lines, all of which increases the evidence that a clinical protocol might be derinved from the results obtained during this project. Although more work is still needed before a clinical scenario using these phytochemicals is ready for testing on patients.

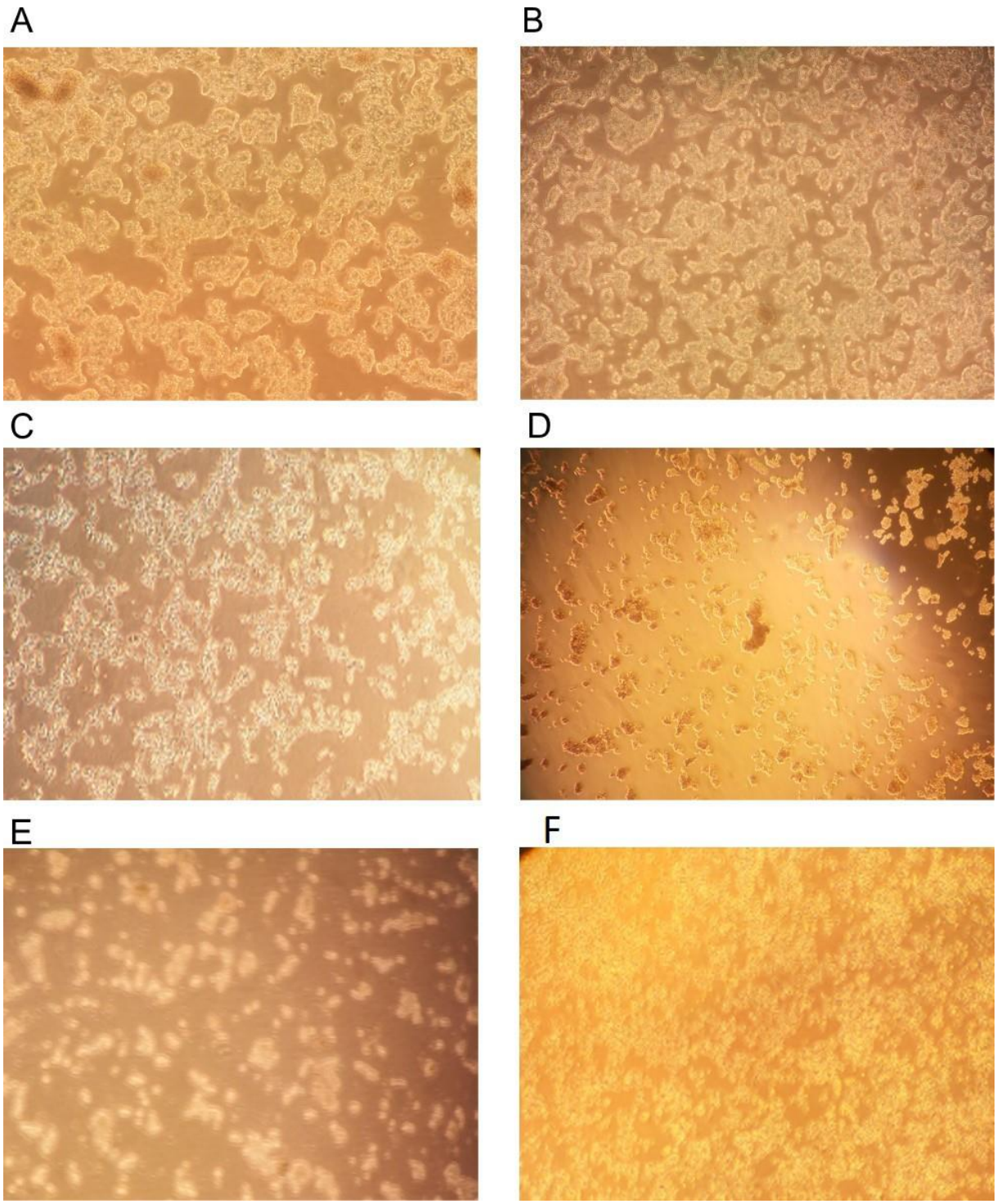


Figure 5.2. Microscopic images of HepG2 cells treated with resveratrol, berberine, combined resveratrol/berberine or cystamine for 24 hours.

5.2 A: Untreated control. **5.2B:** Resveratrol treated (30 uM). **5.2C:** Berberine treated (30 uM). **5.2D:** Resveratrol/berberine combined treatment (20 uM R/30 uM B). **5.2E:** Berberine/resveratrol combined treatment (20 uM B/30 uM R). **5.2F:** Cystamine treatment (2 mM).

5.2.3 Measurement and comparison of trypsinisation times in Caki-2 and HepG2 cells treated with berberine, resveratrol, combined resveratrol/berberine treatments or cystamine

Caki-2 and HepG2 cells were seeded in 6 well plates and 1 mM stock solutions of berberine and resveratrol and a 12 mM stock solution of cystmaine were prepared (see Materials and Methods 2.2.1.5). Wells were then treated with either 30 uM resveratrol (30 uM R), 30 uM berberine (30 uM B), 20 uM R/30 uM B, 20 uM B/30 uM R or 2 mM of Cystamine for 24 hours. Following incubation, all wells were washed with PBS and incubated with 500 ul of trypsin. At 5 minute intervals, the cells were observed under the microscope to check if the cells had lost adherence to the base of the wells. Wells were either scored as having 0% floating (trypsinised) cells, 50% or 100% at each 5 minute inspection interval (see Figure 5.3).

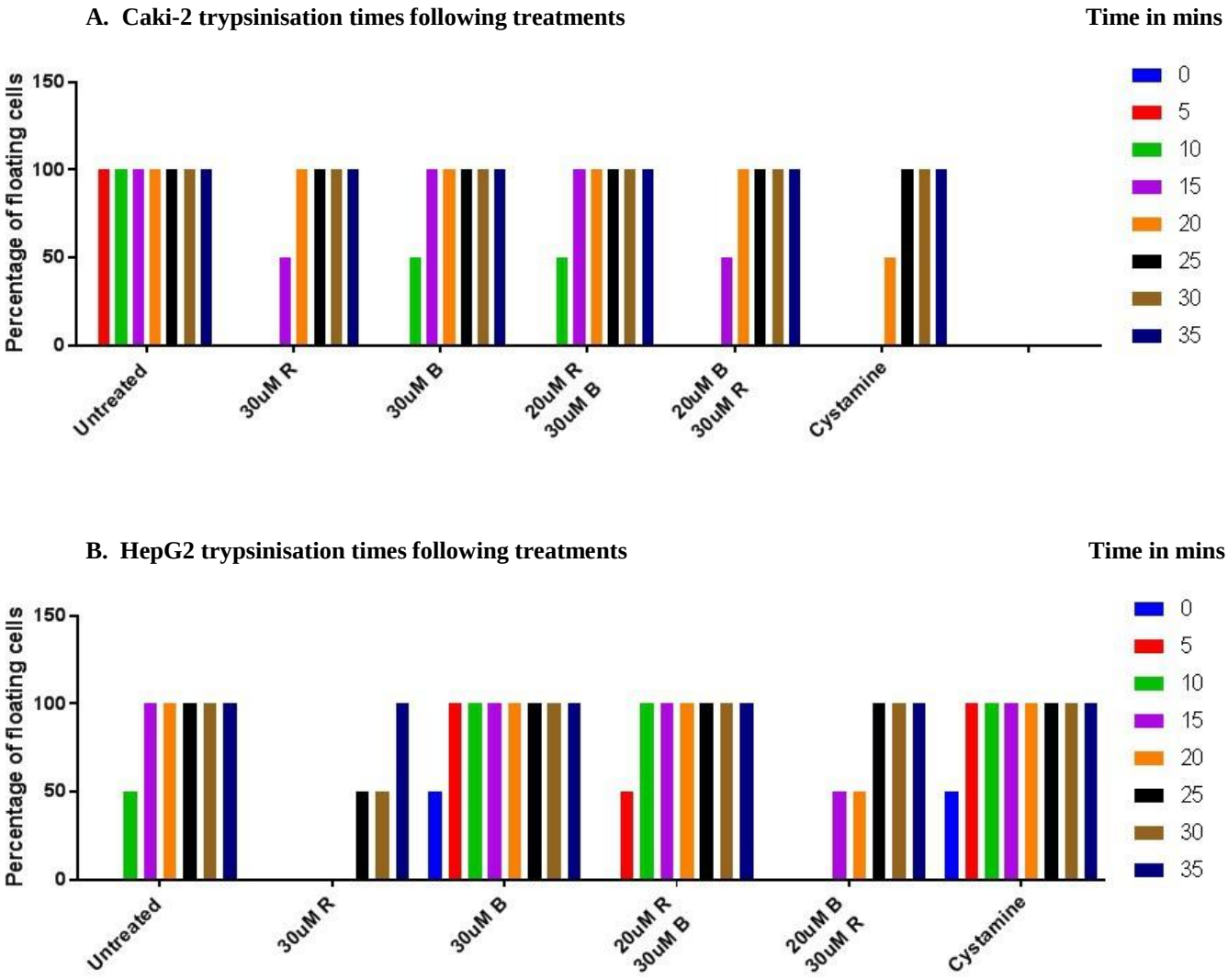


Figure 5.3. Trypsinisation times of Caki-2 and HepG2 cells in minutes following 24 hour incubation with phytochemicals or cystamine.

5.3A: Caki-2 cells were treated with either resveratrol (30 uM), berberine (30 uM), 0uM R/30 uM B, 20uM B/30 uM R, or cystamine (2 mM) for 24 hours. Wells were then washed with PBS and trypsin was added to each well. Observations were taken of each well every 5 minutes and cells were scored as having 0% floating cells, 50% (an intermediate mixture of attached and trypsinised cells) or 100%. Measurements were taken over 35 minute at 100X magiification and repeated 3 timess. The results show that resveratrol seems to reduce the effects of trypsin. Even though the resveratrol was washed from the wells prior to treatment with trypsin.

From Figure 5.3A, it can be observed that untreated Caki-2 cells lose adhesion to the well plates following only 5 minutes incubation with trypsin. When Caki-2 cells are treated with berberine, trypsinisation takes 15 minutes, whilst when they are treated with resveratrol, trypsinisation time increase to 20 minutes. The combined berberine/resveratrol treatments show an intermediate trypsinisation time with trypsinisation taking the 20 uM B/30 uM R treatment also taking 20 minutes and the 20 uM R/30 uM B taking 15 minutes. Cystamine treatment results in a trypsinisation time of 25 minutes. This result shows that resveratrol and cystamine both increase

cellular adhesion in a cell line that typicaly trypsinises within 5 minutes. Figure 5.3B, shows that untreated HepG2 cells lose adhesion to the well plates following 15 minutes' incubation with trypsin. When HepG2 cells are treated with berberine, trypsinisation takes only 5 minutes, whilst when they are treated with resveratrol, trypsinisation time increase to 35 minutes. The combined 20 uM B/30 uM R treatment results in a trypsinisation time of 25 minutes and the 20 uM R/30 uM B takes 10 minutes for 100% trypsinisation of the cells. Cystamine treatment results in 5 minutes for trypsinisation. This result shows that resveratrol increases trypsinisation time in HepG2 cells (a cell line that is already particularly adherent) and that berberine decreases adherence. Cystamine results in approximately 50% (intermadiate) loss of adherence as the cells at the 0 time point. The combined 20 uM B/30 uM R treatment had an intermediate effect on adherence, between that of resveratrol and that of berberine.

5.3. Microscopic analysis of cellular migration

5.3.1 Effects of resveratrol and berberine on cellular migration in the Caki-2 cell line

Caki-2 cells were seeded in 6 well plates and 1 mM stock solutions of berberine and resveratrol and a 12 mM stock solution of cystmaine were prepared (see Materials and Methods 2.2.1.5). Wells were treated with either 30 uM resveratrol (30 uM R), 30 uM berberine (30 uM B), 30 uM B/20 uM R, 20 uM B/30 uM R or 2 mM of cystamine. A P200 pipette tip was then used to clear a lane of cells along the base of each well by applying a single scratch along the base of each well (see Figure 5.4). Cells were then incubated for 24 hours and images and observations were recorded (see Figure 5.5) (Liang, 2007).

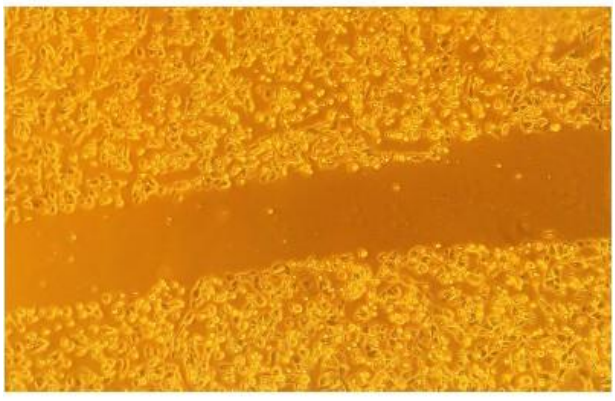


Figure 5.4. Caki-2 cells in a 6 well plate following clearing of a lane of cells by scratching a path with a P200 pipette tip.

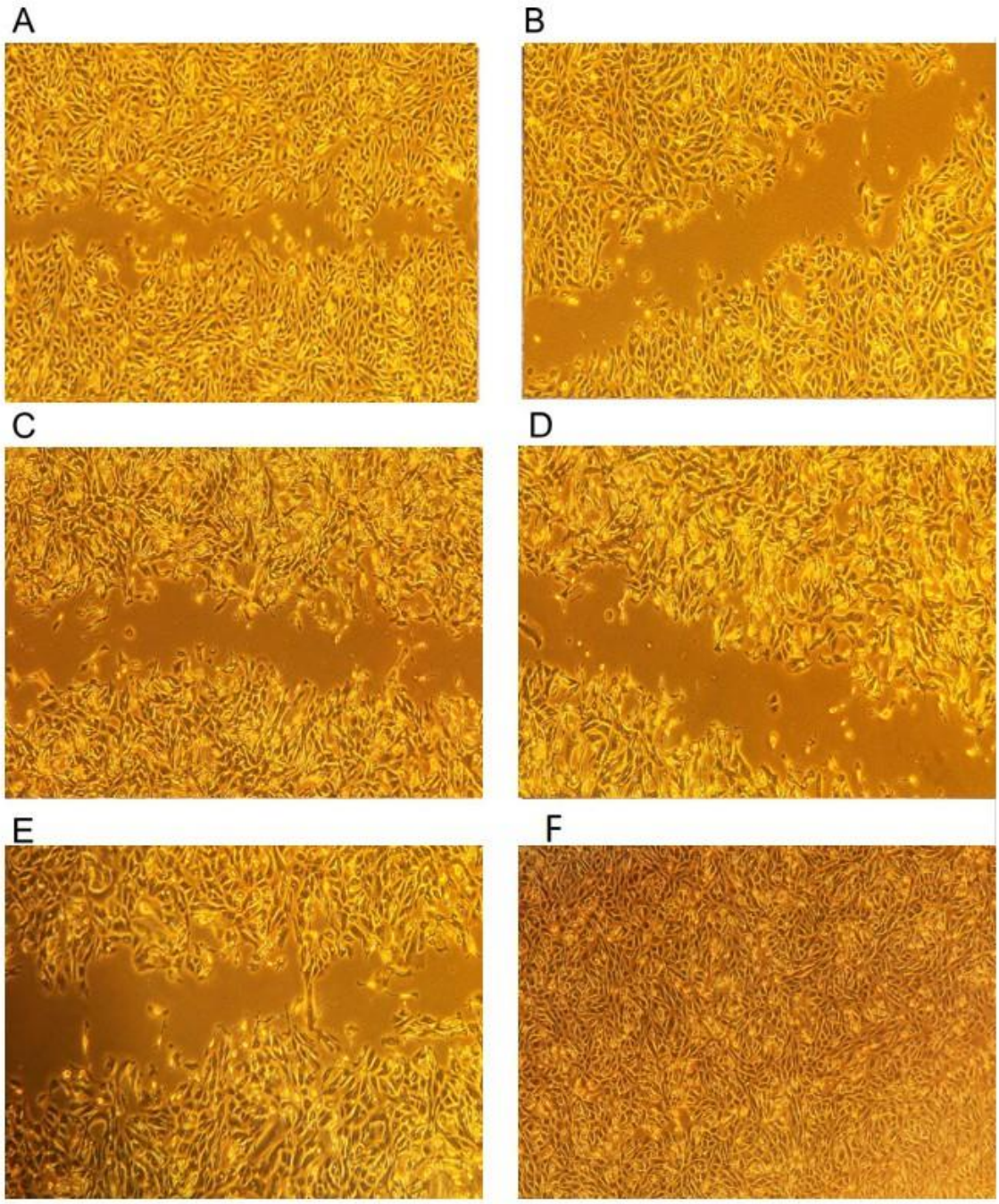


Figure 5.5. Caki-2 scratch test assay

Microscopic images of Caki-2 cells treated with resveratrol, berberine, combined resveratrol/berberine or cystamine for 24 hours following clearance of cells by scratching with a P200 pipette tip.

5.5A: Untreated control. **5.5B:** Resveratrol treated (30 uM). **5.5C:** Berberine treated (30 uM). **5.5D:** Resveratrol/berberine combined treatment (20 uM R/30 uM B). **5.5E:** Berberine/resveratrol combined treatment (20 uM B/30 uM R). **5.5F:** Cystamine treatment (2 mM).

As can be observed in Figure 5.5A, the untreated Caki-2 cells have migrated almost completely over the cleared lane of the 6 well plate following 24 hours of incubation. The resveratrol treated cells

(5.5B) can be observed to be at least equally con fluent compared with the control cells, however they have failed to migrate over the cleared lane to the extent of the untreated cells. Berberine treatment alone (5.5C) induced the same loss of shape of the cells compared with the untreated controls as was observed in Figure 5.1C, and the cells were observed to have migrated partially over the cleared lane to a greater extent than the resveratrol treated cells, but to a lesser extent than the untreated controls. A combined 20 uM R/30 uM B treatment (5.5D) induced the expected morphologic changes that combined the larger size and clearer nucleus of the resveratrol treatment with the more hairlike morphology of the berberine treatment and resulted in a migration pattern similar to that of the resveratrol treated cells. The combined 20 uM B/30 uM R treatment (5.5E) produced a similar morphology to 5.5D, with the migration pattern being observed to be similar to the berberine treated cells. Finally, cystamine treatment (5.5F) resulted in cells that were similar in morphology to the untreated controls, but more densely con fluent, with the cells being observed to have migrated completely over the cleared lane. These results suggest that resveratrol inhibits cell migration in the Caki-2 cell line, whilst the TG2 inhibitor cystamine promotes migration. Berberine treatment inhibits migration compared with controls, but has a much stronger inhibitory effect when combined with a higher concentration of resveratrol. This will be discussed further in section 6.5 and its significance relating to the initial aims/objectives of this project discussed in section 1.5.1.

5.3.2 Effects of resveratrol and berberine on cellular migration in the HepG2 cell line

Following seeding of HepG2 cells in 6 well plates and the preparation of 1mM stock solutions of berberine and resveratrol and a 12 mM stock solution of cystmaine (see Materials and Methods 2.2.1.5) wells were treated with either 30 uM resveratrol (30 uMR), 30 uM berberine (30 uM B), 30 uM B/20 uM R, 20 uM B/30 uM R or 2 mM of Cystamine. A P200 pipette tip was then used to clear a lane of cells along the base of each well (see Figure 5.6). Cells were then incubated for 24 hours and images and observations were recorded (see Figure 5.6 below).

Figure 5.6. HepG2 cells in a 6 well plate following clearing of a lane of cells by scratching a path with a P200 pipette tip

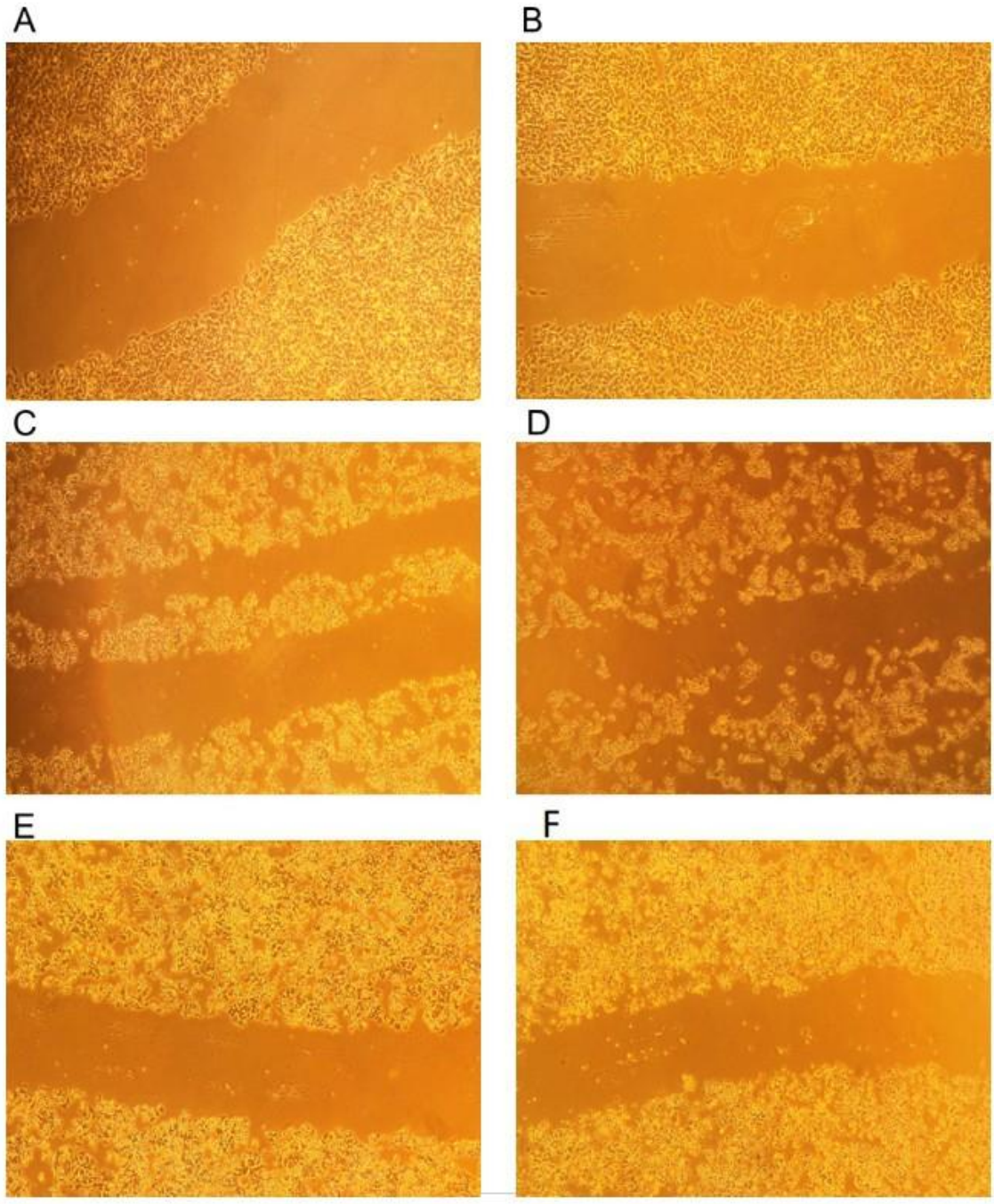


Figure 5.7. HepG2 scratch test assay

Microscopic images of Caki-2 cells treated with resveratrol, berberine, combined resveratrol/berberine or cystamine for 24 hours following clearance of cells by scratching with a P200 pipette tip.

5.7A: Untreated control. **5.7B:** Resveratrol treated (30 uM). **5.7C:** Berberine treated (30 uM). **5.7D:** Resveratrol/berberine combined treatment (20 uM R/30 uM B). **5.6E:** Berberine/resveratrol combined treatment (20 uM B/30 uM R). **5.7F:** Cystamine treatment (2 mM).

In Figure 5.7A, the untreated HepG2 cells and the resveratrol treated cells (5.7B) failed to migrate over the cleared lane of the well plate following 24 hours of incubation. The resveratrol treated cells

also were observed to be slightly less con fluent compared with the control cells. Berberine treatment alone (5.7C) induced cell death and a change in morphology, with many of the cells being observed to have migrated over the cleared lane of cells. The berberine treated cells were also only loosely attached to the well with many cells visibily floating in the media. A combined 20 uM R/30 uM B treatment (5.7D) was observed to inhibit cell migration and reduce both cell proliferation and viability. The cells did however remain mostly adherent to the well plate. The combined 20 uM B/30 uM R treatmed cells (5.7E) failed to migrate over the cleared lane, although they migrated further than the untreated or the resveratrol treated wells; this treatment also resulted in a change in morphology of the cells and a reduction in cell number compared with the controls. Finally, the TG2-inhibiting compound cystamine treated cells failed to migrate over the cleared lane and appeared non-viable and only loosely adhered to the well plates. These results indicate that the compounds affect HepG2 cells very differently to how they affect the Caki-2 cells. Whilst resveratrol appeared to increase cellular adhesion and inhibit migration in both Caki-2 and HepG2 cells, resveratrol and cystamine were observed to stimulate cell proliferation in Caki-2 cells, but inhibit it in HepG2 cells.

5.4. Cell cycle analysis

The observed effects of resveratrol in the cell adhesion and scratch test assays indicate that it modulates cell proliferation in by an unknown mechanism. One possible mechanism will be proposed in section 6.6 using protein modelling software to help to explain this behaviour. Cell cycle analysis was also performed on both Caki-2 and HepG2 cells using 30 uM of resveratrol to determine if resveratrol modulated the cell cycle. Cells were fixed in ice cold methanol for 10 minutes at -20°C and were then washed twice in PBS before being resuspended in 1 ml of PBS and incubated with the DNA-binding fluorescent compound Propidium Iodide (PI) for 15 minutes in a dark cupboard. DNA content of cells was then measured on the FL2 channel (695/40 nm) of a FACSCalibur flow cytometer.

5.4.1 Effects of resveratrol on the cell cycle in the Caki-2 cell line

Caki-2 cells were seeded in T-25 flasks in four replicates of untreated control flasks and four replicates of flasks treated with 30 uM of resveratrol. Cells were incubated for 24 hours and prepared for flow cytometry analysis, 10, 000 events were recorded and PI fluorescence was measured on the FL2-W channel. All data were analysed using the FlowJo software (LLC) and cell cycle phases were determined using the Watson model (LLC) (see Figures 5.7 and 5.8 for FlowJo

outputs).

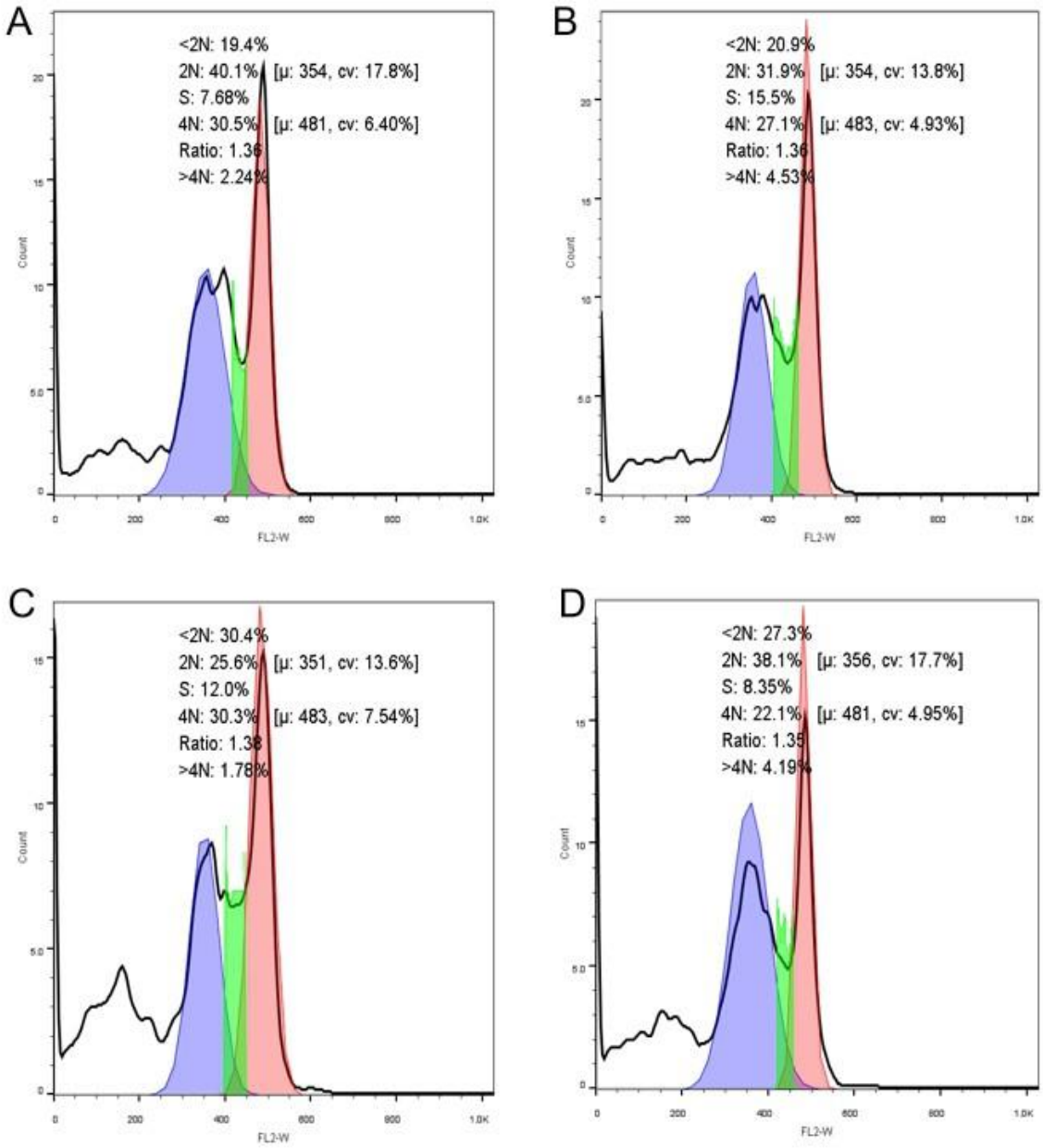


Figure 5.7. The cell cycle of untreated Caki-2 cells

FlowJo outputs of cell cycle analysis of untreated Caki-2 cells.

5.7A-D show four replicates of Caki-2 cells that were fixed in ice cold methanol, had their DNA stained with propidium iodide and were run through a FACSCalibur flow cytometer (BD Biosciences). In each case 10, 000 events were recorded and the percentage of cells at each stage of the cell cycle determined by DNA content and cell size using the Watson model (LLC). **Key** - <2N = debris and apoptotic cells, 2N = G0/G1, S = S phase, 4N = G2/M phase.

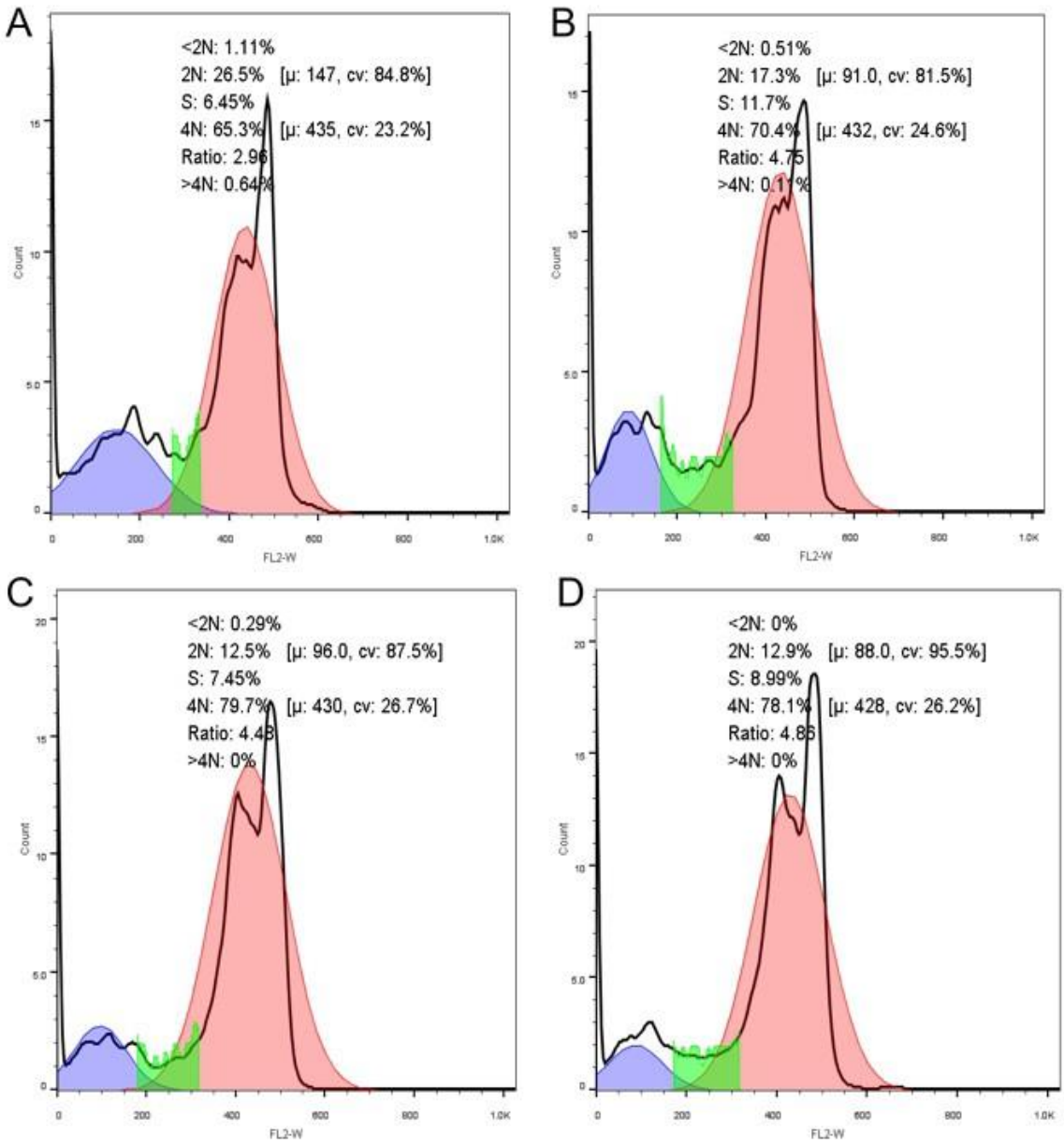


Figure 5.8. The effects of Resveratrol on the cell cycle of Caki-2 cells

FlowJo outputs of cell cycle analysis of resveratrol treated Caki-2 cells (30 uM for 24 hours.

5.8A-D show four replicates of Caki-2 cells that were fixed in ice cold methanol, had their DNA stained with propidium iodide and were run through a FACSCalibur flow cytometer (BD Biosciences). In each case 10, 000 events were recorded and the percentage of cells at each stage of the cell cycle determined by DNA content and cell size using the Watson model (LLC). **Key** - <2N = debris and apoptotic cells, 2N = G0/G1, S = S phase, 4N = G2/M phase.

5.4.2 Effects of resveratrol on the cell cycle in the HepG2 cell line

HepG2 cells were seeded in T-25 flasks in with four replicates of untreated control flasks and four

replicates of flasks treated with 30 uM of resveratrol. Cells were incubated for 24 hours and prepared for flow cytometry analysis as described in section 2.2.16. 10, 000 events were recorded and PI fluorescence was measured on the FL2-W channel. All data were analysed using the FlowJo software (LLC) and cell cycle was determined using the Watson model (see Figures 5.9 and 5.10 for FlowJo outputs).

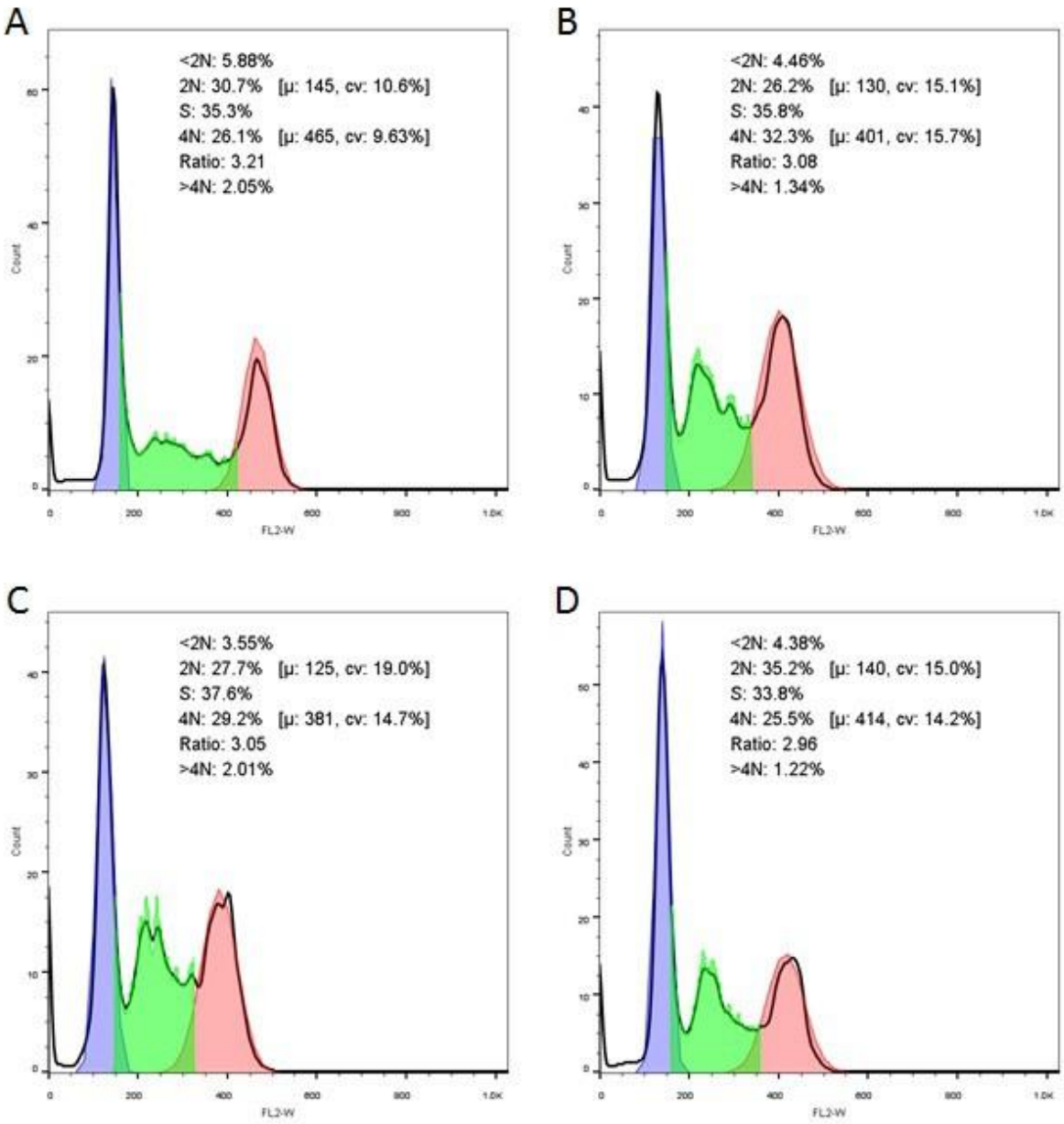


Figure 5.9. The cell cycle of untreated HepG2 cells

FlowJo outputs of cell cycle analysis of untreated HepG2 cells.

5.9A-D show four replicates of HepG2 cells that were fixed in ice cold methanol, had their DNA stained with propidium iodide and were run through a FACSCalibur flow cytometer (BD Biosciences). In each case 10, 000 events were recorded and the percentage of cells at each stage of the cell cycle determined by DNA content and cell size using the Watson model (LLC). **Key** - <2N = debris and apoptotic cells, 2N = G0/G1, S = S phase, 4N = G2/M phase.

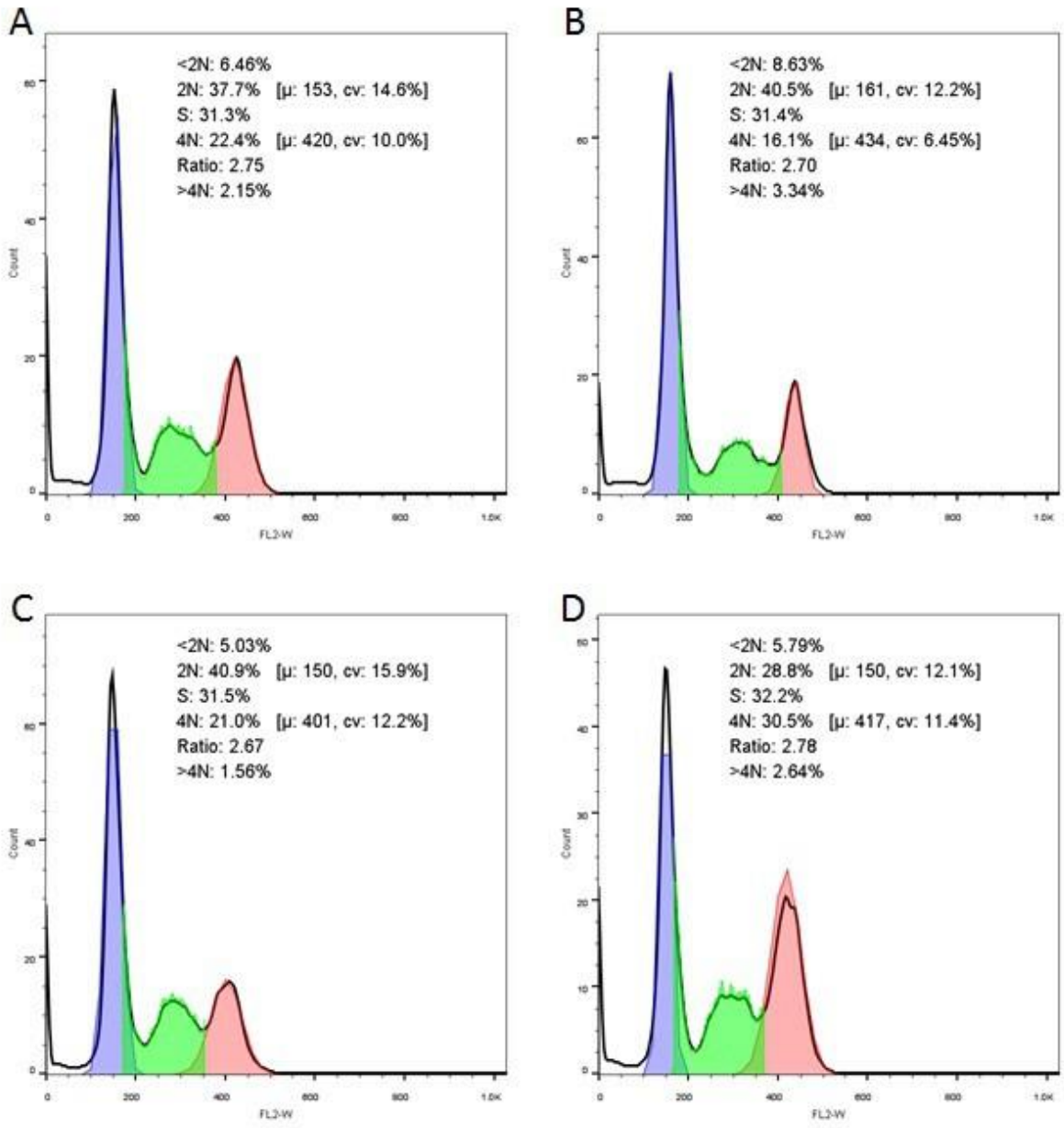


Figure 5.10. The effects of Resveratrol on the cell cycle of HepG2 cells

FlowJo outputs of cell cycle analysis of resveratrol treated HepG2 cells (30 uM for 24 hours.

5.8A-D show four replicates of HepG2 cells that were fixed in ice cold methanol, had their DNA stained with propidium iodide and were run through a FACSCalibur flow cytometer (BD Biosciences). In each case 10, 000 events were recorded and the percentage of cells at each stage of the cell cycle determined by DNA content and cell size using the Watson model (LLC). **Key** - <2N = debris and apoptotic cells, 2N = G0/G1, S = S phase, 4N = G2/M phase.

5.4.3 Statistical analysis of the effects of resveratrol on the cell cycle in the Caki-2 and HepG2 cell line

The affects of resveratrol on the cell cycle for Caki-2 cells and HepG2 cells were compared graphically (see Figures 5.11 and 5.12) and statistical analyses were performed.

An independent-samples t-test was conducted to compare the effects of 24 hours' exposure to resveratrol (30 uM R) on the percentage of Caki-2 cells in the G0/G1 stage of the cell cycle (5.11A). There was a signi ficant difference in the scores for the untreated group (M=33.93, SD=3.278) and the resveratrol treatment group (M=17.3, SD=3.254) conditions; t (3.599)=6 p = < 0.0114. When comparing the percentage of cells in S phase (5.11B), t-test did not reveal a signi ficant difference between untreated treatment group (M=10.88, SD=1.808) and the resveratrol group (M=8.648, SD=1.144) conditions; t (1.045)=6, p = < 0.3365. A t-test analysis of the effects of resveratrol on the percentage of cells in G2/M phase (5.11C) revealed a signi ficant difference in scores between the untreated group (M=27.5, SD=1.961) and the treated group (M=73.38 SD=3.371) conditions; t (11.76)=6, p = < 0.0001. These results show that resveratrol either stimulates the cell cycle and therefore proliferation in the Caki-2 cell line or results in an accumulation of cells in the G2/M phase, without the cell cycle completing and mitosis occurring. A check of cell counts obtaining during flow cytometry for the resveratrol-treated cells showed that the cell number were the same as for the controls, (backed up by the images taken in the scratch test assay), which shows that the later explanation is correct (see section 5.2.1).

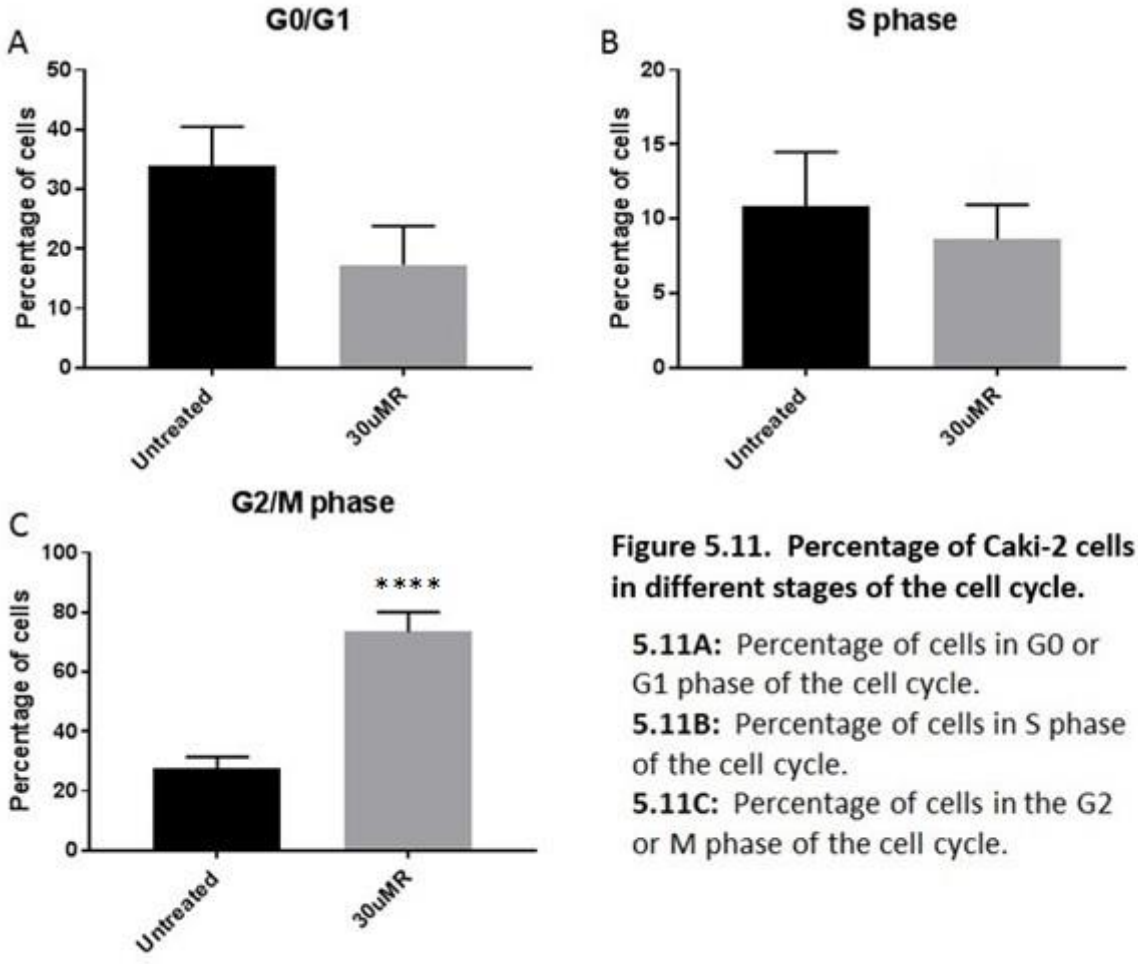


Figure 5.11. Percentage of Caki-2 cells in different stages of the cell cycle.

5.11A: Percentage of cells in G0 or G1 phase of the cell cycle.
5.11B: Percentage of cells in S phase of the cell cycle.
5.11C: Percentage of cells in the G2 or M phase of the cell cycle.

From the results illustrated in Figure 5.12 and analysed below, it can be seen that the combined resveratrol treatment has a small inhibitory effect on the cell cycle. Although this is only a slight effect, it is in stark contrast to the observations in Figure 5.11 which showed that when Caki-2 cells are treated with resveratrol, there is an increase in the cell cycle. This indicates that resveratrol might help to initiate the cell cycle in Caki-2 cells, yet inhibit it in HepG2 cells. This could relate to the differing levels of the TG2-isoforms observed in section 4.4. Although the data outlined in that section requires is only partially complete.

An independent-samples t-test was conducted to compare the effects of 24 hours exposure to resveratrol (30 uM R) on the percentage of HepG2 cells in the G0/G1 stage of the cell cycle (5.12A). There was no signi ficant difference in the scores for the untreated group (M=29.95, SD=1.984) and the resveratrol treatment group (M=36.98, SD=2.816) conditions; t (2.039)=6 p = < 0.0876. When comparing the percentage of cells in S phase (5.12B), t-test revealed a significant difference between the untreated group (M=35.63, SD=0.7836) and the resveratrol treatment group (M=31.6, SD=0.2041) conditions; t (14.971)=6, p = < 0.0025. A t-test analysis of the effects of resveratrol on the percentage of cells in G2/M phase (5.12C) did not reveal a signi ficant difference in scores between the untreated group (M=28.28, SD=1.568) and the treated group (M=22.5 SD=2.989) conditions; t (1.711)=6, p = < 0.1379. These results show that resveratrol has a small, but signi ficant inhibitory effect on the cell cycle.

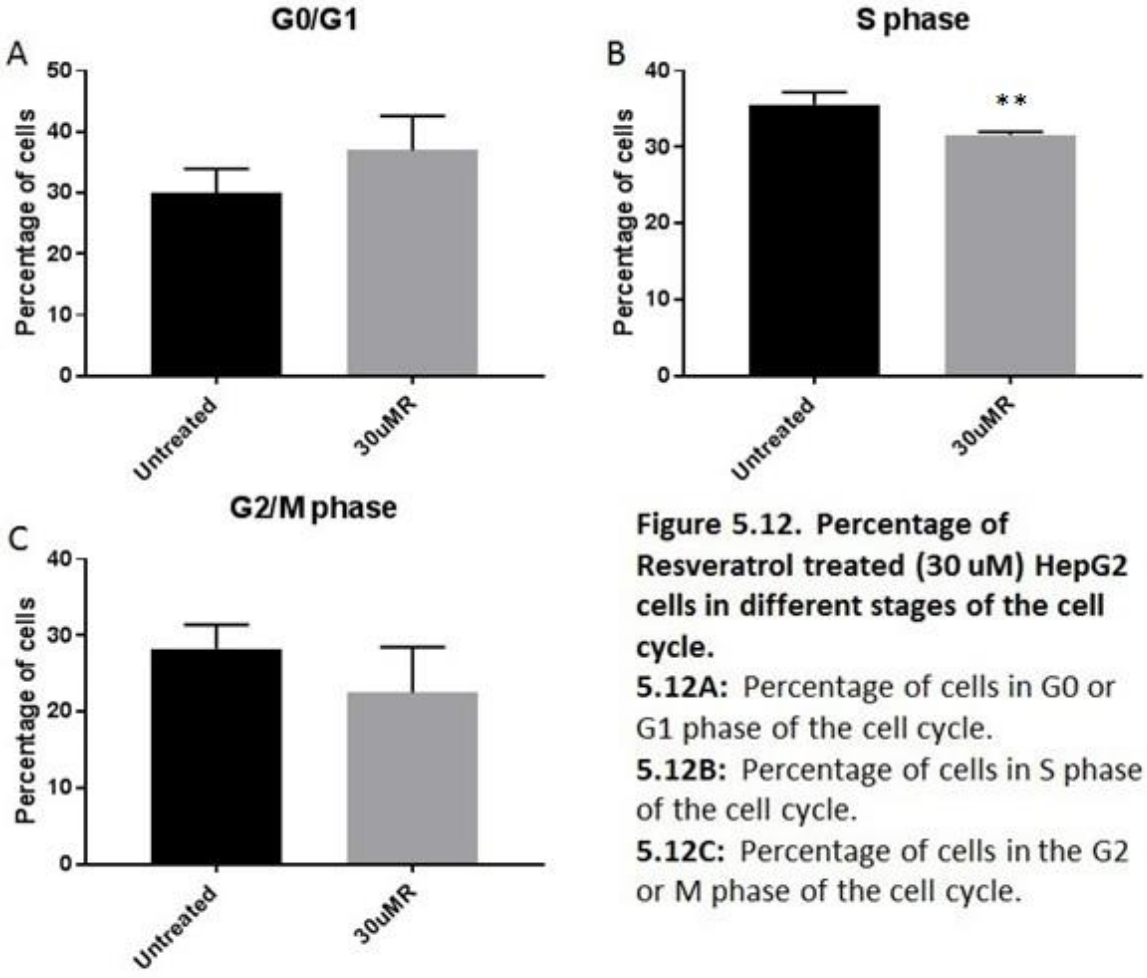


Figure 5.12. Percentage of Resveratrol treated (30 uM) HepG2 cells in different stages of the cell cycle.

5.12A: Percentage of cells in G0 or G1 phase of the cell cycle.

5.12B: Percentage of cells in S phase of the cell cycle.

5.12C: Percentage of cells in the G2 or M phase of the cell cycle.

5.5. Summary

The series of experiments outlined in this chapter show that resveratrol increases adherence in both Caki-2 cells and in the HepG2 cell line. The TG2 inhibitor cystamine notably results in a loss of cell adhesion in HepG2, whilst it increases adherence in Caki-2. The effects on the cell cycle are also of note, with resveratrol modulating the cell cycle in both Caki-2 and HepG2 cells. The scratch test assays show that both cell lines reduce their speed of migration when treated with resveratrol. The trypsinisation times show that the increase in adherence is relatively stronger in Caki-2 cells than in HepG2 cells.

HepG2 cells show a far more favourable and possibly chemotherapeutic response to resveratrol and to berberine, with the combined treatment both slightly inhibiting the cell cycle and also increasing adherence, and thus potentially preventing metastasis.

Chapter Six Interpretation of results and future work

6.1. Introduction and reiteration of objectives

The aim of this project was to evaluate the natural phytochemicals berberine and resveratrol, in isolation and in combination to determine whether they showed any promise as chemotherapeutic agents and whether these chemotherapeutic effects related to the modulation of transglutaminase 2 (TG2). The model systems used were initially the 1321N1, Caki-2 and HepG2 cancer cell lines. These were chosen as they represented cancers from different types of tissues, expressed TG2 and exhibited different sensitivities to a variety of chemotherapeutic agents. The cells' organs of origin were - 1321N1 (astrocytoma from the brain), Caki-2 (renal carcinoma with an origin in the kidney) and HepG2 (a cancer cell line originating from the liver). These cell lines were used as model systems to evaluate the effects of the phytochemicals on cell viability - particularly apoptosis and necrosis - TG2 activity, to determine how they interacted with the cell cycle (particularly in relation to cellular proliferation) and to measure their effects on both cellular adhesion and cell migration.

6.2. Why phytochemicals?

Phytochemicals have become a subject of particular interest in recent years to scientists researching a variety of diseases, including cancer and mutagenesis (Young-Joon Surh, 2003). There are a number of reasons for this, including (but not limited to) their proven individual effects on the behavior of a variety of cancer cell lines (see section 1.4 for further details on these previous studies). The easy availability of phytochemicals due to the fact that there are no patents associated with the pure forms of these naturally occurring products was also a factor in choosing these compounds to study further in this research project; this means that they can usually be purchased for a low price or extracted directly from source material by the research scientists themselves. Phytochemicals can often be easily extracted, for example resveratrol can be extracted directly from the skins of red berries using a protocol such as that used at the Nutrició i Bromatologia at the University of Barcelona (Romero-Pérez, A.I, 2000) with very little associated cost and time.

Another reason why phytochemicals have become such a prominent topic of research in recent years is due to the fact that they do not require FDA (Food and Drug Administration) approval in the U.S

or NICE (National Institute for Medical Excellence) approval in the UK in order to be used therapeutically. Also many of these compounds do not fall into regulatory acts such as the Control of Substances Hazardous to Health (COSHH) resulting in far less administration overheads for companies and academic institutions working with phytochemicals.

Taken together, using phytochemicals, rather than regulated or novel compounds in research and medicine means that the months and sometimes years taken to complete the required paperwork and to pass research proposals through the various ethics panels can be somewhat shortened. Although due diligence and a thorough review of any ethical conciderations is still required when considering any novel compound or combination of compounds for clinical use. These factors, together with the results of some in fluential papers into the efficacy of phytochemicals such as curcumin, a compound found to have anti-inflammatory and anti-cancer affects (Aggarwal, 2003) have lead to increase in interest in this group of compounds.

6.3. Why resveratrol and berberine?

There has been an increasing body of work published in recent years into the compounds berberine and resveratrol (see sections 1.4.2 and 1.4.3) particular in relation to their potential use as chemotherapeutic agents (Lizuka, 2000 and Zhang, 2013). Research into these compounds has lead not only to interest from the academic community, but also from the pharmaceutical industry. An example of the extent of this interest was the purchase of the small Harvard spin-off company Sirtris in 2008 by GlaxoSmithKline for $720 million dollars. GSK, by acquiring Sirtris gained access to the expertise that Sirtris had built up through initially studying the anti-in flammatory, metabolic, neurologic and immunologic affects of resveratrol; speci fically relating to the interaction of resveratrol with a class of enzymes known as sirtuins (Howitz, 2003) that are are amongst other things thought to be involved in the process of ageing. Interest in berberine began to increase when it was found to inhibit proliferation of several oesophageal cell lines (Lizuka, 2000) and much research has been done into this phytochemical since in relation to cancer and in flammation (Mantena, 2006).

Resveratrol and berberine are structurally related polyphenol compounds and both have been shown to affect cells in a variety of ways (see section 1.4.1). For example resveratrol has been shown to induce the formation of autophagosomes (Zhang, 2013) and berberine has been shown to increase the expression of the pro-apoptotic Bcl-2 protein Bax (Wang, 2010). Although berberine had previously been used as a chemotherapeutic agent and was proven to induce apoptosis in the DU145 cancer cell line (Mantena, 2006) at a range of 10-100 uM and had also been observed to counteract

the upregulation of TG2 in astroglial cell cultures; no work had yet been done relating to the regulation of TG2 by this phytochemical. This clear gap in knowledge required further in-depth investigation, which was the purpose of much of the work conducted during this project (see sections 3.1-5.5). The results discussed in those sections showed that these phytochemicals show great potential as clinical chemotherapeutics.

Resveratrol was also known to interact with cells by inhibiting migration in TG2 expressing cells (Kumar, 2014), but it was still unclear how either of these compounds interacted with TG2 and no research had been published into the affects of berberine and resveratrol on the TG2-S and the TG2-L isoforms of transglutaminase 2. To this end it was decicded to use Real-time PCR following treatment of the HepG2 and Caki-2 cell lines with the phytochemicals to determine if any modulation of TG2-S and TG2-L expression was induced.

*** Note.** A modulation of the TG2-S isoform was indeed observed and found to be significant for berberine-treated HepG2 cells (see section 4.4). These results point towards a possible mechanism of action for the observed effects of berberine on apoptosis in HepG2 (Hepatocarcinoma), specifically that it might induce apoptosis via the upregulation of TG2-S, a known pro-apoptotic protein (Antonyak et al., 2006). Further elucidation of this pathway of action could lead to novel clinical therapies for patients suffering from a variety of liver cancers.

As modulation of TG2 is known to both induce apoptosis (Fesus, 1992) and to inhibit it (Hermans, 2006) this was the initial motivation for deciding to use these structurally related compounds when developing the model system. Also, as these compounds had never been used in combination and both have known, but differing chemotherapeutic affects, resveratrol and berberine were tested on the model system both in isolation and in combination to determine if the combined affect of these phytochmeicals differed from their individual affects.

6.4. Phytochemicals and the 3 main aspects of cancer

Cancer is a disease that results from a variety of genetic and epigenetic causes (see section 1.2) but goes through several key developmental stages. If a cell is to become cancerous, it needs to develop the ability to evade the mechanism of apoptosis. When a cell becomes damaged beyond the ability of its DNA repair enzymes to repair and is no longer able to function correctly apoptosis is usually triggered. The damaged cell may initiate the intrinsic pathway of apoptosis (section 1.1.2) resulting in activation of the caspases, DNA fragmentation, breakdown of the cellular components, which are then accumulated in vesicles; then once the cell is dead the vesicles diffuse into the surrounding

tissues where they are removed by macrophages. Alternatively, if the intrinsic pathway fails, apoptosis can be triggered externally by patrolling cells of the immune system when they recognise damage to the cell, via the extrinsic pathway of apoptosis (section 1.1.3). The extrinsic pathway also results in the activation of caspases, the formation of vesicles and then the disintegration of the cell; the debris of which is also removed by cells of the immune system. To become cancerous, a cell must also achieve self-suf ficiency in proliferation signalling, so that it can divide despite the absence of extracellular growth factors and then to transition from a benign to a malignant tumour, the cell must acquire the ability to invade other tissues (metastasis).

The phytochemicals resveratrol and berberine have both been shown to interact with one or more of these pathways (see section 1.4), however they had not previously been used in combination. TG2 has been shown to be involved in *all* 3 of these pathways (see section 1.3.2).

6.5. Summary of findings and analysis of results

The work undertaken during the course of this project can be divided into 5 categories, which were intended to answer the primary research questions listed in section 1.5.1 and summarized in the decision tree (Figure 1.8). The research questions will be restated below, followed by a summary of the findings and how they relate to these "specific" research questions.

Question 1: Can a novel phytochemical drug therapy be developed that effectively kills neoplastic cells in any or all of the cancer cell models used in this research? The models studied represented 3 disperate forms of cancer; these were liver cancer (using the HepG2 cell line as a model), renal cancer (using Caki-2) and a form of brain cancer known as astrocytoma (using 1321N1).

Question 2: If a phytochemical drug therapy can be developed and is proven to induce a substantial and statistically significant degree of cell death in one or all of the model systems, can the cell death be induced at clinically achievable doses?

Question 3: Do the phytochemical treatments tested exert their effects on cancer by modulating TG2 expression and/or activity? If so, then what are the possible mechanisms by which this modulation might be occurring?

The research was divided into the below categories:

- **Cell viability assays.** CCK-8 was used to determine general cell viability, followed by Annexin V/Propidium Iodide flow cytometry analysis to determine the percentages of apoptotic and necrotic cells in treated and untreated populations. This helped to answer research questions 1 and 2 (see sections 3.2 and 3.4).
- **TG2 activity analysis.** Incubation of cells with monodansylcadaverine was used to measure TG2 activity using flow cytometry to determine relative TG2 activity in treated and untreated populations of cells. This answered research question 3 (section 4.2).
- **TG2 inhibition analysis.** Following incubation of treated and untreated cells with cystamine to inhibit TG2 activity, Annexin V/Propidium Iodide was used in combination with flow cytometry to determine how inhibition affected cell viability. Helping to answer question 3 (section 4.3).
- **Cell adhesion and migration assays.** Trypsinisation timing was used to measure the affects of treatments on cellular adhesion and scratch test assays were used to measure cell migration. Elucidating answers to questions 1 and 2 (sections 5.2 and 5.3).
- **Cell cycle analysis.** Treated and untreated cells were fixed and incubated with the DNA-binding dye Propidium Iodide and the stage of the cell cycle that populations of cells were in was determined using flow cytometry. Answering questions 1 and 2 and helping to elucidate question 3 (section 5.4).

In Chapter One the literature review revealed gaps in current knowledge relating to the phytochemicals berberine and resveratrol and also in relation to the TG2 enzyme and the mechanisms by which it in fluences cell viability. The literature showed that the effects of berberine on TG2 activity are unclear and poorly researched. Resveratrol had been observed to inhibit tumour formation in breast cancer (Venugopal and Liu, 2012) and also to inhibit cell proliferation in the A549 lung cancer cell line, but the mode of action of this compound was also unclear. Following the literature review and with the aid of decision trees (Figures 1.8 and 2.1) it was decided to test the effects of resveratrol and berberine in isolation and in combination on model cancer cell lines to explore the effects of these compounds further.

In Chapter Three, the results of a number of cell viability assays were presented and statistical analysis was conducted to determine whether resveratrol, berberine or a combination of the two had any effect on the percentages of healthy cells. The first series of assays (section 3.2.1-3.2.3) used the CCK-8 protocol (see Materials and Methods section 2.3.4.2) to measure and compare the

relative numbers of viable cells present in 96 well plates treated with a concentration curve of resveratrol, berberine or combinations of the two. The initial series of CCK-8 assays revealed that the 1321N1 cell line was highly susceptible to cell death induced by berberine at doses as low as 5 uM. 1321N1 was however not shown to be susceptible to resveratrol at any of the range of concentrations used (0-75 uM). When exposed to either berberine or to resveratrol, there was a small, but statistically signi ficant effect on cell viability in the Caki-2 cell line, but when exposed to a combined treatment, no effect on cell viability was observed. The HepG2 cell line showed a highly signi ficant loss of cell viability when exposed to either the individual phytochemicals or to a combined treatment.

Analyses of the results of the CCK-8 data provided the basis for choosing fixed concentrations of the compounds to use for treating the cell lines in a downstream flow cytometry Annexin V/Propidium Iodide assay (see Materials and Methods 2.3.4.3). This assay determined the percentages of viable, early apoptotic, late apoptotic and necrotic cells in a population of treated cells and allowed for the comparison of these percentages with those of untreated controls (section 3.3.2). 1321N1 cells showed a highly signi ficant movement of almost 100% from a viable to an apoptotic and necrotic state when exposed to 10 uM of berberine and showed a signi ficant decrease in cell viability when exposed to resveratrol at concentrations of 50 uM and above (approximately 10% decrease at 50 uM). It should be noted that 50 uM of resveratrol is a concentration not achievable in the CNS either via oral or IV infusion.

Flow cytometry revealed that Caki-2 cells moved from a viable to an apoptotic state when exposed to berberine, except at the higher concentration (120 uM) were there was an increase in the number of viable cells. This observation may be of note to future studies, but could possibly be an experimental artefact due resulting from damage to the cells. The change in viability of the Caki-2 cells at none of the concentration ranges used was greater than approximately 6-8% however. When exposed to resveratrol, the Caki-2 cells seemed to reduce their levels of apoptosis and necrosis and increase their viability by approximately 5-7%, which may indicate that resveratrol has a protective effect on this cell line. When Caki-2 cells were exposed to a steady 30 uM of resveratrol together with a concentration range of berberine, there was a statistically signi ficant, but small increase in viability of 1-2%, but only at the higher (60 uM B/30 uM R) concentrations. For HepG2 cells, it was shown that there was a steady decrease in the percentage of viable cells and an increase in the number of apoptotic and necrotic cells as the concentration of berberine increased, with an approximate decrease of 10-15% in the number of viable cells being observed following a 30uM treatment and a decrease of up to 60% at 90 uM. When treated with resveratrol, there was also shown to be a reduction in viability as the concentration range was increased, but

this reduction only became signi ficant at concentrations of 60 uM and above. When HepG2 cells were exposed to a steady 30 uM of resveratrol together with a concentration range of berberine, there was a large and significant increase in the percentage of apoptotic and necrotic cells of up to 40% for combined treatments.

Following analyses of the flow cytometry cell viability results, it was decided that, as resveratrol has low bioavailability in the CNS following either following oral or IV administration in animal models, (Asensi, 2002) and has resveratrol had already been observed to have little effect on the viability of 1321N1 cells using the CCK-8 assay at concentrations below 75 uM, this cell line was not subjected to any further study. It was decided however that although Caki-2 was only moderately susceptible to these compounds, it might be useful to analyse this cell line further as there was a small, but signi ficant response to both compounds some response so it would provide a useful comparator for the HepG2 cell line, which showed a highly signi ficant response to berberine, resveratrol and to the combined treatment.

In Chapter 4, changes in TG2 activity relative to untreated controls were measured using monodansylcadavarine as an indicator of activity, with higher fluorescence indicating higher activity (section 4.2). In Figures 4.1 and 4.3 it can seen that when treated with a 30 uM dose of berberine both Caki-2 and HepG2 responded by increasing TG2 activity compared with untreated controls, but TG2 activity remained roughly at the level of untreated controls in both cell lines when treated with 30 uM of resveratrol. When challenged with a combined 20 uM R/30 uM B combination, Caki-2 cells the increase in TG2 activity was similar to that seen in the berberine treatment group, but when treated with 20 uM R/30 uM B Caki-2 cells showed a smaller increase in TG2 activity, although the activity was still much higher than in untreated controls. For HepG2 cells, both combined treatments gave a similar increase in TG2 activity compared with controls. As HepG2 cells had been shown to show a large decrease in viability when treated with 30 uM of berberine (Figure 3.1) it was decided to measure the activity of TG2 at a range of berberine concentrations around the 30 uM point (0 uM, 10 uM, 20 uM, 30 uM and 40 uM) and when doing so it was shown that a spike of TG2 activity was observed at 10 uM, where activity was observed to almost double compared with controls before reducing to a level around 5 times higher than the controls at 20 and 30 uM treatments. As this 10 uM spike stood out from the activity observed with other berberine concentrations, this series of experiments was repeated and the 10 uM spike was again observed. This increase in TG2 activity at a concentration just below the level required to induce a high level of apoptosis may show that the high expressing TG2 cell line reacts when challenged with berberine by increasing its TG2 activity, which may help the cells to tolerate the compound. This would agree with the theory that TG2 when active increases drug resistance

(Herman's, 2006 and Kumar, 2012), but as the Caki-2 cell line shows more tolerance to berberine, despite having a lower TG2 expression, it may be that the increase in TG2 activity that was observed acts to initiate cell death, which would agree with other published results (Melino, 1994 and Rodolfo, 2004). As the high-expressing 1321N1 cells also showed a low tolerance to berberine (section 3.3.2) perhaps it is a feature of cancers that express high levels of TG2 that any substantial increase in its activity reduces cell viability.

To determine whether the observed increase in TG2 activity was causal or merely correlated with a loss of cell viability, TG2 was inhibited with the competitive inhibitor cystamine (section 4.3). The results of this inhibition did not signi ficantly affect the levels of apoptosis in the low TG2 expressing Caki-2 cells following a 24 hour 2 mM treatment. (Figure 4.7), however the response to TG2 inhibition in the HepG2 cell line was dramatic, with almost 100% apoptosis (and some necrosis) being observed. These results disagree with the previously outlined hypothesis that increased TG2 activity induces cell death and supports the idea that TG2 is protective of HepG2 cells and the observed increase in activity just prior to cell death is a mechanism that the cell uses to protect itself from berberine. This behaviour may be a feature of other high TG2 expressing cancers, although this is speculative, based on one series of experiments performed and further research would need to be done to prove this hypothesis.

The above results lead to determining whether the transglutaminase 2 short and long forms (TG2-S and TG2-L) were present in the Caki-2 and HepG2 cell lines. Previous studies had indicated that the isoforms, which are derived by alternative splicing of the primary mRNA transcript were linked to susceptibility to cell death in cancer (see section 1.3.4.3). The TG2-S isoform was thought to be pro-apoptotic whilst the TG2-L isoform was believed to be involved in cell survival (Tee, 2009). At the time of this research, there were not yet antibodies available for these newly-discovered TG2 isoforms, so protein levels of the isoforms could not be measured, however relative mRNA levels could be determined using qPCR (see section 4.4.2). Before performing qPCR however, primers were designed that were specific for the isoforms by exon jumping (section 2.3.7.2) and both cell lines were tested for the presence of TG2-S and TG2-L (section 4.4.1). The isoforms were identi fied in both Caki-2 and HepG2, a novel discovery which may indicate that these isoforms are more prevalent than was previously thought to be the case. The qPCR results following treatments with the phytochemicals were inconclusive for Caki-2 cells, which may have been due to the relatively small replicate number, or that the method used was the one proposed by Bustin (2000), a method that is best practice so followed in all qPCR assays. Bustins method does however generate a large standard deviation when applied to the Delta Delta CT method used in this qPCR analysis. An alternative approach from using Bustins technique is to

generate the error bars from the final fold change calculations, when this is done (data not included), then there is a significant difference in isoform expression for both cell lines between untreated controls and the phytochemical treatments. As Bustins method has become almost a standard however, this method was used and despite the generally large standard deviation the TG2-S isoform was shown to be overexpressed in the HepG2 cell line when exposed to 30 uM of berberine, a concentration that induces approximately 50% cell death following 24 hours exposure. This may indicate that this TG2 isoform when overexpressed is involved in the process of cell death in HepG2 cells.

In Chapter 4 the microscopy-based cellular adhesion and migration assays showed that berberine affected the morphology of both Caki-2 and HepG2 cells at 30 uM. In Caki-2 the berberine treatment resulted in a more elongated, thinner cell (Figure 5.1C) whilst in HepG2 the morphology granular appearance than in untreated controls (Figure 5.2C).
These changes in morphology were also present when cells were challenged with combined 20 uM B/30 uM R and 20 uM R/30 uM B treatments, to a greater extent when the berberine concentration was higher and to a lesser extent when the resveratrol concentration was higher (see Figures 5.1D-E and 5.2D-E). Relating the morphological changes to the cell viability studies performed in Chapter 3, this change in morphology does not correlate with a decrease in cell viability in the Caki-2 cell line, however it does correlate with such a decrease in the HepG2 cell line (see sections 3.3.2-3.3.4). There is however a correlation between the change in morphology in Caki-2 cells when treated with berberine and their ability to migrate over a cleared lane of cells (Figure 5.5C) with berberine-treated cells migrating slower than untreated controls, a correlation that was not observed in HepG2 cells (Figure 5.7C). Resveratrol also induced a change in morphology in the Caki-2 cell line (Figure 5.1B) with the treated cells showing a larger nucleus when challenged with 30 uM of resveratrol. The HepG2 cells were not shown to change in morphology, however the observed populations appeared less densely clustered and the layers of cells did not seem as deep as in untreated controls (Figure 5.2B).

When treated with resveratrol, both Caki-2 and HepG2 cells became more adherent, as was evidenced by the trypsinisation timing assay (section 5.2.3). This was an observation of particular note in the Caki-2 cell line which normally trpsinises in less than 5 minutes when untreated, but took 20 minutes to trypsinise when exposed to 30 uM of resveratrol. HepG2 cells increased their average trypsinisation time from 15 minutes in untreated controls to 35 minutes when treated with 30 uM of resveratrol. The scratch test assay results (section 5.3) showed that resveratrol inhibited cellular migration in both cell lines, with the effect being greater in HepG2. Combined 20 uM B/30 uM R and 20 uM R/30 uM B treatments intermediately affected migration with the higher

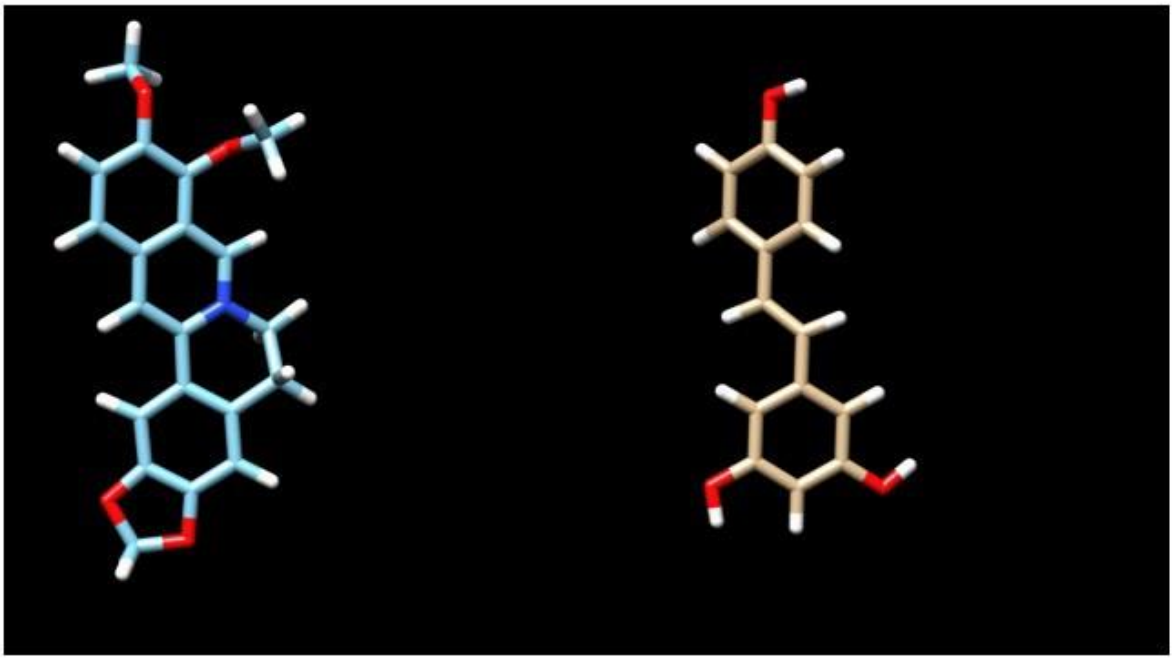
resveratrol combination inhibiting migration to a greater extent than the lower concentration. As resveratrol had shown a particularly strong effect on inhibiting cellular migration in both cell lines, and to determine if this lack of cell migration resulted at least partially from an inhibition of the cell cycle, both cell lines were treated for 24 hours with 30 uM of resveratrol and the effects on the cell cycle were analysed using flow cytometry (section 5.4). The results showed that treated Caki-2 cells accumulated in the G2/M phase stage of the cell cycle, whilst the HepG2 cells accumulated in G0/G1.

6.6. Introduction of a proposed mechanism for interpreting the observed results

From the results discussed in section 6.5 it is clear that resveratrol and berberine have very different effects on on the model systems. Resveratrol inhibits both the cell cycle and cellular migration, whilst increasing cellular adhesion in both cell lines. Berberine however slightly inhibits migration in Caki-2 cells, but in the HepG2 cell line has a strong effect on cell viability. TG2 activity is increased in both models when challenged with berberine, but not when challenged with resveratrol, despite both compounds having very similar structures (see Figures 6.1 and 6.2).

A. Berberine 3D model

B. Resveratrol 3D model



C. Left: Superimposition Berberine (light blue) and Resveratrol (light brown). Centre and right: Rotation of 90° (centre) and 180° (right) from left image

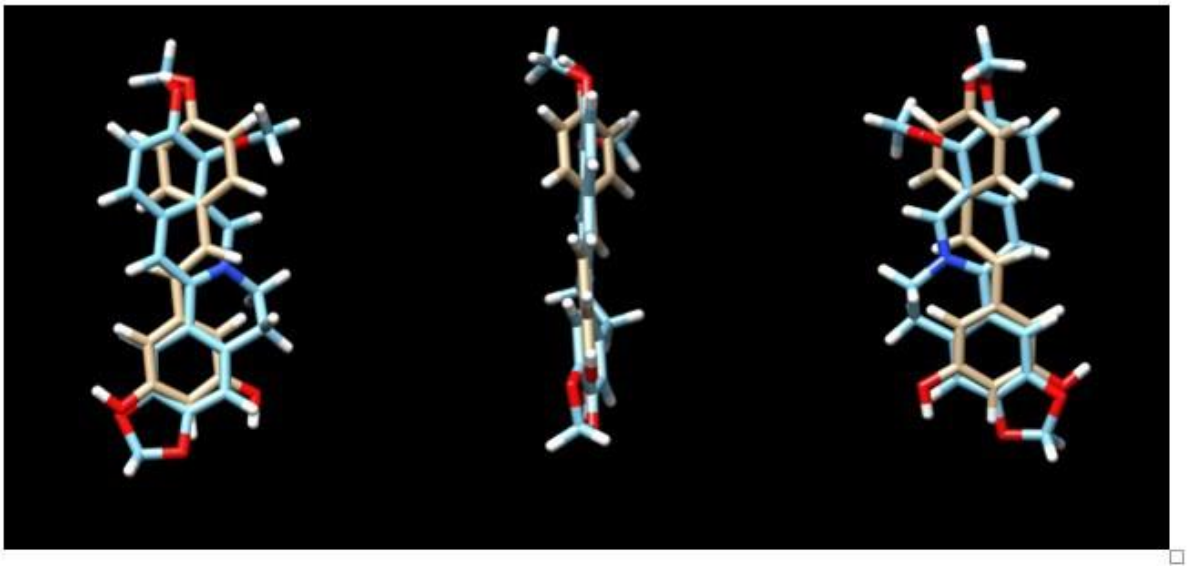
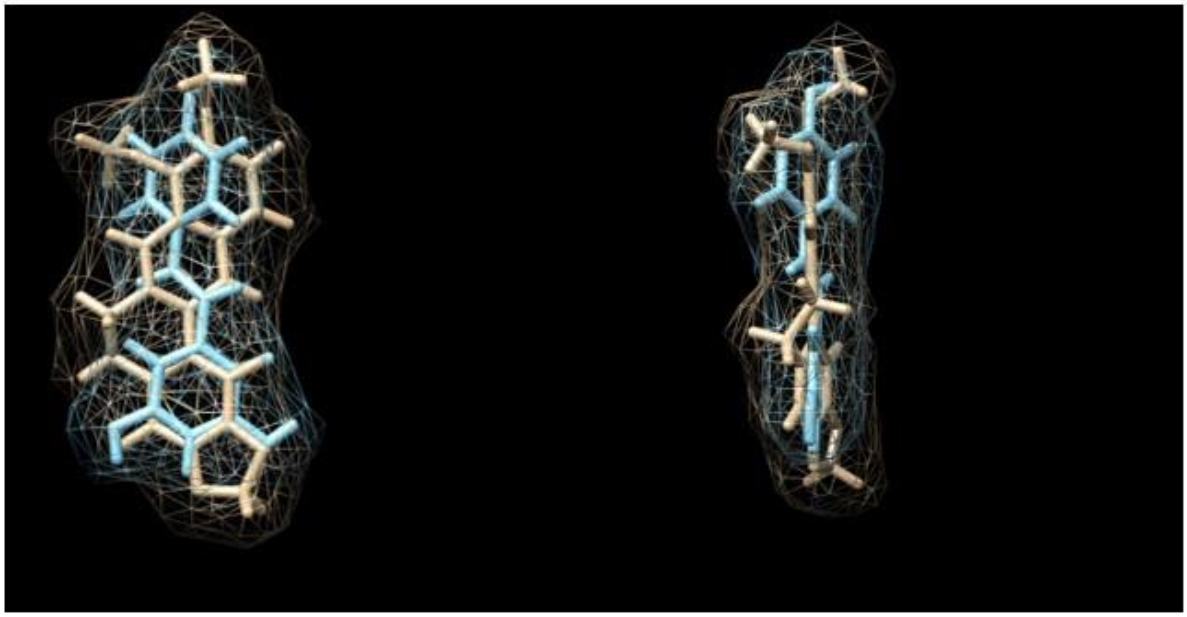


Figure 6.1. A comparison of the structures of berberine and resveratrol.

The structural information (3D conformer) for berberine and resveratrol was downloaded from the PubChem open chemistry database as structure-data files (SDF).

The SDF files were analysed using the UCSF Chimera molecular modelling software. (Images courtesy of Dr Cristina Fanutti, UEA, Norwich, UK)

A. Three-dimensional mesh representations of molecular surfaces. Superimposition of Berberine (light brown) and Resveratrol (light blue) - rotation of 90° (left)



B. Three-dimensional space fill representations of molecular surfaces. Superimposition of Berberine (light brown) and Resveratrol (light blue) - rotation of 90° (left)

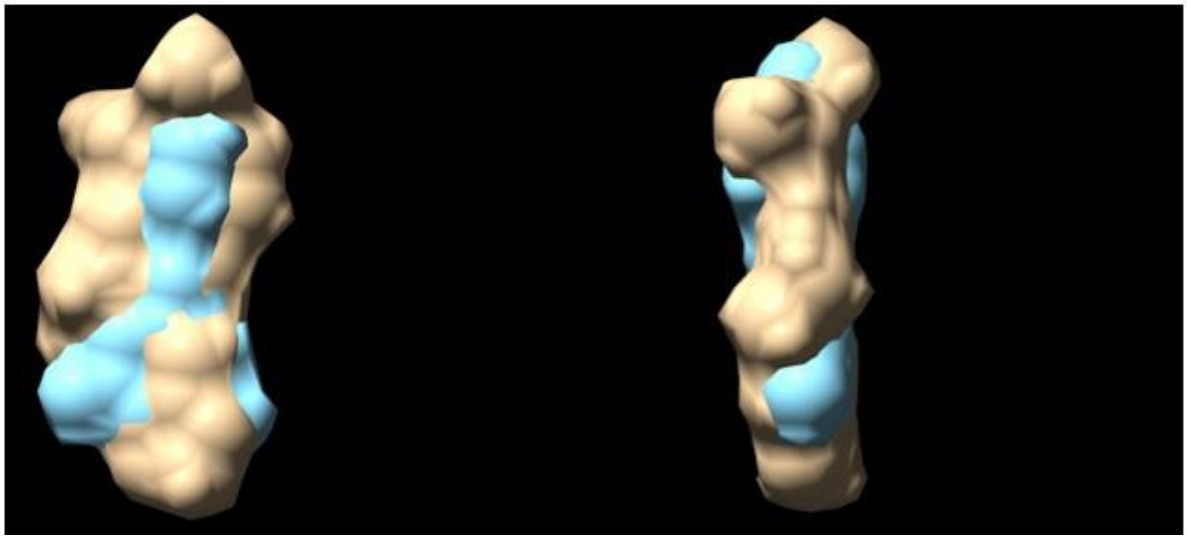


Figure 6.2. A comparison of the structures of berberine and resveratrol.

The structural information (3D conformer) for berberine and resveratrol was downloaded from the PubChem open chemistry database as structure-data files (SDF).

The SDF files were analysed using the UCSF Chimera molecular modelling software. (Images courtesy of Dr Cristina Fanutti, UEA, Norwich, UK)

To determine if there was any direct interaction between TG2 and the phytochemicals, the interaction between berberine and resveratrol with TG2 were modelled using the swissdock

protein-ligand modelling tool (swissdock.ch). No high probability binding sites were found on TG2 for berberine, however potential binding sites were found for resveratrol near the catalytic triad binding site responsible for TG2's transamidation role (see Figure 6.3).

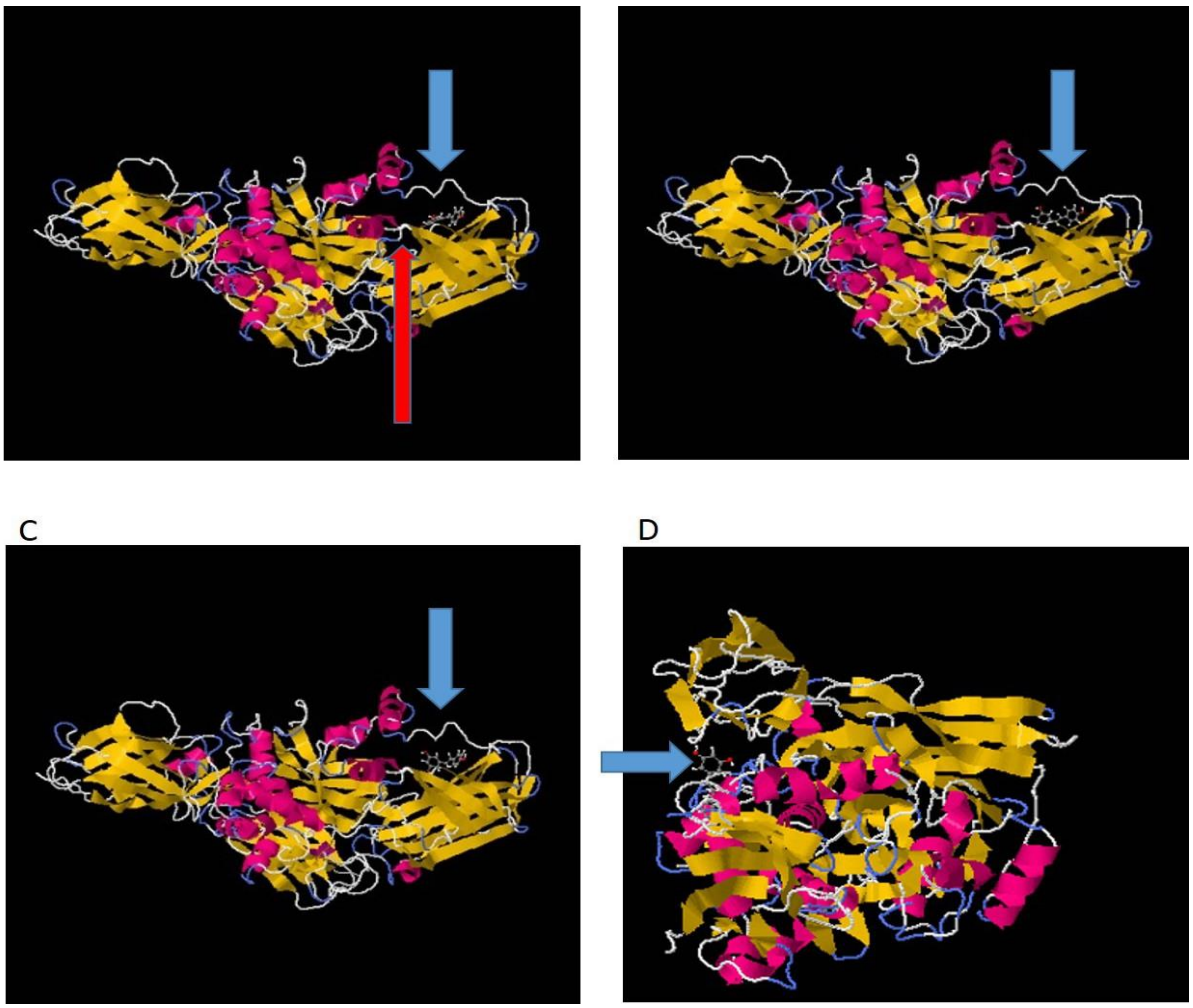


Figure 6.3. A swissdock model of the interaction between resveratrol and TG2.

6.3A: This shows the open form of TG2 with the blue arrow indicating the most likely predicted binding site for resveratrol. The red arrow indicates the transamidation active site.

6.3B: This shows the open form of TG2 with the blue arrow indicating the second most likely binding site for resveratrol.

6.3C: This shows the open form of TG2 with the blue arrow indicating the third most likely binding site for resveratrol.

6.3D: This shows the closed (catalytically inactive) form of TG2 with the blue arrow indicating the most likely predicted binding site for resveratrol. In this con figuration, the transamidation site is closed and therefore inaccessible.

From Figure 6.3, it can be seen that in all 3 of the most likely binding models predicted by the swissdock software, resveratrol has a binding site very close to the catalytic triad transamidation binding site (Cys^{277}, His^{335}, Asp^{358}). If these models prove to be true, then resveratrol by binding so close to the site active site, may modify the 3 dimensional structure of this region of TG2 and therefore may inhibit the transamidation activity. This is again speculative, but offers one

explanation for the observed behaviour, as if transamidation is blocked, then inhibition of apoptosis as predicted by Antonyak (2006) may occur and a slight inhibition of apoptosis was observed in the Caki-2 cell line and to a lesser degree in the HepG2 cell line. As the GTP-binding site of TG2 is directly opposite from the transamidation site, any change in con firmation of the protein by resveratrol, may effect the GTP-binding part of the enzyme, perhaps allowing easier activation of the GTP pathway and thus increasing cellular adhesion by the following mechanism. Resveratrol by binding so close to the transamidation site could be holding the TG2 in its GTP-binding conformation, which would allow the TG2 to activate FAK and P13K/Akt1, which are known to protect cells from apoptosis (Verna aMehta, 2007) and also to bind with both fibronectin (Fn) in the extracellular matrix (ECM) and with beta and alpha integrins. Alpha5/beta1 integrins, when induced to combine as a heterodimer by the GTP-binding activity of TG2, is associated with the activation of Bcl2, a pro-survival (anti-apoptotic) mediator. This integrin activating explanation also elucidates why cystamine inactivation of transamaidation alone did not prevent apoptosis in HepG2 cells and is also a possible explanation for the increased cellular adherence observed in both cell lines when treated with resveratrol; as the resveratrol could be locking the TG2 in its GTP-binding con firmation by closing the transamidation catalytic triad. As the swissdock models did not show any high probability binding sites for berberine with TG2, it may be that the increase inTG2 activity observed when cells were treated with berberine is a defensive measure (particularly in the HepG2 cell line). This would explain the observation that TG2 activity increased sharply when cells were treated with sub-lethal concentrations of berberine, and activity remained higher than untreated controls as the cells became less viable (section 4.2.2.1).

The exact mechanism by which berberine increases TG2 activity needs to be investigated further. TG2 activity has generally been found to increase the levels of inflammation in a variety of cell lines, which is a marker of many cancers (see section 1.4). However berberine has been shown to increase the levels of IκBα, an inhibitory subunit of the pro-inflammatory cytokine NF-κB (Kaipang, 2014), which results in a reduction of inlammation. One possible mechanism of action for the observed effects of berberine on HepG2 cells in this project could be that in this high-expressing TG2 cancer model, the presence of TG2 in its general TG2-L form, which has been shown to be involved in providing drug resitence in certain forms of cancer (section 1.3.4.3) is responsible for maintaining cell viability. When treated with berberine however, the TG2 increases its transamidation activity (see section 4.2.2), which inhibits the GTP-binding activity. This makes the cells less adherent (as was observed in section 5.2). Another side effect of this phenomenon would be an increase in the TG2-S levels (as was observed in section 4.4), which is a known pro-apoptotic isoform of the enzyme (discussed in section 1.3.4.1). This explains and ties

together many of the observed effects of berberine on the HepG2 human hepatocarcinoma cell model and further manipulation of this model could lead to potentially effective clinical cancer therapies.

6.7. Significance of results

The berberine-induced increases in TG2 activity in both the low-expressing Caki-2 cell line (used as a model for renal carcinoma) and in the high-expressing HepG2 cell line (used as a model for hepatocarcinoma) is a highly significant observation, as following a spike in TG2 activity there was a movement in the HepG2 model from a viable state to a apoptotic or non-viable state, a phenomenon which was not observed in Caki-2 cells. Resveratrol treatment was shown to increase cellular adhesion, to inhibit the cell cycle and to prevent cellular migration in both model systems, but to a greater extent in the HepG2 model. In combination, the compounds affect both cell viability and cellular adhesion in both model systems, but to a greater extent in the high-TG2 expressing HepG2 model. If this response was reflected *in vivo* with other forms of hepatocarcinoma, then the result might be a modulation of the cell cycle and metastasis together with an induction of apoptosis/necrosis of other types of hepatocarinoma cells. It should be stated that this is speculative as all results have been gathered in a model cell line system and results may differ *in vivo* and when directed to target active cancers. Work has been previously performed using this type of model with nude mice to test how effective chemotherapeutics are on various forms of cancer *in vivo* following on from initial cell culture studies (Wenhui, 2014).

Another significant result of this research was to identify the presence of the TG2-S and TG2-L isoforms in both model cell lines for the first time, which indicates that these isoforms are more prevalent than is currently understood to be the case. Research into the isoforms (section 1.3.4.3) shows that TG2-S is generally pro-apoptotic and TG2-L is anti-apoptotic (or pro-survival). The general body of work relating to TG2-S and its relationship with TG2-L is to date limited, leaving the details of the mechanisms and relationships between the isoforms unclear. Antonyak and his team first discussed how the TG2 isoforms have opposing effects in 2006 (Antonyak et al., 2006) and the qPCR analysis results obtained in this study built on his teams results. To summarise, the results outlined in section 4.4 showed a statistically significant increase in TG2-S in HepG2 cells during berberine-induced apoptosis. This opens up the possibility that berberine induction of TG2 activity could be used in a clinical setting to decrease cell viability of TG2 positive hepatocarcinomas and reduce the severety of tumours. In combination with resveratrol to reduce

cell migration (invasion/metastasis), tumours may even be elimated, however at this stage this is speculative and further work is required to develop this idea furtherhis gene expression analysis strengthens the case for further research into the roles of the isoforms in cancer development and in cancer therapy.

This investigation has shown that although in isolation both berberine and resveratrol are potentially useful chemotherapeutic tools in a variety of model systems, which had been previously noted (see section 1.4.1) these phytochemicals have now been shown to be a more potent chemotherapeutic treatment in the HepG2 hepatocarcinoma model when used in combination than when used in isolation. As a combined treatment, they may hold the potential to inhibit metastasis and the cell cycle, together with inducing cell death in some high TG2-expressing cancers.

6.8. Limitations

One limitation of this study was the lack of availability of antibodies for the TG2-S and the TG2-L isoforms. If these isoforms had been available, then Western blots, ELISA or flow cytometry could have been used to measure their relative levels. This may have provided more representative results than the qPCR.As a cell culture study, the results obtained have clarified the role of TG2 in cancer and cell viability and have also highlighted the potential for the phytochemicals berberine and resveratrol as chemotherapeutics; particularly as a potential ‘combined therapy’ for the treatment of hepatocarcinoma. A limitation of this work was however the lack of primary cell lines to work with. As an initial test for modelling the effects of these phytochemicals on cancer, immortal cancer cell lines are an invaluable tool, however a comparison with primary cells would be useful to both expand knowledge and to act as an additional control.

6.9. Future work

To build on the results of this study, other hepatocarcinoma and renal carcinoma cell lines could be investigated to determine if the results observed with HepG2 and Caki-2 cells can be repeated in other model cell lines. Also, as discussed above, a repeat of the experiments conducted on the cancer cell lines could be performed using primary cell lines. Using non-cancerous primary cells obtained from healthy liver and kidney would no doubt generate more data that would help to

elucidate further the mechanism of action of this combined phytochemical treatment and determine if this therapy has clinical merit. Following on from further cell culture studies, it is suggested that investigation into the effects of the modulation of TG2 activity using berberine, resveratrol or similar phytochemicals on high TG2-expressing cancers be performed. Hepatocyte-derived cancers may be a good initial target as these are known to express high levels of transglutaminase 2 and may therefore respond in the same way to TG2-modulating compounds as the HepG2 model did in this study. One suggested avenue of research would be to inoculate nude mice with HepG2 cells followed by treatment with resveratrol and berberine in isolation and in combination. HepG2 cells have previously been transfected into nude mice to test chemotherapeutic compounds *in vivo* (Wenhui, 2014) so this would be a logical study to perform and would help to determine if this chemotherapeutic approach has the potential to be a clinically effective treatment for cancer. Another avenue of research that it would be prudent to explore would be to use 3D growth chambers to determine the migration of treated cells more thoroughly than was able to be accomplished using scratch test assays alone. This would help to determine more accurately how these treatments might effect the invasiveness and metastasis of cancers in vivo. This, together with the fluorescent labeling of berberine, reseveratrol and transglutaminase 2 would allow for microscopic analyses (using fluorescent and/or confocal microscopy) of the interactions between the phytochemicals and transglutaminase 2. Additinally, using such fluorescent staining technologies, the exact location of the phytochemicals and TG2 within the cells could be determined, which would elucidate even further the mechanism of action of these compounds. As an adjunct to this experimental protocol, CFSE labelling of cells could be a useful technique to separate dividing from non-dividing cells. Finally, to analyse the results of the scratch test assays further, the software ImageJ could be used to generate numerical and statistical data relating to the migration patterns of the cells.

6.10. Final conclusions

In section 1.5.1 the below hypothesis were stated:

Hypothesis 1: A novel phytochemical drug treatment can be developed to effectively induce cell death in one or more of the cancer cell models used in this project.

Hypothesis 2: The phytochemical treatment/treatments developed in this project induce their effects in the cancer cell model/models by modifying the behavior and/or the activity of the enzyme transglutaminase 2.

Hypothesis 3: The phytochemical treatments developed in this project induce their effects at doses that are clinically achievable.

For a combined resveratrol/berberine treatment in the HepG2 cell line (a model for human hepatocarcinoma) these hypothesis have been validated.

Resveratrol and berberine have in this study proven an effective chemotherapeutic tool to significantly reduce viability and inhibit cell migration, particularly in the HepG2 cell line. This research has contributed to the body of work and shows promise if progressed further. If taken further by performing the suggested further experiments discussed in the previous section, then this combined phytochemical therapy could potentially be used in some future form as a chemotherapeutic tool for the treatment of cancer in human patients. The greatest potential suggested from the research conducted in this project would be for the treatment of hepatocarcinoma (liver cancer), a cancer that currently effects millions of people worldwide and that current therapies have only a moderate success rate in treating.

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