Investigating the effects of curcumin and resveratrol on pancreatic cancer stem cells

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Abstract

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Anti-proliferative and cancer stem-cell targeting abilities of curcumin and resveratrol individually have been shown in different cancers. This project aimed to assess the activity of these compounds, alone and in combination in pancreatic cancer cell lines (PCCLs) and stellate cells.

Anti-proliferation assays were performed for curcumin and resveratrol alone and in combination, combined with end point markers of activity including apoptosis and cell cycle arrest. Pancreatic cancer stem cell populations were defined using the cell surface markers CD44, CD24, ESA, CD133, ALDH-1 activity or sphere forming ability, and finally Nanog expression was assessed. The intracellular uptake of curcumin and its metabolites was analysed by HPLC.

The PCCLs were more sensitive to curcumin than resveratrol, and combinations of these compounds showed anti-proliferative efficacy through apoptosis and cell cycle arrest at low, clinically achievable concentrations (CACs) in 2 out of 4 cell lines. Capan-1 cells exhibited the highest sensitivity to curcumin, which was able to enhance the effectiveness of resveratrol treatments in targeting cancer stem-like populations. Spheroid growth was significantly inhibited by curcumin and resveratrol combinations in Capan-1 cells, correlating with decreased ALDH1 activity and Nanog expression. In human pancreatic cancer tissue, various stem-like populations were identified based on expression of ALDH1 or $CD24^{+}/CD44^{+}$, which may provide a suitable target *in vivo*. Capan-1 cells metabolised curcumin to detectable amounts of curcumin glucuronide. However, curcumin metabolites did not show any significant activity at CACs. Curcumin alone may have activity against pancreatic cancer stem cells, and enhances efficacy at low concentrations when in combination with resveratrol. Capan-1 cells are able to internalise curcumin, and this cell line exhibited the greatest sensitivity to treatment.

Overall, the results suggest that curcumin and resveratrol warrant further investigation as combination therapies for targeting cancer stem-like cells and stellate cells responsible for the dense stroma observed in pancreatic cancer.

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List of Abbreviations

5FU	5-Fluorouracil
ABC	Adenosine triphosphate–Binding Cassettes
ABCG2	ATP-binding cassette transporter G2
AD	Alzheimer's disease
ALDH1	Aldehyde dehydrogenase-1
AMPS	Ammonium Persulphate
APC	Allophycocyanin
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
bDMC	Bisdemethoxycurcumin
CDK4 and CDK6	Cyclin D-associated kinases
C	Curcumin
CDKs	Cyclin-dependent kinases
Chk1	Checkpoint Kinase 1

C_{max}	Maximum Concentration
COG	Curcumin-O-glucuronide
COS	Curcumin-O-sulfate
COX2	Cyclooxygenase-2
CRP	C-reactive protein
CSCs	Cancer stem cells
CTP	Cytidine triphosphate
CXCR4	CXC chemokine receptor 4
dCK	Deoxycytidine kinase
DEAB	Diethylaminobenzaldehyde
dFdCTP	Di-fluorodeoxycytidine triphosphate
dFdCTP	gemcitabine triphosphate
dFdUMP	2′,2′-difluoro-2′-deoxyuridine monophosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix

EGCG	Epigallocatechin Gallate
ENT1	Equilibrative nucleotide transporter 1
EpCAM	Epithelial Cell Adhesion Molecule
ESCs	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein Isothiocyanate
GEM	Gemcitabine
GPIS	Glycosyl phosphatidylinositols
HHC	Hexahydrocurcumin
hNT	Human nucleoside transporter
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish peroxidase
IGFBP3	IGF-binding protein 3

IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IMS	Industrial Methylated Spirits
IPMNs	Intraductal papillary mucinous neoplasms
KRAS2	Kirsten ras-2 gene
LIF	Leukemia inhibitory factor
LOD	Llimit of detection
LOQ	Limit of detection quantification
LPE	Liquid Phase Extraction
MCNs	Mucinous cystic neoplasms
MDR	Multidrug Resistance
MRP5	Multidrug Resistance Protein 5
MUC1/4	Mucin1/4 Protein
NF-κB	Nuclear Factor Kappa B
NICE	National Institute for Health and Care Excellence

NOS	Nitric oxide synthetase
NOD-SCID	Nonobese Diabetic/Severe Combined Immunodeficiency
NPC	Nasopharyngeal carcinoma
OATPs	Organic anion transporting polypeptides
OHC	Octahydro-curcumin
OATs	Organic anion transporters
OATPs	Organic anion–transporting polypeptides
PCSC	Pancreatic cancer stem cell
PCCLs	Pancreatic cancer cell lines
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline – tween-20
PDAC	Pancreatic Ductal Adenocarcinoma
PDGF	Platelet–derived growth factor
PEG	Polyethylene glycol
ROS	Reactive oxygen species
R	Resveratrol

SDF-1	Stromal cell-derived factor 1
SDS	Sodium Dodecyl Sulfate
SMAD4	Signaling effectors mothers against decapentaple protein 4
Sox2	Sex determining region Y
SP	Side populations
STAT3	Signal transducer and activator of transcription 3
SULTs	Sulfotransferases
TAMs	Tumour associated macrophages
TGFβ	Transforming growth factor β
TICPs	Tumour initiation cell populations
T_{max}	The time after administration of a drug when the maximum concentration is reached
TNF	Tumour necrosis factor
UGTs	Uridine diphosphate glucuronosyl transferases
UV	Ultraviolet

Chapter One: Introduction

1.1. Cancer

Cancer can be defined as a group of diseases, which occur in consequence of an extended process beginning with one cell being damaged beyond repair, or changed in a manner that makes them depart from normal cellular control mechanisms (Figure 1.1). The cells then follow an agenda for uncontrolled proliferation, leading to the production of a mass of cells (tumour) (1-5). A tumour is considered benign only if the neoplastic cells are clustered in a single mass which does not have the ability to spread into surrounding tissues or distant organs. Once these invasive characteristics are acquired, the tumour is considered cancerous (Figure 1.1). Cancer can arise by hereditary means, due to inherited genetic or epigenetic alterations to the genome, or sporadically, as a result of somatic mutations, which may be caused by the action of environmental mutagenic agents (6-9).

Even though cancer is a group of diseases characterised by dysregulation of proliferation, leading to invasion and metastasis, they differ in various characteristics including the tissue of origin, causal factor(s) and molecular mechanisms leading to tumour development. The origin of a tumour (primary tumour) is classified based upon the tissue from which it comes; for example, carcinomas arise from epithelial cells, sarcomas from mesenchymal cell types**,** leukemia from haemopoietic cells**,** neuroectodermal from the nervous system and melanomas are of neural crest origin. The majority (~90%) of solid tumours occur in epithelial tissues (10-13). In addition to this, there are tumours whose origins are untraceable and are therefore known as tumours of unknown origin.

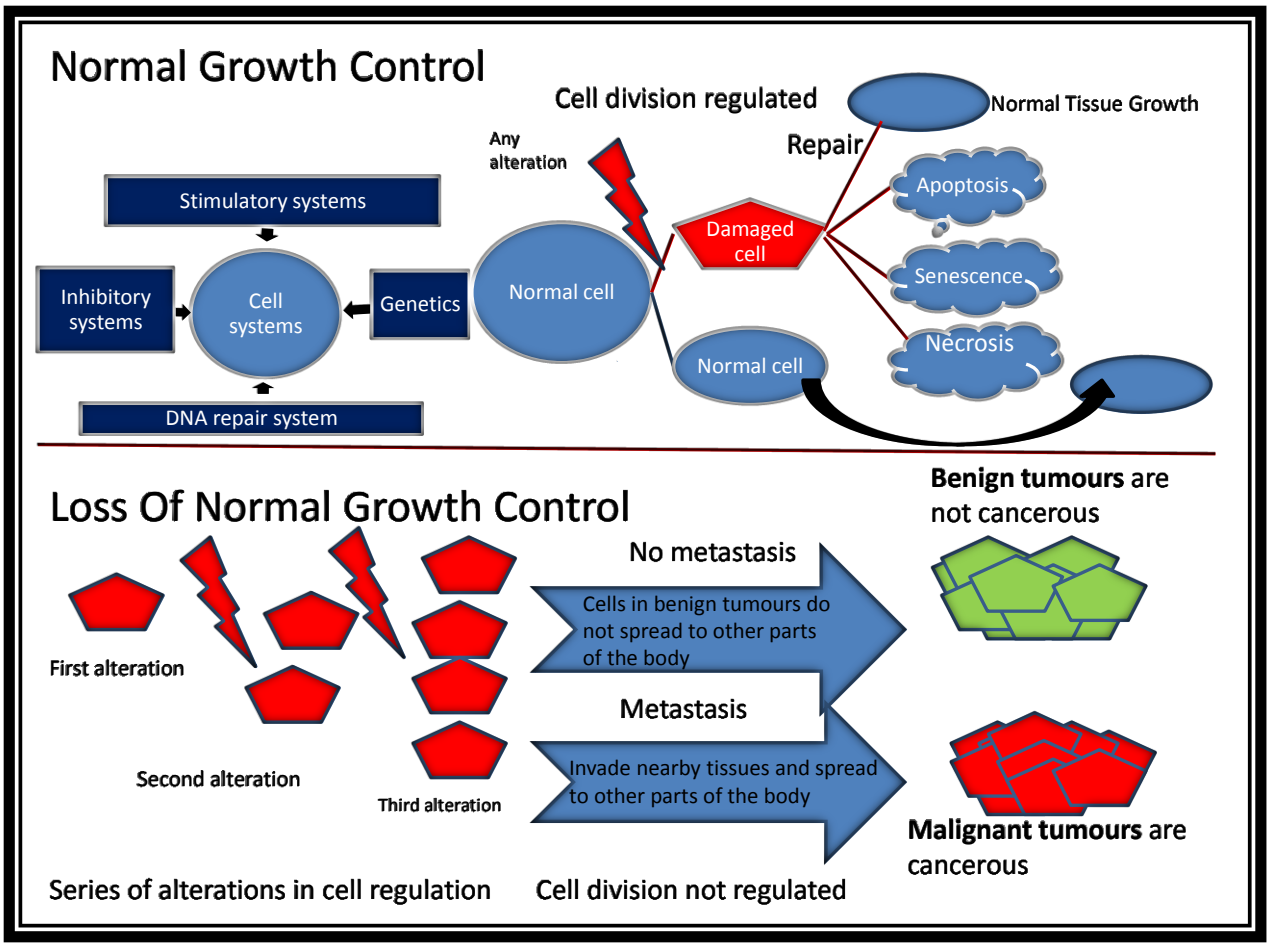


Figure 1.1 Model depicting the role of the microenvironment in normal and tumour cells.
Upper Panel: A schematic illustration of normal cells where growth is usually tightly regulated by physiological systems, allowing a balance between programmed cell death (apoptosis) and the proliferation of cells. Lower panel: a series of alterations lead to accumulation of mutations and ultimately in tumour formation, adapted from (4, 14).

1.2. Carcinogenesis

Carcinogenesis, oncogenesis or tumourigenesis is the multi-step process whereby cells in a normal state undergo transformation into cancer cells. Carcinogenesis is classified into three stages: initiation, promotion and progression (10, 12, 13, 15) (Figure 1.2). The driving forces behind carcinogenesis include environmental stresses and genetic factors which may occur over a prolonged period, resulting from one or a combination of, chemical, physical, biological, and/or genetic changes in the normal cells (12).

In the initiation stage, irreversible genetic mutations occur in the DNA sequence (12). These can be due to DNA replication errors in the synthesis phase of the cell cycle, or from intrinsic cellular metabolism, for example, the release of reactive oxygen species (ROS) and free radicals (16-18).

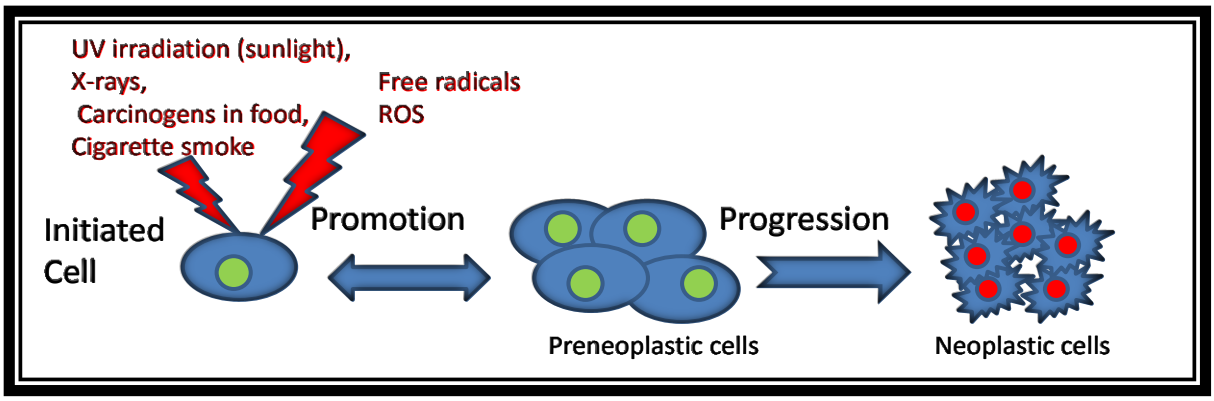


Figure 1.2 Illustration depicting initiation, promotion and progression of cancer.
Cancer can be induced by various mutagens such as UV light, X-rays and free radicals adapted from (12, 18, 19).

Additionally, environmental mutagens (17) may have a huge impact on DNA sequence and structure, most commonly through carcinogens in food, cigarette smoke or through UV irradiation (sunlight) and X-ray exposure (16-18). Any environmental mutagen which becomes covalently bound to the DNA structure is known as a DNA adduct, and could be the starting point for the initiation stage of carcinogenesis. These DNA adducts may cause mutations if not eliminated by DNA repair systems prior to replication. There are three classifications of DNA damage: breaks in the DNA backbone; loss, addition or substitution of bases and chemical modification of bases (10-13).

The second stage, which can lead to pre-malignant tumour growth, is believed to involve epigenetic mechanisms and is referred to as cancer promotion (Figure 1.2). Many cellular functions are negatively impacted at this stage, with the loss of cell cycle checkpoints, alteration to the regulatory proteins involved in apoptosis and an increase in cell proliferation. The third stage of cancer progression is characterised by the formation of neoplastic cells by further DNA alteration and epigenetic changes. These cells have a higher potential rate of replication and a greater chance of metastasising (10-13).The de-regulation of

many cellular processes are required for metastasis to take place including, cell migration, matrix degradation, angiogenesis, and host immune response avoidance.

Progression through these stages can take a considerable amount of time, giving ample opportunities for medical intervention in an attempt to delay, reverse or prevent progression of the disease. The three stage model for carcinogenesis, in some cases, is not adequate to describe the carcinogenic process (10, 12, 13, 15). The detailed hallmarks of cancer as described by Douglas Hanahan and Robert Weinberg (5) includes 6 primary modifications in physiological regulation of a cell that, in aggregate, determine the extent of malignant growth: growth signal self-sufficiency, growth inhibition signal insensitivity, apoptosis evasion, unlimited potential for replication, angiogenesis sustainability and invasion or tissue and metastasis. These were reviewed in 2011, and a further 4 hallmarks added: tumour-promoting inflammation, mutation and instability in genome, immune destruction avoidance and cellular energetics deregulation (20) (Figure 1.3).

Figure 1.3 The hallmarks of Cancer by Douglas Hanahan and Robert A. Weinberg.
Biological characteristics of cancer include: growth signal self-sufficiency, growth inhibition signal insensitivity, apoptosis evasion, unlimited potential for replication, angiogenesis sustainability and invasion or tissue and metastasis plus tumour-promoting inflammation, mutation and instability in genome, immune destruction avoidance and cellular energetics deregulation (20).

1.2.1. Apoptosis and the cell cycle in cancer

Evasion of apoptosis (resisting programmed cell death) and limitless replicative potential can be considered as an imbalance between cell death and cell division, which will alter an organism's internal state and eventually cause disease. Therefore, apoptosis is needed in order to maintain homeostasis and a constant number of cells. A rate of division faster than that of cell death will cause tumours to develop, whilst the reverse will cause a loss of cells, ageing and degenerative diseases (5).

For cancer cells, a critical stage is evasion of apoptosis, achieved via genetic alterations in genes for tumour suppression and oncogenes. The extrinsic and intrinsic signaling pathways are both involved in apoptosis and are relatively separate. Caspases are executioner enzymes which are responsible, in both pathways, for cell death (21). Apoptosis will not occur if these pathways function improperly and tumours can consequently form (21, 22).

Triggering of the intrinsic pathway (mitochondrial or Bcl-2 (B-cell lymphoma 2) inhibited pathway) occurs in response to several intracellular factors, such as stimulation of death receptor, growth factor withdrawal, radiation, viral infections, hypoxia, deprivation of nutrients, endoplasmic reticulum stress and DNA damage (23). Induction of the extrinsic pathway can occur via extracellular signalling, including signalling via growth factors, nitric oxide, cytokines or hormones (21, 22).

Caspases can be categorised into 2 groups, with approximately 14 caspases in total. Caspases 2, 8, 9 and 10 trigger mechanisms but don't have a direct part to play in cell execution, and are termed initiator caspases (24). Effector caspases have been implicated as having a direct role in DNA degradation and nuclear shrinkage, and include caspases 3, 6 and 7. At least 7 of the caspases, are thought to be directly involved in cell death and are activated by proteolytic cleavage. Caspases 8 and 9 both activate caspase 3. Caspase 8 is activated upon an extracellular signal while caspase 9 is activated when it is engaged with cytochrome c (25, 26).

Another aspect of cellular regulation to be considered is that of the cell cycle, as the quality and rate of cellular division are tightly monitored by the cell-cycle checkpoints. There are four phases to the cell cycle: G1, S phase, G2 and M phase. CDKs (Cyclin-dependent kinases) promote the cell's progression through this cycle and these are regulated negatively and positively (27, 28). Driving progression of the cell through G1, cyclin D isoforms interact with CDK6 and CDK4. At the G1-S transition, cyclin E associates with CDK2, directing entry into S-phase. For entry into mitosis, CDK1/cyclin B is required. Similarly,

the cyclin A and CDK1 complex is important during G2 while the complex of cyclin A/CDK2 directs S-phase progression (27, 28).

Research has indicated that correcting defects in the G_1 arrest checkpoint could induce apoptosis and retard growth, and correcting errors in the G_2-M checkpoint could increase the cytotoxicity of chemotherapy, providing examples of how targeting the cell cycle could be used in cancer treatments (29).

1.3. The Pancreas

1.3.1. Anatomy of the Pancreas

The pancreas is an organ of many purposes and has both endocrine and exocrine capabilities. It is made up of 3 regions known as the head, body and tail. The length of the pancreas is traversed by a main pancreatic duct which serves to drain pancreatic fluid and deliver it to the duodenum. This main pancreatic duct merges with the bile duct and forms a structure known as the ampulla of Vater, which is effectively a terminal widening of the duct at the point immediately before entering the duodenum (30).

1.3.2. Function of the pancreas

The function of the pancreas can be broadly divided into endocrine and exocrine. Endocrine functions of the pancreas centre on the cell clusters known as islets of Langerhan; these islets contain four primary types of cell, with the essential ones being α and β cells, which secrete glucagon and insulin, respectively. These play the vital role of regulating blood glucose levels and glucose metabolism.

Exocrine functions of the pancreas involve the release of enzymes which help in the digestion of food, such as lipase, amylase and proteases. These

enzymes are contained within the pancreatic fluid which are passed to the duodenum and small intestine (30).

1.4. Pancreatic cancer – the scale of the problem

Among all types of cancers, pancreatic cancer is known as a silent and significant killer, due to the fact that it is amongst the most aggressive of the solid malignancies with an extremely high mortality rate (31-36). Tellingly, the incident and death rates are very similar; in 2011, 8,773 people in the UK were diagnosed with pancreatic cancer and 8,662 people died from pancreatic cancer in 2012. Only 3.7 % of those diagnosed in Britain will survive for five years – the worst prognostic outcome of any of the cancers. Furthermore, only 20% will live for a year (31, 36).The main reason behind this outcome is a poor detection rate leading to late diagnoses once the cancer has already metastasized. With early diagnosis comes a real chance to reduce the death rate. Pancreatic cancer shows resistance to chemoradiotherapy treatments, and late discovery is common as the cancer is of a silent nature (31, 36). On an international level, 338,000 pancreatic cancer cases were reported worldwide in 2012 (2% of the total cancers). The highest incidence rate was recorded in Europe and Northern America, with distinctly higher rates among males within Eastern and Central Europe. In contrast, the lowest rate of occurrence were in Africa and Asia.

1.5. Characteristics, Pathogenesis and Management of pancreatic cancer

1.5.1. Characteristics of pancreatic cancer

Pancreatic cancer is characterised by weakly-vascularised, dense, stroma existing in a micro-environment with interactions between cellular and non-cellular elements. The paracrine and autocrine release of growth factors including transforming growth factor β (TGF-β) and platelet–derived growth factor (PDGF), as well as cytokine action leads to a constant interaction between cancer cells and their stromal counterparts. A main cellular component of the stroma are pancreatic stellate cells. These cells are characterised by intracellular fat droplets, glial fibrillary acidic protein and the production of desmin, and can produce α-smooth muscle actin and express excessive collagen fibres when stimulated by growth factors. The desmin phenomenon contributes to tumour hypoxia (37, 38). Emerging studies have shown that a typical mature pancreatic cancer cell contains an average of 63 genetic alterations compared to a normal cell, which can be grouped together in 12 core signalling pathways (Table 1.1). Inhibition of tumour suppressor genes and oncogenic activation have a vital role in the progression of early lesions to metastasis in pancreatic cancer as well as other cancers (39).The accumulation of genetic alterations in the process of pancreatic carcinogenesis is often classified into early (mutation activation in *KRAS2* (*Kirsten ras-2 gene*), shortening of telomeres, p21 and Mucin-1), intermediate (mutation inactivation or epigenetic silencing of *CDKN2A,* Hes1, COX2 and Notch-1) and late (mutation inactivation of *TP53, Brac2* and *SMAD4*) events (Figure 1.5). Additional genetic mutations could, similarly, occur during PanIN (Pancreatic Intraepithelial Neoplasia) formation which is a precursor to cancer but are not illustrated here (40-42).The most common alterations in genes and gene products are shown in Table 1.1. In addition, recently epigenetic abnormalities (SOCS-1, TSLC) (43) and miRNA alterations (miR-107, miR132) (44) are associated with pancreatic cancer.

Table 1.1 Common genetic alterations in pancreatic cancer signalling pathways (42).				
Gene type	***Gene***	**Intracellular function**	**Frequency in PDAC (%)**	**Reference**
Oncogenes	*KRAS2*	ERK–MAPK signalling	>90	(45)
	Cyclin D	Cell cycle progression	65	(42)
	BRAF	ERK–MAPK signalling	~5	[40]
Tumour suppressor genes	*CDKN2A*	G1/S phase	>95	(46)
	SMAD4	TGF-β-signalling	50	[40]
	TP53	Cell cycle arrest	~75	(47)
Genome maintenance genes	*MLH1*	DNA damage repair	5	(48)
	BRCA2	DNA damage repair	~10	[40]
Developmental signalling pathways	*GLI1, SOX3, CREBBP*	Hedgehog (Hh) signalling pathway		[40]
Developmental signalling pathways (Notch)	*HES family* *P21, TCF4*	Cell death through crosstalk with NF-κB signalling		[40] [44]
Developmental signalling pathways	*WNT9A, MYC*	Wnt signalling pathway		(33)

1.5.2. Pathogenesis

The precursor lesions which lead to metastatic pancreatic cancer include pancreatic intraepithelial neoplasia (PanIN) which occurs in 90% of all pancreatic cancer (49), intraductal papillary mucinous neoplasms (IPMNs) (found in approximately in 5-8%) and mucinous cystic neoplasms (MCNs) (very rare type, found in approximately 1%). Each lesion type would have different pathological progression (Figure 1.4)) (50, 51). PanINs are divided into early and late lesions, beginning with PanIN-1A, 1B (minimally dysplastic epithelium) developing to PanIN-2 and subsequently to PanIN-3 (severe dysplasia) or carcinoma *in situ*, then the final stage of invasive carcinoma (31-33, 35, 36). There are three distinct pathways for the progression of normal pancreatic tissues to malignant tissues which vary depending on the location (head, neck or tail) and type of pancreatic cancers (Figure 1.4). In many cases pancreatic cancer's pathological and physiological characteristics are correlated to different mutations which occur during pancreatic carcinogenesis (Figure 1.5).

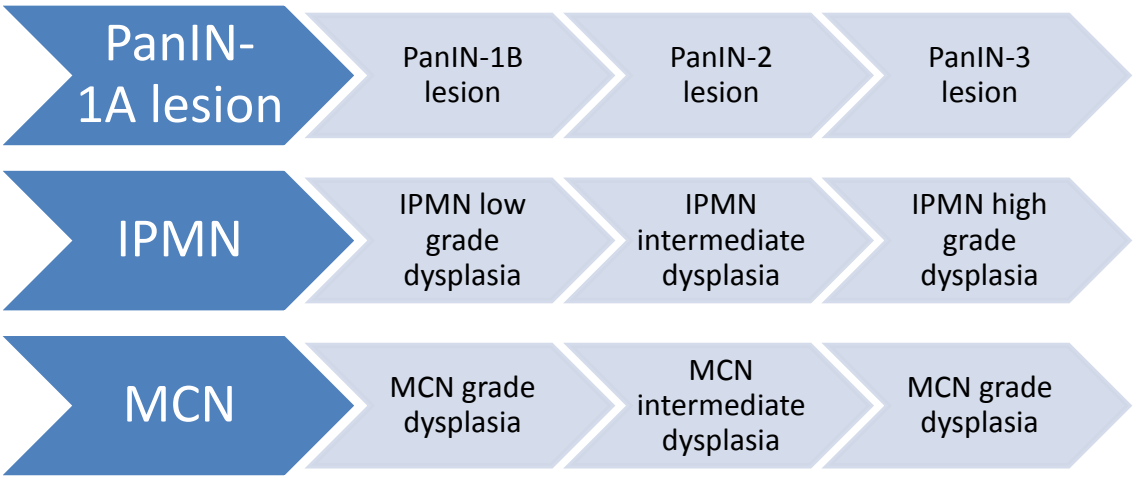


Figure 1.4 Three distinct morphological pathways leading to invasive pancreatic carcinoma. PanIN-1A lesion= Pancreatic Intraepithelial Neoplasia 1-A, IPMN= Intraductal Papillary Mucinous Neoplasms, MCN= Mucinous Cystic Neoplasm (50).

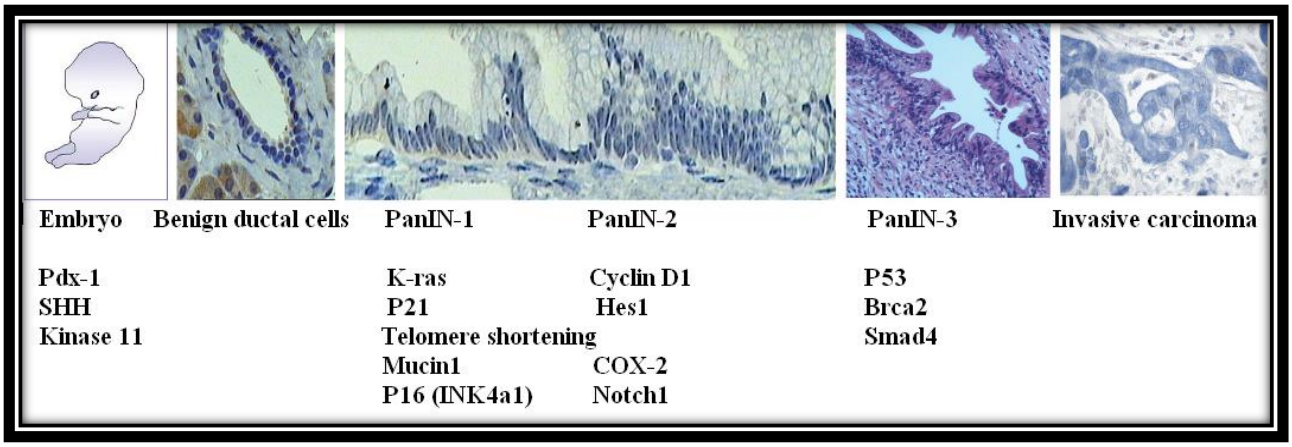


Figure 1.5 Histological sections depicting precursor lesions which are known to adopt invasiveness in pancreatic cancer. Pancreatic intraepithelial neoplasia (PanIN) are categorised into early (PanIN-1A, 1B (hyperplasia)) and late (PanIN-2 and PanIN-3) lesions, with invasive carcinoma following on (31). Abbreviations: Pdx-1: Pancreatic and duodenal homeobox 1, SHH: sonic hedgehog, K-RAS: Kirsten rat sarcoma viral oncogene homolog, P21: Harvey rat sarcoma viral oncogene homolog, Mucin1: cell surface associated, Hes1: hairy and enhancer of split-1, COX2: cyclooxygenase-2 or COX-2, in humans, is an enzyme encoded by the PTGS2 gene, Notch1: Notch homolog 1, translocation-associated (Drosophila) encodes a single-pass transmembrane receptor and is a human gene p53: tumour suppressor, Brca2: gene belongs to a class of genes known as tumour suppressor genes, taken from (31, 32).

1.5.3. The involvement of the stroma cells in pancreatic cancer cell growth and invasion

One of the hallmarks of cancer is the requirement for tumours to evade immune destruction (20). To deliver this, the tumour needs to destroy any immune response to generate an environment that fosters tumour growth and progression (20, 52). As well as immune cells (Figure 1.6), key roles in PDAC pathogenesis are played by other stromal elements. One example from stromal cells are PSCs; are activated as a result of this interaction, which is the main contributor to the extensive fibrosis observed in PDAC (52). PSCs are involved in tumour growth locally and metastases due to their mobility and capacity to assist in the formation of metastatic growths (52, 53).

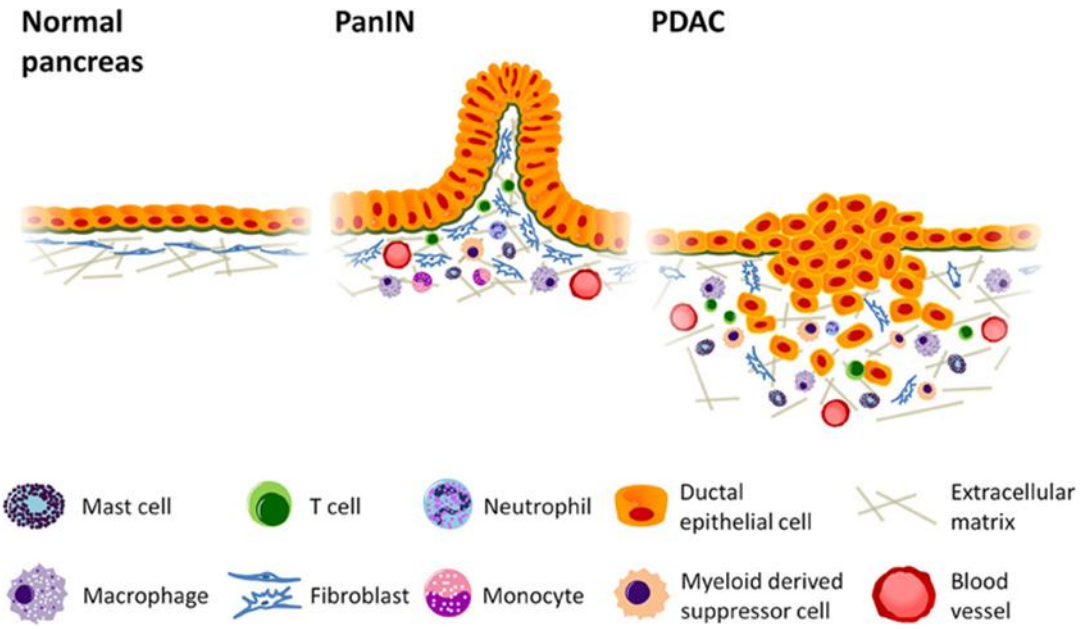


Figure 1.6 Extracellular Matrix in pancreatic cancer.

Tumourigenesis progresses desmoplastic stroma accumulates, increasing the production of collagen and vascular formation while recruiting immune cells to enhance tumour growth. Activated tumour associated macrophages (TAMs) and mast cells localize at the leading edge of the tumour; and can speed up tumour invasion, lymphatic metastasis and angiogenesis, taken from (53).

1.5.4. Inflammation and pancreatic cancer

Recently, there has been clarification of the involvement of inflammation in the development of PDAC. It has been suggested that pancreatic inflammation could be considered as a pre-existing condition for PDAC initiation (54, 55). Some inflammation markers such as C-reactive protein (CRP) are used clinically, as indicators of systemic inflammation, whilst others such as COX-2, production of NF-κB, nitric oxide synthetase, TNF-α and formation of free radical oxygen were reported to be directly associated with PDAC growth (56).

1.6. Management of pancreatic cancer

The mainstay of treatment for pancreatic cancer, is that of surgical resection. Surgical resection is usually most effective in stages I and II of the disease and can yield 5-year survival rates approaching 25 to 35% (57, 58). Following surgical resection, post-operative (adjuvant) chemotherapy or chemo-radiotherapy may be offered to prevent recurrence and to maximise therapeutic effects. So far Gemcitabine (GEM) and 5-Fluorouracil (5FU) are the only two drugs that have been shown to reliably advance the chances of survival in patients (59). Initially, 5FU was tested as an adjuvant and neoadjuvant therapy for pancreatic cancer (60-63). GEM can increase patient's median survival by up to six month (64, 65). 5FU is a pyrimidine analogue related to uracil with an extra fluorine at the fifth position; it acts as a thymidylate synthase inhibitor, which is an enzyme that converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), thereby causing DNA damage (66, 67). Also, phosphorylation of 5-FU leads to integration into RNA, at which point it can cause miscoding and stop protein expression (68) (Figure 1.7). Gemcitabine (GEM) or (2',2'-difluorodeoxycytidine) acts via three mechanisms; first, through the activity of deoxycytidine kinase (dCK), which is an enzyme essential for the phosphorylation of deoxyribonucleosides. dCK converts GEM to di-fluorodeoxycytidine triphosphate (dFdCTP) which the same site that cytidine triphosphate (CTP) does, for incorporation into DNA (59, 69). Next, its diphosphate metabolite (dFdCDP) prevents the action of ribonucleoside diphosphate reductase hindering the creation of the triphosphate nucleotide. Thirdly, the triphosphate metabolite (dFdCTP) stops DNA polymerases which are essential in the repair of DNA (59, 69, 70), these all result in DNA replication errors and arrest DNA replication via "masked chain termination" mechanism (70) (Figure 1.8).

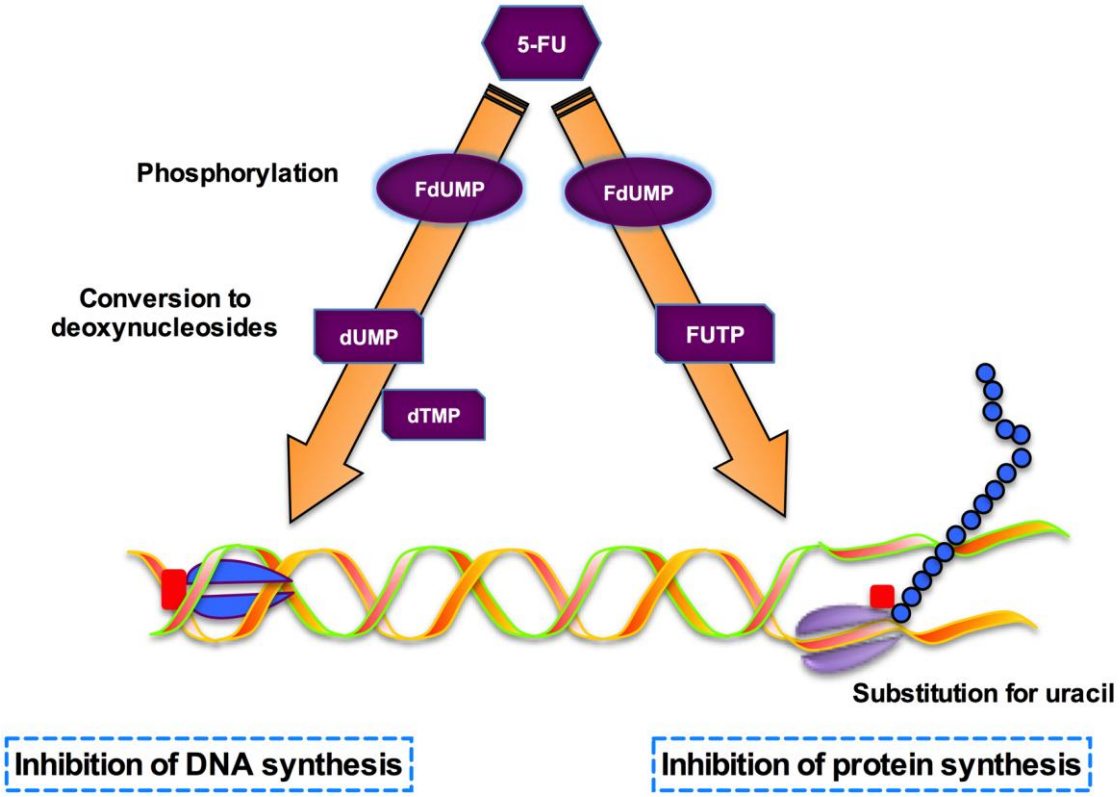


Figure 1.7 5-FU mechanism of action.

Inhibition of DNA synthesis: the 5-FU, when phosphorylated, is transformed to its deoxynucleoside, and synthesis of DNA is inhibited due to the functions of an important DNA-replication enzyme, thymidylate synthetase, being blocked. Inhibition of protein synthesis: Phosphorylation of 5-FU takes place and it is incorporated into RNA, wherein miscoding results, so halting protein synthesis. Adapted from (68).

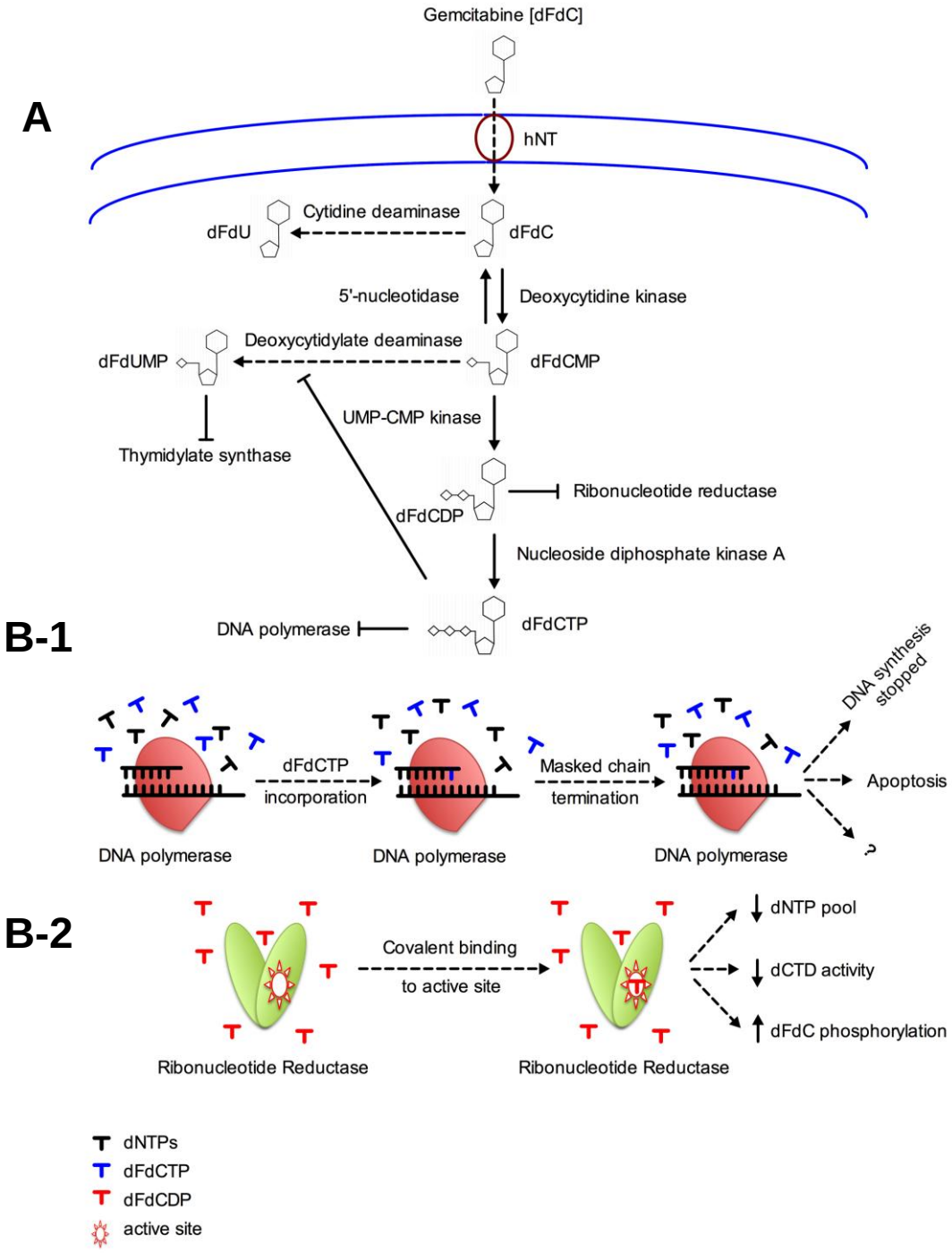


Figure 1.8 Gemcitabine cellular metabolism and mechanism of action.

(A) dFdUMP: 2′,2′-difluoro-2′-deoxyuridine monophosphate; dFdCTP: gemcitabine triphosphate; dFdCMP: gemcitabine monophosphate; hNT: human nucleoside transporter; dFdCDP: gemcitabine diphosphate, dFdU: 2′,2′-difluoro-2′-deoxyuridine. **(B-1)** Gemcitabine mechanisms of action. (B-1) Representation of the masked chain termination. In this mechanism, (gemcitabine triphosphate (dFdCTP), (nucleotide triphosphate (dNTP). **(B-2)** Gemcitabine self-potentiation. Covalent binding of gemcitabine diphosphate (dFdCDP), adapted from (70).

The equilibrative nucleoside transporter 1 (ENT1) facilitates the uptake of GEM (71). Interestingly, when pancreatic cancer patients were treated with GEM it was shown that the absence or lower levels of this nucleoside uptake mediator (ENT1) in patients, correlates with notably shorter survival compared to those with detectable or higher levels of expression (72). 5FU in combination with radiation improved the survival of pancreatic cancer patients from six to ten months compared to treatment with 5FU alone (60, 61).

The United Kingdom National Institute for Health and Care Excellence (NICE) provide recommendations for advanced pancreatic cancer treatment (73). NICE recommend the use of GEM if the patient shows a Karnofsky performance (a normal measurement method regarding cancer patients' capability for carrying out normal activities. The marks are between 0 and 100. Higher marks indicate that a patient is more functional in their daily tasks and this can inform the prognosis given as well as being used in clinical trials to score of 50 or more. It should also be used as a first line treatment. NICE also recommended that GEM is not appropriate for patients who are fit for surgery that could be curative, nor for patients who show a Karnofsky performance score of less than 50 (73). The European Society for Medical Oncology (ESMO) recommends 6 months of 5-FU chemotherapy or GEM postoperatively (74). Furthermore, patients can also be given erlotinib and GEM in combination as a first line treatment and this can be followed by 5FU combined with oxaliplatin as a second line treatment (74). Also lately, the combination of GEM and *nab*-Paclitaxel demonstrated a significant survival advantage over single agent GEM statistically and clinically (75).

1.7. Stem cells

1.7.1. Defining stem cells

There are several defining characteristics of stem cells, which include the ability to proliferate, self-renew, and maintain an undifferentiated state capable of generating a variety of cell lineages (76). Stem cells can be further classified according to their ability to differentiate, referred to as pluripotency, multipotency, unipotency and totipotency. The strength of this ability determined by stem cells' location and the duration of time from the point when the sperm fertilized the egg (zygote); this potentially can be the source of any tissues in the developing embryo. Adult stem cells do not possess the same potency as ES (Embryonic stem cells) cells, but are required to maintain tissue homeostasis and to affect repair in response to injury. These cells are resident in a specific stem cell niche within each organ, where they are maintained in their undifferentiated state. This niche mediates stem cells' homeostatic regulatory action, in order to ensure a continuous replacement of cells at a rate similar to that at which they are lost.

1.7.2. Long-term survival of embryonic stem (ES) cells and embryonic transcription factors (Nanog, Oct4 and Sox2)

ES cells maintain their pluripotency via expression of the transcription factor Oct 3/4 (Figure 1.9). Over-expression of Oct 3/4 makes cells differentiate into mesoderm and endoderm, while under-expression stimulates the creation of trophectoderm (trophoblasts differentiate from this layer of cells). Other significant regulatory elements, such as Nanog and Sox2 , have recently been found to be involved in the control of pluripotency and maintenance of stem cell identity (77) (Figure 1.9).

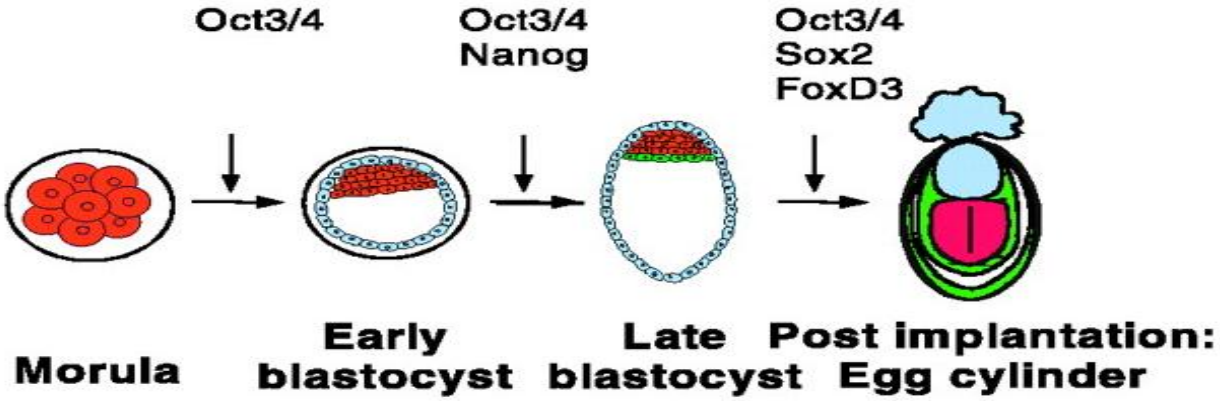


Figure 1.9 Role of Nanog, Oct4 and Sox2 in embryonic stem cells.

These transcription factors involved in maintenance of pluripotency in the developing embryo (77).

1.7.3. Stem cells and cancer

The most important similarity between cancer cells and stem cells is their ability to self-renew, suggesting that they may share some common signalling pathways. Self-renewal in cancer cells is unregulated, and so perhaps if there was a greater understanding of ES cell self-renewal processes, this could be translated to controlling self-renewal in the deregulated cancer cell (77, 78). Signalling pathways common to both cancer and stem cells include Notch, Shh and Wnt and the transcription factors Nanog, Oct 3/4 and Sox2.

1.8. The cancer stem cell (CSC) hypothesis

In all tumour types, heterogeneity is observed across all aspects including morphology, proliferation rates, genetic alterations, and therapeutic response. This heterogeneity intimates that there is much cellular diversity within a tumour, even one that has arisen clonally, and it is this heterogeneity which becomes the fundamental problem facing cancer researchers. Sequential mutations (Darwinian) provide a fair explanation for this heterogeneity. However, the accepted theory of clonal evolution could not answer all questions, such as the reappearance of metastatic lesions after removing of tiny tumours and metastases of unknown primary tumour. These unanswered questions strongly motivated cancer researchers to look at cancer from a different perspective; by comparing the functional hierarchy of embryonic and

adult stem cells with cancer cells. This approach brought about a new hypothesis, known as the cancer stem cell hypothesis (79-84). According to this hypothesis, cancer stem cells (CSCs) can generate differentiated progeny for maintaining the tumour, self-renewal and survival, even after exposure to chemo-radiotherapies, leading to reoccurrence of the tumour (Figure 1.10).

The first experimental evidence for the CSC hypothesis came from observations made by Furth and Kahn in 1937 (85), when a single leukemic cell from a mouse was injected into an inbred mouse, resulting in transmission of the leukaemia. In 1994, the first experimental proof regarding the identity of the tumour-initiating cell was reported. Specific cell-surface marker profiles were utilised in conjunction with fluorescence activated cell sorting (FACS) to identify populations with cancer stem cell characteristics; these cells were defined by a $CD34^{+}$ $CD38^{-}$ surface phenotype.

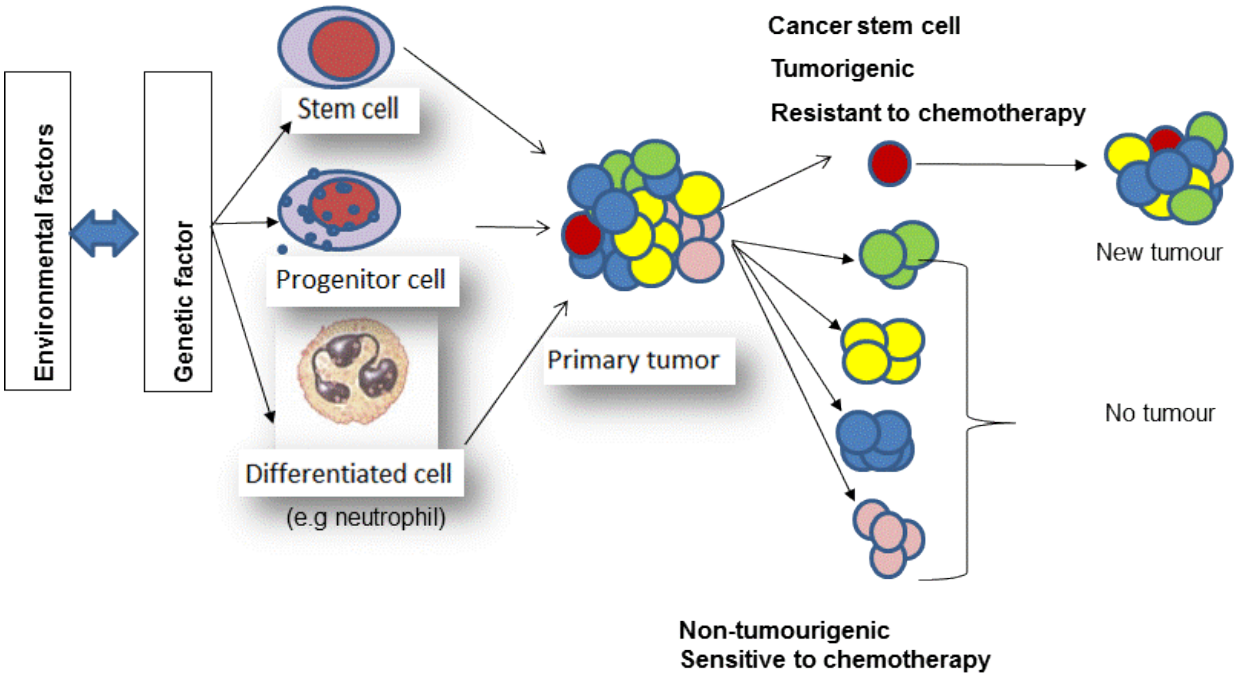


Figure 1.10 Hypothesis for the origin of the cancer stem cell.

Long-lived stem cells may gain mutations to become cancerous stem cells. Alternatively, more differentiated cells may gain mutations to give them a more stem-like phenotype. The CSC theory explains the existence of heterogeneous cells within a tumour, with a subset of cells that mimic the behaviour of stem cells and are ultimately responsible for tumour initiation, relapse, and chemo and radioresistance (86).

More recently, cancer stem cells have been identified in several tumour types, including colon, breast, prostate, liver, brain, melanoma, multiple myeloma and pancreatic cancer (87-91). It is now accepted that the majority of tumours have small subpopulations of cells with characteristics of stem cells that have the capability to generate the entire population of distinct cell types found in the original tumour (92). The stem-like properties of cancer stem cells have classically been demonstrated in the NOD-SCID (Nonobese Diabetic/Severe Combined Immunodeficiency) mouse model, by showing that transplantation of isolated pure populations can regrow tumours repeatedly on serial passage, and maintain the same morphological characteristics as the original tumour. According to the CSC hypothesis, CSCs have generally been shown to constitute only a small fraction of the cells within the tumour but provide the driving force behind malignancy. Tumours that contain higher CSC burdens are associated with higher rates of metastasis, poor patient prognosis and increased resistance to chemoradiotherapeutic agents (92). From a clinical perspective, the main issue, currently, with CSCs is that they are resistant to approved chemo-radiotherapies; a characteristic proposed as the fundamental reason for the recurrence of tumours (Figure 1.10).

It is still not clear whether CSCs are derived from transformation of specific stem cells, their progenies, or more closely-related dedifferentiated descendants of mature neoplastic cells. The origin of cancer stem cells in each specific malignancy has yet to be truly defined. The CSC model is expanding from its original definition of a small and distinct subpopulation, and it has been hypothesised that the CSC may encompass more common and heterogeneous cells. Additionally, CSCs are now thought to be dynamic and reversible entities in cancer, governed by the tumour microenvironment (92).

Understanding the behaviour of this small population of cells, which are known by many different names (tumour initiating cells, cancer stem cells and cancer stem-like cells) has become the main objective of stem cell researchers focussed on the discovery and development of drug therapies for cancer. For the sake of simplicity and without any prejudice, cancer stem cells (CSCs) will be used as the preferable terminology throughout this thesis.

1.8.1. Cancer stem cell niches

The tumour microenvironment is similar to the niche of normal stem-cells and it is thought that the tumour microenvironment provides a cancer stem cell niche. The microenvironment is comprised of diverse stromal cells, for example immune and mesenchymal cells, a vascular network, soluble factors and components of an extracellular matrix (ECM) (93). These microenvironments play a very important role in the interactions between non-malignant cells that comprise the micro-environment and CSCs. CSCs rely on these niches for their self-renewal and differentiation, and for the maintenance of tumour growth. The ECM could also have a protective role for CSCs against genotoxic insult from therapeutic interventions (94, 95).

1.8.2. Methods for identification of CSCs and markers for CSCs

CSC identification from a heterogeneous population of tumour cells is most commonly undertaken using Fluorescence Activated Cell Sorting (FACS). Until more recently, CSC populations were identified by the use of side populations (SP) which identify a cellular subset that have a high ability for effluxing drugs. This method has now largely been overtaken by the identification of specific markers which can be used in combination to identify CSC subsets of varying potency. Of particular interest within the context of this thesis are the markers CD133, CD24, CD44 and activity of the intracellular enzyme, aldehyde dehydrogenase (ALDH1). Commonly used markers for defining CSCs in solid tumours can be found in (Table 1.2) (86, 92). These markers have limitation as they are not expressed for all patients which could be different from one patient to another patient.

Table 1.2 Cancer Stem Cell markers for various types of solid tumours. This table lists some of the salient markers for the better-characterised solid tumours. Markers are not listed in order of importance, and some data originates from cell line only studies (86, 96).

Breast	Colon	Glioma	Liver	Lung	Melanoma	Ovarian	Pancreatic	Prostate
$ALDH1^{High}$	ABCB5	CD15	CD13	ABCG2	ABCB5	CD24	ABCG2	ALDH1
$CD24^{Low}$	ALDH1	CD90	$CD24^{Low}$	ALDH1	ALDH1	CD44	ALDH1	$CD44^{High}$
$CD44^{High}$	β-catenin	$CD133^{High}$	$CD44^{High}$	CD90	CD20	CD117	$CD24^{Low}$	CD133
CD90	CD24	α6-integrin	CD90	CD117	CD133	CD133	$CD44^{High}$	CD166
CD133	CD26	nestin	CD133	CD133	CD271		CD133	α2β1-integrin
Hedgehog-Gli activity	CD29		OV6				c-Met	α6-integrin
α6-integrin	CD44						CXCR4	Trop2
$CD49f^{+}DLL1^{high}DNER^{high}$	CD133						Nestin	
$CD133^{+}CXCR4^{+}$	CD166						Nodal-Activin	
Lin^{low}	LGR5							

1.8.3. Sphere forming characteristics of CSCs

Culture of cells under low attachment conditions, provides a sphere forming environment which enriches for the CSC. A single cancer stem or progenitor cell has the ability to proliferate and produce a spheroid, relying on its self-renewal capacity. This assay was used for the first time to demonstrate the existence of adult neuronal stem cells (97), and the method subsequently adopted to probe stem cells and progenitors in a variety of normal and neoplastic tissues. Nowadays, in studying CSCs, sphere-forming assays are utilised as indicative parameters (98, 99). However, there are limitations in this

methodology, including the fact that sphere-forming assays cannot detect stem cells which are not proliferating and which are in a quiescent state; they are not located in their true niches. Furthermore, the markers are dynamic and there is a possibility of bias in differentiation potential as a result of being cultured with exogenous growth factors (100).

1.8.4. Tumour drug resistance and membrane transporters in CSCs

Clinical drug resistance can occur due to alterations to drug targets, inactivation/detoxification of the drug, reduced uptake of the drug, higher drug efflux and dysregulation of apoptotic pathways. Many models have been suggested to explain the origin of multidrug resistance: the cancer stem cell model of drug resistance, the acquired-resistance stem cell model and the intrinsic resistance model (101-103).

For the sake of our limitation in this thesis, only more detail will be addressed on the cancer stem cell model; according to the cancer stem cell model of drug resistance, the original tumour has a small population of cancer stem cells and the progeny thereof, following differentiation. After exposure to the drug, only the cancer stem cells survive. These stem cells divide and restore the tumour's population with both CSCs and differentiated cells which are the progeny of the CSCs. It has been shown that cellular membranes and their constituents play a very important role in drug resistance in cancer cells, particularly the Adenosine triphosphate–Binding Cassettes (ABC). ABC transporters enforce the transport of substrates through biological membranes against a concentration gradient, via hydrolysis of ATP. Inactivation of ABC efflux pumps in order to reinstate drug sensitivity of CSCs, holds great promise for tackling various cancers (103, 104). Initial compounds that were used experimentally for this purpose include drugs such as verapamil and cyclosporine, which were capable of inhibiting the ABCB1 multidrug efflux pump (102).

1.8.5. Characteristics of CSCs in pancreatic cancer - markers and heterogeneity

The pancreatic cancer stem cell (PCSC) population has been commonly defined using the cell surface markers CD44+CD24+ESA+ (Epithelial Cell Surface Antigen), which are expressed in only a small set of cells, representing only ~0.2 to 0.8% of the primary tumour (91). Compared to their non-CSC counterparts, these cells are highly tumourigenic and have stem cell characteristics, including the ability to self-renew by symmetric division and produce differentiated progenies by asymmetric division (105, 106). In addition to the cell surface markers, the sphere formation assay (Clonal Colony-Forming Assay) was used to mark a cell sub-population within pancreatic cancer that have characteristics of stem cells (91, 107). The cells, which have the ability to form spheres in appropriate media, were highly tumourigenic when injected through intraperitoneal into 4 week-old Nude mice. Hermann *et al*. (2007) showed another subpopulation with high $CD133^+$ expression to be chemoresistant, and Hermann concluded that the $CD133^+CXCR4^+$ cells to be responsible for metastasis. Also other populations of PCSCs such ($CD44^+$c-Met^+) and ($ALDH^{high}$ activity and $CD44^+CD24^+$) have been subsequently reported to have metastatic ability in pancreatic cancer (106-109).

1.8.6. Roles of CSC markers in pancreatic cancer

The potential role of PCSCs stem cells in the initiation and recurrence of pancreatic cancer has recently been explored (91). Samples from human pancreatic adenocarcinomas were sorted for expression of the cell surface markers $CD24^+$, $CD44^+$ and ESA^+ and the isolated cells implanted into NOD/SCID mice (91). Dose limiting dilutions in this model revealed that the $CD44^+/CD24^+/ESA^+$ population had the highest tumourigenic potential, with cell numbers as low as 100 able to generate tumours. Cells which did not express these markers could not develop tumours in mice following injection of the same cell number. For additional markers associated with pancreatic cancer see Table 1.3.

Table 1.3 Pancreatic cancer stem cell markers.			
Marker (expression unless stated otherwise)	**Definitions**	**Functions**	**References**
CD44:	Class 1 transmembrane glycoprotein	Cell adhesion, proliferation, growth, survival, motility, migration angiogenesis, and differentiation	(110, 111)
CD24:	Heat-stable antigen consisting of a small extracellular protein held by GPIS (glycosyl phosphatidylinositols)	Role in cell-cell and cell-matrix interactions	(112)
CD133:	A cholesterol interacting penta-span transmembrane glycoprotein (120 kd). An associate of the prominin family, is defined in many of tissues with at least three variants.	Gives pancreatic cancer cells a high migration and invasion potential	(113-115)
ALDH-1 activity:	Has the ability to metabolise and neutralise cytotoxic alkylators such as cyclophosphamide causing high resistant cells	Cellular population exhibiting chemotherapy resistance	(106, 107, 116-118)
C-Met:	c-Met, a member of the receptor tyrosine kinase family.	Motility, invasion and metastasis	(108, 118)

1.8.7. Role of Nanog, Oct4 and Sox2 in pancreatic cancer

The major regulatory roles of the transcription factors Nanog, Oct4 and Sox2 in pancreatic cancer have yet to be fully elucidated. However, over-expression of these transcription factors individually or in combination has been linked to the transformation from pre-malignant to malignant conditions, poor differentiation of tumours, recurrence, metastasis and poor prognosis in pancreatic cancer, as well as other cancers (119). The degree of expression of Nanog, Oct4 and Sox2 can be highly heterogeneous in different tumours and sometimes within the same tumour (120).

To validate the importance of Nanog and Oct4 in PCSCs for proliferation, migration, invasion and the self-renewal processes, both genes were knocked down and chemotherapeutic resistance assessed in Panc-1 cell lines (121). It was observed that concurrent with knockdown of Nanog and Oct4, expression levels of CXCR4, MMP2, MMP9 and ABCG2 were also significantly decreased. The role of Nanog and Oct4 was also explored in the early stages of pancreatic carcinogenesis and correlated K-RAS mutation as Oct4 expression was increased in advance of K-RAS mutation, with high expression of Oct4 and *Nanog* genes in metaplastic ducts (119), indicating that targeting these proteins could be an ideal approach towards pancreatic cancer prevention and treatment. The clinical implications of targeting those CSCs with a distinct high expression Oct4 and Nanog is a subject of ongoing investigation (121, 122).

The *Oct4* gene has a pro-oncogenic role and is overexpressed in 69% of PDAC cases and in human pancreatic cancer cell lines (122-124). Particularly in the former, this induces cell proliferation, migration and invasion and correlates with clinical staging of cancers indicating a worse prognosis, whilst in human cell lines it contributes to metastasis and drug resistance (122). The *Nanog* gene is overexpressed in ~54% of PDAC (of 43 cases, 23 (53.5%) indicated that Nanog expression in the cancer tissue was strong, inducing proliferation, migration and invasion; it is associated with early-stage carcinogenesis, a worse prognosis and a negative impact on overall survival (121). Nanog is overexpressed in cells

which are capable of initiating spheres and promotes resistance to 5-FU treatment (125).

The *Sox2* gene is overexpressed in poorly-differentiated human tumours, correlating with aggressiveness (126) and also its ectopic expression in 19% of PDAC, promotes proliferation of cancer cells and their dedifferentiation, correlating with poor differentiation and rapid tumour progression (120, 127). Similar to Oct4, its induction of tumorigenic capacity can result in chemoresistance (128).

A few suggestions have been put forward to nominate which transcription factor or combination of factors should be targeted for clinical therapies; Wang *et al*. (128) suggested targeting Sox2/Oct4/c-Myc markers would be a possible strategy worthy of further study in preclinical settings, but Wen *et al*. (119) and Lu *et al*. (121) suggested Oct4 and Nanog co-expression could be a useful marker in forming a prognosis and could be targeted by pancreatic cancer therapies. It is likely that the PCSC population will not be defined by a single marker, but by a combination of the markers previously described. Expression of different sets of markers may be indicative of the degree of potency, tumour location and chemoresistance associated with CSCs.

1.8.8. Targeting PCSCs as a therapeutic approach for pancreatic cancer

Therapeutic strategies that selectively target CSCs have been investigated, some of which have been evaluated in pre-clinic (Table 1.4).
Significantly, such results suggest that clinical outcomes could be improved as a consequence of developing novel therapies that can eliminate or inhibit CSC proliferation or self-renewal capacity. A variety of possible approaches targeting surface antigens specific to CSCs have been detailed in preclinical studies, as well as those targeting cellular pathways related to cell differentiation, survival, adhesion and self-renewal. The recent attempts to target PCSCs are listed in (Table 1.4).

Table 1.4 Agents used to target PCSCs. Adapted from (118).			
Agents	**Target receptor/pathway or mechanism of action**	**Population markers**	Reference
XL184 (Cabozantinib)	c-Met	c-Met^{high}CD44^{+} CD44^{+}CD24^{+}ESA^{+}	(129)
DR5 Agonistic monoclonal antibody	DR5	ALDH^{+} CD44^{+}CD24^{+}ESA^{+}	(130)
SB431542	ALK4/7	CD133^{+}	(125)
Cyclopamine, IPI269609	Hedgehog	ALDH^{+} CD44^{+}CD24^{+}ESA^{+}	(131-133)
GSI-18 MRK-003, a potent and selective γ-secretase inhibitor	Notch Nuclear Notch1	ALDH^{+} CD44^{+}CD24^{+} and ALDH^{+}	(134) (134)
Salinomycin	EMT	CD133^{+}	(135)
Metformin	indirect activation of AMP-activated protein kinase	CD133, CD44, CXCR4 and SSEA-1 and Nanog, Oct-4 and Sox2	(125)
XL184 (Cabozantinib)	c-Met	SOX2, c-Met and CD133	(129)
Antibody-directed chemotherapeutics	Monoclonal antibodies against CD44^{+}	CD44^{+}	(136)
Antibody-directed chemotherapeutics	RON	CD44^{+}CD24^{+}ESA^{+}	(137)
Sorafenib (BAY 43-9006 or Nexavar) Sulforaphane	ALDH activity NF-κB	ALDH+	(138)
Anti-DLL4	DLL4 blocking antibody (Notch) and EMT	*in vitro* sphere-form CD44+/CD24+/ESA+	(139)

1.8.9. Crosstalk between pancreatic stellate cells (PSCs) and PDAC cells

PSCs initially were identified by Watari *et al*. (1982). They are mainly located in the periacinar region of the pancreas and constitute between 4 and 7% of parenchymal cells. PSCs, in a normal pancreas, are found in a condition of quiescence and their cytoplasm has an abundance of vitamin A as lipid droplets. There has been considerable study of PSCs in tumours (140). Understanding the interaction between PSCs and PDAC cells *in vitro* and *in vivo* is vital in tackling pancreatic cancer. PSCs play a key role in stroma formation to form a physical barrier preventing radiochemotherapies getting to the tumour site, enhancing resistance to therapeutic intervention. In addition, PSCs participate in tumour growth, invasion and metastasis as PSCs disseminate with cancer cells to distant metastatic sites, promote angiogenesis and have the ability to migrate over the endothelial barrier to and from blood vessels. The process of stromal formation by PSCs is not clear but it was reported (52, 140-142) that PSCs create fibrosis via excessive extracellular matrix (ECM) deposition, changing formation of intratumoural vasculature, creating a hypoxic state. Moreover, PSCs not only survive, but actually thrive and proliferate allowing the tumour microenvironment to take over up to 90% of the tumour total volume (52). PSCs and pancreatic cancer cells exist in a dynamic state, with each cell type influenced by paracrine signalling from the other, enhancing tumour growth and proliferation. Changes to signalling pathways are effected by signalling via cytokines and growth factors including fibroblast growth factor-2 (FGF-2), TGF-β1(Transforming growth factor beta 1), and platelet-derived growth factor.

In addition, it is thought that PCSCs interact with stromal cells mediated by factors such as SHH (Sonic Hedgehog), which is associated to PCSCs (143). PSCs also secrete SDF-1(stromal cell-derived factor-1), the ligand for CXCR4, to stimulate migration, invasion, and proliferation of pancreatic cancer cells *in vitro* (144). SDF-1 is also produced by other stromal cells, and so may provide an attractant for migratory PCSCs in other tissues via $CD133^{+}/CXCR4^{+}$

expression in PSCSs, to facilitate metastasis. Research has been done into blocking PSC activity in pancreatic cancer. For example, halofuginine, a smad3-phosphorylation-inhibitor, decreases PSC activation and inhibits pancreatic xenograft tumour development (145). Retinoic acid can also stop PSC activity and decreases wnt-β-catenin signalling in cancer cells and their invasive capability. Key signalling pathways between PSCs and cancer cells have been proposed, including sonic hedgehog, which could be targeted for potential therapeutic drugs (146, 147).

1.9. Cancer Chemoprevention

Cancer chemoprevention is a pharmacological approach, originally described by Sporn in 1976, which is an interventional attempt to stop or reverse the process of carcinogenesis (148). Since then, over nearly four decades, cancer chemoprevention has changed from a concept to an achievable reality (149). Chemoprevention might include retarding many steps in tumour initiation, promotion and progression and falls into three broad categories (150, 151):

A- **Primary Prevention** aims at preventing the initial development of tumours, both in healthy individuals, and to a greater degree in those predisposed towards cancer, due to genetics or personal history.

B- **Secondary prevention** relates to individuals with pre-cancerous conditions, attempting to prevent their development into cancer.

C- **Tertiary prevention** aims to prevent new cancers from forming in those already cured of pre-existing cancers, and to prevent disease recurrence or the development of metastases (111, 150, 152, 153).

1.9.1. Chemopreventive agents

Chemopreventive agents can be natural, synthetic, biological or chemical agents could possibly reverse, suppress, prevent or delay carcinogenic progression. The effective application of a chemopreventive substrate decisively relies upon the pinpointing of its mechanism of action at different

levels which ideally should be specific and effective, they should be easily administered (preferably orally), have little or ideally no toxicity and be affordable (111, 150, 152, 153).

Chemopreventive agents can be generically grouped into 2 categories according to their mode of action; blocking agents and supressing agents, however, in reality, most chemopreventive compounds identified to date have the capacity to act as both blocking and supressing agents. The aim of blocking agents is to prevent the initiation step of carcinogenesis; these compounds inhibit DNA damage and or enhance repair through a variety of processes, including free radical scavenging, phase II drug-metabolising enzyme induction, antioxidant action (154), and the promotion of DNA repair, inhibiting of phase I drug-metabolising enzymes, or prevention of carcinogen uptake (155). Tumour suppressing substrates may exert their effects through altering genetic regulation, halting of cell proliferation, causing of terminal differentiation, senescence, triggering of apoptosis in pre-neoplastic lesions and alteration of signal transduction (150, 151). The most credible target human populations for intervention are those individuals who have greater risk of carcinogenic progression or who are found to have premalignant lesions. None of the existing chemopreventive agents are perfect so far, which may be due to a variety of reasons, including a lack of efficacy or potency, lack of defined biomarkers, or due to unacceptable side-effects.

1.9.2. Current clinical use of cancer chemopreventive agents

Synthetic chemopreventive agents are already in use clinically for patients at increased risk of cancer development or recurrence. In breast cancer the chemopreventive drug, tamoxifen (156), is used for its oestrogen blocking properties. The concept of cancer chemoprevention has gained increased recognition since the approval of tamoxifen for use in primary (156) and tertiary chemoprevention (156). Fisher *et al*. showed a 49% decrease in metastatic breast cancer and a 50% reduction in non-invasive disease, but the danger of endometrial carcinoma doubled and an incident rate of thromboembolic occlusions increased with taking tamoxifen over a period of 5 years, in

comparison with taking a placebo. The primary action of tamoxifen is via the oestrogen receptor, and so its protective effect is limited to those tumours which are oestrogen receptor positive. Its side effects, including blood clot formation and an increased risk of endometrial cancer (149), have limited the use of tamoxifen by healthy high-risk women for prevention purposes. Two other prevention trials have reported (157) similar findings to those described by Fisher in terms of the protective effects and side effect profile of tamoxifen (151).

In prostate cancer, finasteride has been evaluated for chemoprevention, as an inhibitor of the 5-alpha-reductase enzyme (158). Although effective at reducing the occurrence of cancer, initial results from clinical prevention trials suggested that there was a greater prevalence of high-grade prostate cancer in men that took finasteride compared to those receiving placebo, but a follow-up study conducted 18 years later did not show a significant difference in the overall survival rate (158). Despite this, the ambiguity around the prevention of prostate cancer remains high, even following the introduction of more effective second generation 5α-reductase inhibitors such as dutasteride (159, 160).

Notable benefits have also been observed for aspirin in the prevention of colorectal cancer in patients with HNPCC, with aspirin reducing the average polyp number in patients by 28% (161-164) and lowering the risk of death from cancer by nearly 20 percent. Aspirin also reduced spontaneous intestinal tumour formation in a mouse model (165) also at low-dose aspirin could decrease the risk of spontaneous colorectal cancer death in human (166).

1.9.3. Dietary-derived cancer prevention agents

In the last two decades there has been increasing concern regarding the impact of dietary factors on cancer incidence, 42% cancers thought to be linked to 14 lifestyle modifications (167). It has been shown that fruits and vegetables are rich in antioxidant chemicals (168, 169) and as part of a healthy lifestyle, an intake of 5 portions of fruit and vegetables daily is recommended. It is from these source that many putative cancer chemopreventive agents are derived, including those from turmeric, ginger, onion, soybeans, garlic, grapes, tomatoes, broccoli, brussel sprouts and cabbage. The active components of these foods include genistein (170), resveratrol (171-175), allicin (176) and curcumin (174, 177-186).These substrates are found to inhibit cancer cell proliferation, to promote apoptosis, to suppress growth factor signalling pathways, to deactivate NF-κB, AP-1 and JAK-STAT pathways and to halt angiogenesis. Their value as cancer preventive agents may therefore lie in their ability to target many pathways that contribute to the carcinogenic process. Recently, many clinical trials have been carried out aiming to identify a particular nutritional supplement or modified diet which can be used cancer prevention.

1.9.4. Dietary chemopreventive agents for pancreatic cancer

Many substrates sourced from humans diets have been tested pre-clinically as prospective pancreatic cancer prevention, both alone and in combination with chemotherapy drugs. When developing a substance as a chemopreventive agent, the same substance is often evaluated for potential use as a chemotherapy drug or as a sensitizer to standard chemotherapeutic drugs as many of the mechanisms required overlap (Table 1.5). The two poly-phenolic compounds explored within this project are resveratrol and curcumin (Table 1.6), both of which target multiple pathways in carcinogenesis and have been used in clinical trials for the treatment and/or prevention of a variety of cancers. (187).

Table 1.5 Dietary-derived chemopreventive agents being used as adjuncts for chemotherapeutic drugs in preclinical studies of pancreatic cancer, taken from (188).

Chemopreventive agent	Chemotherapeutic drug	System	Reference
Curcumin	Gemcitabine	*In vitro* & orthotopic xenograft	(182, 189)
Curcumin	Celecoxib	*In vitro*	(190)
Celecoxib & Mucin-1-based vaccine	Gemcitabine	Kras ^{G12D}/ MUC1 animal model	(191)
Sulforaphane	TRAIL	*In vitro* & xenograft	(192)
Genistein	Docetaxel, Cisplatin	*In vitro*	(170)
Genistein	Erlotinib	*In vitro*	(188))
Resveratrol	Gemcitabine	*In vitro* & orthotopic xenograft	(193)
B-Dim	Gemcitabine	*In vitro*	(194)

Table 1.6 The ongoing or completed clinical trials to investigate the value of curcumin or resveratrol (187, 195).					
Identifier no.	**Year started**	**Phase Patient condition**	**Patient condition**	**Dose of curcumin or resveratrol**	**Purpose**
NCT01294072 Curcumin	2011	Phase I	Colon cancer	3.6 g/day for 7 days	Ability of plant exosomes to effectively deliver curcumin to colon tumours
NCT00973869 Curcumin	2009	Phase I	Colorectal cancer	14–28 days, dose not specified	Side effects of curcumin in preventing colorectal cancer in patients undergoing colorectal endoscopy or colorectal surgery
NCT00641147 Curcumin	2010	Not specified	Familial adenomatous polyposis	Twice a day for 12 months, dose not specified	Ability of curcumin to prevent colorectal cancer in patients with familial adenomatous polyposis
NCT01333917 Curcumin	2010	Phase I	Colorectal cancer	4 g/day for 30 days	Identify biomarkers that are modified by curcumin in patients with colorectal cancer
NCT00094445 Curcumin	2004	Phase II	Pancreatic cancer	8 g/day for up to 6 months	Ability of curcumin to shrink or slow the growth of pancreatic cancer
NCT00256334 Resveratrol	2009	Phase I	Colon Cancer	125 mg/day	Targeting Wnt signalling pathway
NCT01476592 Resveratrol	2015	Not specified	Neuroendocrine Tumor	5 mg/day	Effects on Notch-1 signalling
NCT00098969 Resveratrol	2010	Phase I	Unspecified adult solid Tumour	0.5, 1.0, 2.5, or 5.0 g daily for 29 days	Decrease in circulating IGF-I and IGFBP-3

1.9.5. Curcumin (diferuloylmethane)

Curcumin is derived from turmeric (Curcuma long is ginger family) (178) and is the main bioactive component, and is responsible for the yellow pigmentation (196). Curcumin has been used as a medicine in the Asian community for a thousand years but it has only been studied extensively over the last few decades (174, 177-186, 197, 198). During this time, evidence has accumulated to support the notion that curcumin is able of preventing or treating numerous pathophysiological developments, such as cardiovascular disease (199), pulmonary conditions and stroke (184), cancer (183), inflammation (184), liver disorders (200) and Alzheimer's disease (201). Curcumin can be obtained as a food supplement and is a commonly used food additive (E100) where it is often used as a colouring agent.

Curcuminoids is the principle component of turmeric responsible for the yellow colour of turmeric and it consists of a mixture of 75% curcumin, 16% demethoxycurcumin (DMC), 8% bisdemethoxycurcumin (bDMC) and a little quantity of cyclocurcumin (196) (Figure 1.11).These percentages can vary depending upon the formulation and purity of the extraction from turmeric.

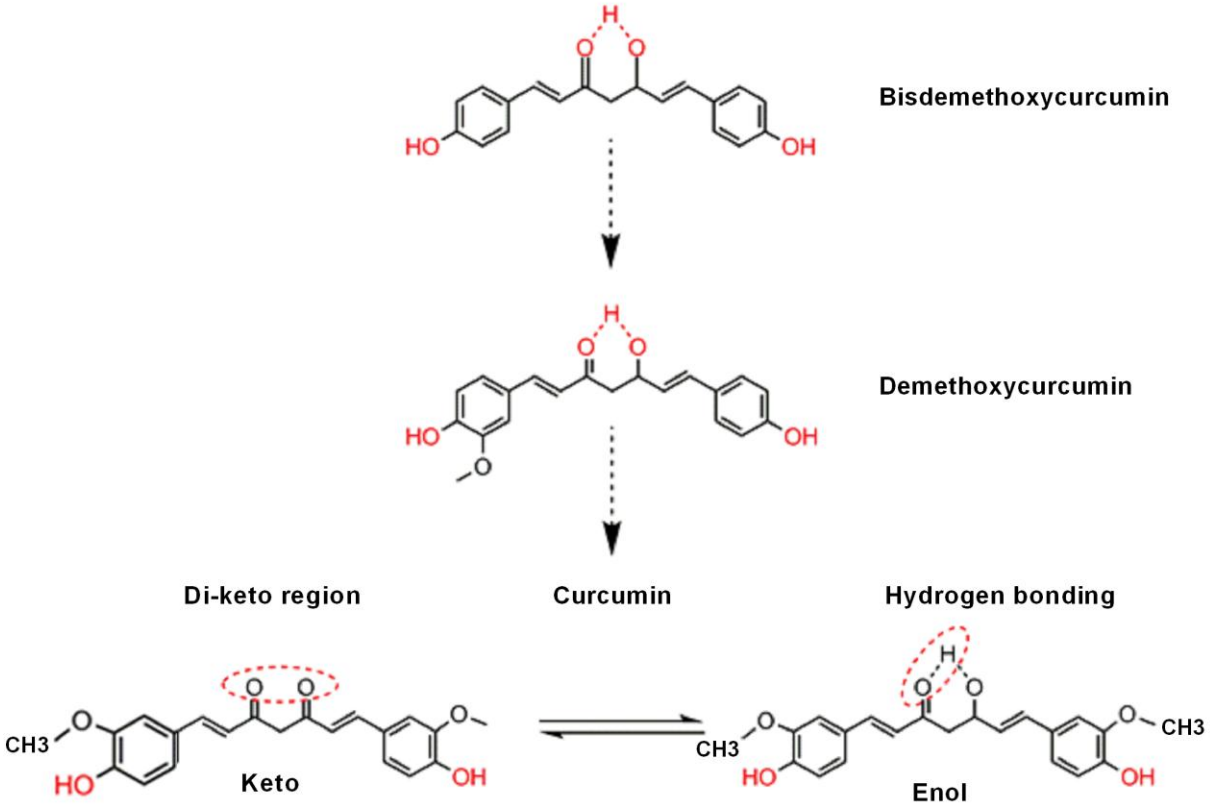


Figure 1.11 The major active constituents of turmeric.

Bisdemethoxycurcumin (bDMC), demethoxycurcumin (DMC), and a small amount of cyclocurcumin. Curcumin exists as keto and enol isomers (7).

There are a number of tautomeric forms of curcuminoids, which are a 1, 3-diketo form and two similar enol forms (Figure 1.11), with the OH functional group easily capable of donating hydrogen ions and undergoing nucleophilic addition. This property allows curcumin to have many biological activities, playing a role in reduction–oxidation and as an anti-oxidant, able to trap and scavenge radicals. Radicals commonly generate reactive oxygen species and nitrogen free-radicals, and are therefore capable of damaging DNA and proteins. An important property of curcuminoids, which can bring beneficial effects, is through the chain-breaking anti-oxidant activity via hydrogen atoms, in most cases originating from the phenol (OH) components (196).

1.9.6. Curcumin: molecular mechanisms of anti-tumour effects

Curcumin has an ability to selectively modify various cell signalling molecules including those relating to invasion, growth, metastasis, inflammation, angiogenesis and survival of cancer cells in various cancers. Curcumin is able to elicit many of these anticancer effects through inhibition of pro-inflammatory proteins: NOS (Nitric Oxide Synthase) and COX-2, growth factors: (VEGF (vascular endothelial growth factor) and HER2 (human EGFR type 2), apoptotic proteins: (survivin, Bcl-2, DNA topoisomerase, p53, hTERT), transcription factors: (NF-κB, Wnt/beta-catenin, STAT3, HIF-1(Hypoxia-inducible factor) and cell cycle proteins (cyclin B, cyclin E, p27, p21, cyclin D1, Chk1) (Figure 1.12). The anti-cancer effects of curcumin across a variety of cellular models are summarised in (Table 1.7) (174, 177-186). Curcumin affects every signalling pathways associated with The Hallmark of Cancer (Figure 1.3).

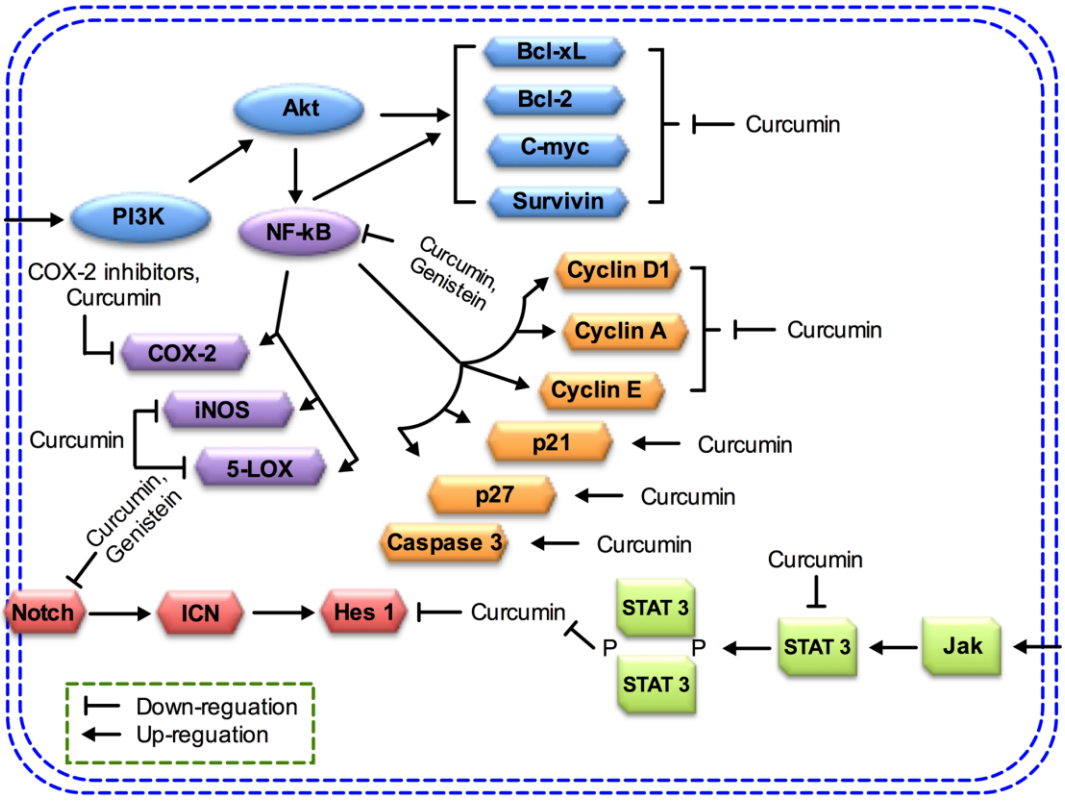


Figure 1.12 Curcumin and pancreatic cancer.

Shows the effect of possible pancreatic cancer chemopreventive substrates of dietary origin, including curcumin, on several pathways engaged in carcinogenesis of pancreatic cancer, adapted from (188).

Table 1.7 Preclinical anti-tumour activity of curcumin reported in various cancers			
Cancer type	**Cellular effects**	**Mechanism of action**	**References**
Colon cancer cells	Inhibition of growth factors	Inhibition of insulin growth factor-1 and EGFR receptor	(202)
Breast cancer cells	Inhibition of growth factors	Inhibition of HER-2	(203)
Bladder cancer	Antiangiogenic agent	Inhibits both VEGFR and VEGF	(204)
Human myelomonoblastic leukaemia cell line	Immune system	Suppressed TNF signalling pathways	(205)
Breast cells	Antioxidant, antiangiogenic, and antiapoptotic by inhibiting B-RAF	Induced heme oxygenase-1 (HO-1)	(206)
Rhabdomyosarcoma cells	mTOR-mediated signaling pathways in the tumour cells	Anti-mTOR agent	(207)
Breast cancer cell	Blocks proliferation	Downregulating ER activity	(208)

1.9.7. Curcumin in pancreatic cancer

In vitro studies have demonstrated that curcumin targets many cellular signalling pathways in pancreatic cancer cell lines such as NF-kB, COX-2, EGFR, ERK1/2, Notch, STAT3 and miRNA-22 (188) (Figure 1.10). Wang *et al*. (1999) and Li *et al.* (2004) demonstrated that curcumin halted the growth of pancreatic cancer cell lines in a time- and dose-dependent fashion by inhibiting NF-κB. It was demonstrated that curcumin suppressed COX-2 and EGFR expression and inhibited ERK1/2 activity in pancreatic cancer cells and consequently curcumin augmented the effects of gemcitabine on pancreatic cancer cell lines (189, 209). Further to this, curcumin (210) also downregulates Notch signalling in BxPC-3 and Panc-1 pancreatic cancer cells and alters

specific microRNAs associated with pancreatic cancer (upregulating miRNA-22 (211).

1.9.8. Clinical potential of curcumin

To date, there are more than 40 clinical trials using curcumin and assessing pharmacological and toxicological outcomes (185). However, most have been early phase trials and the outcomes have not yet shown sufficient clinical efficacy for it to be recommended as a treatment for any indication. The use of curcumin as a chemopreventive agent is supported by both *in vitro* and preclinical *in vivo* studies. Consequently, early-phase clinical trials have explored its safety, efficacy and pharmacokinetics in patients suffering from a variety of cancer types.

For pancreatic cancer, new drug development is acutely necessary as those currently in use (e.g. erlotinib and gemcitabine) elicit responses only in ~10% of patients and promise only a prolongation of their lives by a few weeks. Most patients die less than a year after being diagnosed (64, 197, 212). An 8 week phase 2 trial involving 25 patients with advanced pancreatic cancer saw them receiving an 8 g per day dose of curcumin (197). Curcumin-induced toxicity was not reported, while the disease remained stable in one patient for 1.5 years and a 73% reduction in tumour volume was reported for another patient. One of the suggestions from this study was that more consistent blood curcumin levels and better biological effects may result from improved formulations, since curcumin exhibits poor systemic bioavailability (Section1.9.10).

A Japanese phase I/II trial of 21 participants (Advanced pancreatic cancer) were given 8 g of curcumin per day (213). The disease was stable in 5 participants but none experienced a complete or partial response. However, the dose administered was well tolerated and the results of the clinical study indicate that for patients with pancreatic cancer, the use of 8 g curcumin daily in combination with gemcitabine-based chemotherapy is safe and further trials aimed at evaluating efficacy should be conducted.

Table 1.8 Summary of published clinical trials assessing curcumin on pancreatic cancer patients, taken from (187, 213).

Reference	(197)	(214)	(215)	(216)
Sample size	25	21	17	14
Study design	Phase II	Phase I/II	Phase II	Phase I
Study period	2008	2008-2009	2004-2006	2011-2012
Dose of curcumin	8 g/d	8 g/d	8 g/d	200 mg/d (*n* = 9) 400 mg/d (*n* = 5)
Prior history of chemotherapy	Yes (*n* = 22)	Yes (*n* = 21)	None	yes (*n* = 14)
Concomitant use of anticancer drug	No	Yes	Yes	Yes
Major toxicity associated with curcumin	None	None	Abdominal discomfort (*n* = 5)	Abdominal pain (*n* = 2)
Median survival time (months)	NA	5.4	5	4.4
Finding	No systemic side effect observed; Single case kept stable for over 18 months; Another case had a marked but short tumour response	Dose level well-tolerated; Five cases displayed stable illness; No cases found with a partial or complete response	One of 11 evaluable cases (9%) respond partially, 4 (36%) had stable disease, and 6 (55%) had tumour progression	No systemic side effects observed

1.9.9. Pharmacokinetics and pharmacodynamics of curcumin

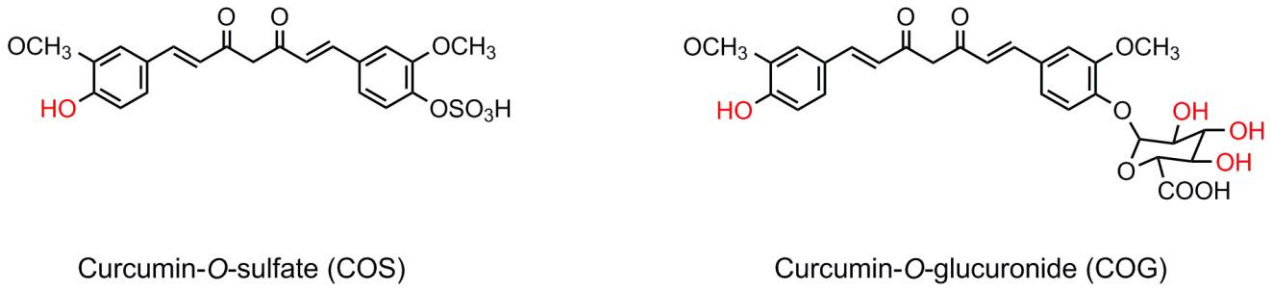
A) Bioavailability

Pharmacokinetic studies of curcumin have shown it has low oral bioavailability, therefore, despite showing promise as a chemopreventive agent, the dose of curcumin required to have a positive impact may potentially be difficult to achieve in the clinic (198). There are several possible reasons for low bioavailability of any compound; weak absorption, a high metabolic rate, metabolic product inactivity and/or fast clearance from the body (179, 217). Research up to the present have shown that the limited bioavailability of curcumin is because of its inadequate absorption and fast metabolism (217). The majority of curcumin ingested orally is excreted unmetabolised and the remaining curcumin is rapidly metabolised and undergoes biotransformation to produce various metabolites (217). It has been reported that typically, following an oral dose of 2.35 g curcumin, mean colonic mucosa levels were 48.4 mg/g (218). Serum concentrations peaked at 1 to 2 h post dose with a decline within 12 h (219). The average peak serum concentrations following ingestion of 4 g of curcumin was 0.51 +/- 0.11 µM; a 6 g dose generated 0.63 +/- 0.06 µM and 8 g curcumin produced concentrations of 1.77 +/- 1.87 µM (219). The highest curcumin levels have consistently been found in the intestinal mucosa, after oral administration (220).

Another formulated curcumin is Meriva (Soybean lecithin-curcumin) which showed higher bioavailability that curcumin, 18-fold more absorption of curcumin in human subjects from Meriva than from unformulated curcuminoid mixture; 29-fold more absorption of overall curcuminoid absorption (187, 221).

B) Curcumin metabolites and chemoprevention

Oral ingestion of curcumin has been shown, by research, to result in its transformation into curcumin-*O*-glucuronide (COG) and curcumin-*O*-sulfate (COS). In a study carried out by Ireson (222, 223) in humans receiving 3.6 g of daily curcumin over 4 months, curcumin glucuronide and sulfate conjugates were detected in plasma. Similarly, in studies carried out in rats, curcumin glucuronide and sulfate were predominantly detected (222, 223) (Figure 1.13).



Curcumin-*O*-sulfate (COS) Curcumin-*O*-glucuronide (COG)

Figure 1.13 Chemical structures of curcumin metabolites (COG, COS), adapted (198).

There are conflicting data on whether the metabolites of curcumin possess any intrinsic chemopreventive activity. In a study carried out by Dempe (224) three cancer cell lines (Ishikawa and HepG2 and HT29) were incubated with different metabolism of curcumin. In Ishikawa and HepG2 cells, curcumin was metabolized by bioreduction to HHC (hexahydrocurcumin) and small amounts of octahydro-curcumin (OHC), whereas the only metabolism in HT29 cells was the formation of curcumin glucuronide. Despite these differences, all cell lines responded to treatment, with G2/M phase arrest and mitotic catastrophe, likely due to the parent curcumin compound rather than its metabolites. The effects of curcumin versus curcumin glucuronide, were compared in HepG2 cells, and the metabolite was found to have much weaker anti-proliferative effects compared to the parent curcumin (225). However, there are studies which suggest that the metabolites are less active than curcumin (222, 223), and there are also studies which come to the opposite conclusion. Overall, the potential

role of curcumin metabolites in mediating any beneficial effects *in vivo* and in humans remains to be determined (226).

C) Improving the bioavailability of curcumin

The low bioavailability of curcumin has been shown to improve by using adjuvants, or by formulating in liposomes, micelles or phospholipid complexes. One such adjuvant, piperine, inhibits hepatic and intestinal glucuronidation and has been shown in various studies to increase serum curcumin levels (217). A study by Suresh and Srinivasan (227) found that absorption was increased from 48.7% to 56.1% when with micelles. Ma *et al*. (2007) found that when compared to dimethylacrylate (DMA), polyethylene glycol (PEG) and dextrose, micellar curcumin resulted in a biological half-life that was 60-fold greater in rats. Phospholipid complexes have also been shown to significantly improve curcumin's bioavailability (228). Furthermore, the bioconjugation of curcumin can help improve its bioavailability by increasing cellular uptake. The bioconjugate BCM-95, when combined with turmeric oil, showed 700% more activity and significantly increased bioavailability compared to curcumin (198, 217).

1.9.10. Curcumin targets stem cells

Curcumin has huge potential in terms of cancer chemoprevention and cancer treatment, particularly through the targeting of CSCs. The effect of curcumin on CSCs has been assessed *in vitro* as well as *in vivo* by utilizing side populations, tumour-sphere formation, cell-surface marker assays and enzyme activity. The findings of these studies are summarised in (Table 1.9).

Table 1.9 Studies demonstrating that curcumin targets CSC populations					
Curcumin or combination	**Cancer cell types**	**CSC populations**	**Mechanism of action**	**Observation**	**References**
Curcumin	Rat C6 glioma cells	Side population	Anti-proliferation	depletion of a side population	(229)
Curcumin	Glioma cells	Nestin and CD133	Anti-self-renewal	Depletion of Nestin and CD133 population	(230)
Curcumin	Breast cancer stem cells	High-ALDH1	Anti-proliferation	Depletion of High-ALDH1 population	(231)
Curcumin	Esophageal squamous carcinomas	ALDH1A1	Anti-proliferation	Depletion of High-ALDH1	(232)
Curcumin in combination with FOLFOX	Colon cancer cells	Lower levels of CD44, CD166	Anti-proliferation		(233)

1.10. Resveratrol as a chemopreventive agent

Resveratrol (Figure 1.14) is a phytoalexin which is naturally-occurring discovered in 1940. The origin, at that time, was the white hellebore lily (*Veratrum grandiflorum* O. Loes), whose roots were used to produce a somewhat white powder. It is naturally found in over 100 plants, such as jackfruit, corn lilies, blueberries, scots pine, mulberries, peanuts, cranberries and grapes (234). Resveratrol is commercially available as a food supplement and the best natural source containing resveratrol is the *Polygonum cuspidatum* root. Resveratrol has also been used as a constituent of traditional Chinese and Japanese medicine (235-238). Plants release greater volumes of resveratrol when stressed due to ultraviolet irradiation, injury, or under the attack of pathogens as defence mechanism.

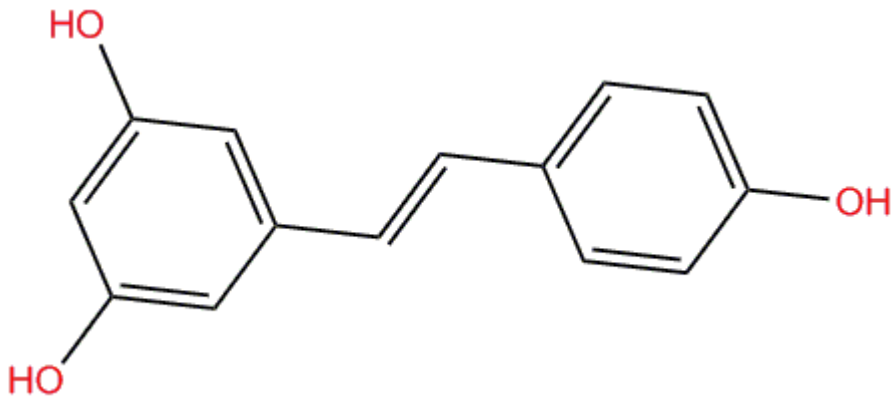


Figure 2.14 Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene), adapted (198).

1.10.1. Health benefits of resveratrol

A growing body of evidence suggests that resveratrol has an impact in reducing the negative effects of a number of pathological processes, with activity such as anti-inflammation, anti-atherosclerosis, inhibition of carcinogenesis, lowering of blood glucose levels, protecting from ischemia and neurotoxicity and cardio-protective effects, observed both *in vitro* and *in vivo* (234). Resveratrol also mimics the effects of caloric restriction in mammals and has been shown to extend lifespan in invertebrates (239, 240).

The characteristics of resveratrol that are thought to contribute to its biological activity are neuroprotection, antioxidant, cyclooxygenase inhibition, lipid modification, antiviral activity, platelet aggregation inhibition and vasodilation, radical scavenging activity, inhibition of tumour initiation, promotion and progression, and mitochondrial oxidative phosphorylation (234). However, the key mechanisms of action for resveratrol required for efficacy in humans at clinically achievable concentrations are not clear, but it has the facility to modulate multiple cellular targets including prostaglandin biosynthesis, gene expression, angiogenesis, cell cycle progression and signal transduction. Like curcumin, resveratrol can manipulate all the cancer phases; initiation, promotion, and progression by modulating signalling pathways. One of the

mechanisms of resveratrol-induced apoptosis which was documented in leukaemia, colon and breast cancer cells is via the recruitment and clustering of Fas receptors (235-238, 241, 242). The Fas receptor is a death receptor which initiates the death-inducing signalling complex (DISC) following ligand binding and this can activate caspase3 which leads to apoptosis. Resveratrol was also shown to repress the products of genes under the control of NF-kB, thus increasing activation of caspase-3, an important player in the caspase cascade leading to apoptosis (243). In a separate report it was shown that knockdown of Bcl-xL enhances the proliferation-inhibiting and apoptosis-inducing effects of resveratrol in H-2452 cells (244). Benitez *et al* (245) found that NF-kB–mediated transcriptional activity was inhibited by resveratrol in prostate cancer cells. Shakibaei *et al* (243) showed that resveratrol could inhibit IL-1β–induced apoptosis via caspase-3 activation which leads to apoptosis.

Translation of the preclinical findings to a clinical setting is essential for determining the potential benefits of resveratrol in humans. The driving force behind taking resveratrol was the “French Paradox” in which epidemiological studies suggested that there was an inverse relation between consuming red wine (a major dietary source of resveratrol) and risk factors for cardiovascular disease (246, 247). Furthermore, the effects of resveratrol on obesity, type 2 diabetes and Alzheimer's disease (AD) from a human perspective are currently under investigation and there is great interest in its potential value for preventing a variety of cancers (248, 249). In 2009 there was the first publication of a small phase 1 clinical trial involving patients, aimed at examining the potential of resveratrol as a cancer treatment (250). Patients scheduled for colorectal surgery received 14 days of oral resveratrol supplementation, at 80 or 20 mg per day (n = 2 and n = 1, respectively) or oral supplementation with grape powder, at 1.20 or 0.80g per day (n = 3 and n = 2, respectively) and paired pre- and post-dose colorectal tissue samples were analysed for evidence of activity. The authors concluded that the most significant effects were noticed with the low-dose grape powder that contained resveratrol in combination with other components, and caused down regulation of the Wnt signaling pathway, which may help reduce the risk of colon cancer progression.

In 2011, a clinical trial conducted in our institution examined treatment of colorectal cancer patients with hepatic metastasis with a micronized formulation of resveratrol (SRT501) or placebo (*n*=6 and 3, respectively) (251). After 10-21 days supplementation with 5 g SRT501, cleaved caspase-3, a marker of apoptosis, was significantly increased in malignant hepatic tissue, compared to tissue from the placebo-treated patients. Another study in healthy volunteers revealed that daily resveratrol intervention (0.5 - 5 g) for one month decreased circulating levels of insulin-like growth factor 1 (IGF1) and IGF-binding protein 3 (IGFBP3) (175), which could contribute to cancer preventive activity.

All evidence to date indicates that resveratrol is generally well-tolerated but mild to moderate gastrointestinal symptoms can occur with repeated high doses of 2.5 g and 5 g. Additionally, there is a report of adverse effects in a particular population of cancer patients that received the SRT501 formulation, which generates higher systemic concentrations than standard resveratrol. At 5 g per day, fatigue, diarrhoea, renal toxicity and nausea, among other symptoms, were reported in a phase II clinical study of relapsed or refractory multiple myeloma patients. One patient's death may have been contributed to by these events, and so the researchers ended the SRT501 trial prematurely (252). However, among healthy subjects and other cancer patients, other trials have found this to be a safe dose (173, 253).

It has been noted that many published preclinical studies have employed resveratrol concentrations significantly higher than those achievable in human plasma (151). Brown *et al*. (175) reported that in healthy volunteers who ingested doses ranging from 0.5 to 5 g daily, the maximum plasma concentrations achievable increased proportionately with dose and ranged from 0.19–4.24 μM. However, since doses exceeding 1 g cause gastrointestinal side effects, it has been suggested that this intake represents the upper limit for repeated administration in healthy populations for prevention purposes (151) and this dose generates maximum resveratrol concentrations of ≤1 μM. In colon tissues Patel *et al*. showed that resveratrol levels were extremely variable but could be recovered at much higher concentrations than in plasma,

with maximum mean levels of 674 nmol/g in patients that took 1 g daily for a week prior to surgery. At doses of 0.5 and 1 g resveratrol decreased tumour cell proliferation by 5%; the authors concluded it has potential as a colorectal cancer chemopreventive agent and that more clinical investigation is warranted (173).

1.10.2. Resveratrol targets CSCs

Resveratrol regulates multiple cellular pathways related to carcinogenesis (171-175, 254). In pancreatic cancer cell lines, resveratrol has many potential anti-tumour activities including induction of apoptosis, inhibition of cell proliferation and angiogenesis inhibition (172, 188). In addition to this, resveratrol in combination with gemcitabine potentiates antitumor activity *in vitro* and in the orthotopic mouse model of human pancreatic cancer (193). Furthermore, it has been shown that chemo-radiotherapies can be enhanced by resveratrol as a result of increasing sensitivity of malignant cells to the treatments (235, 255).

Recently, it was explored whether resveratrol could target CSC populations. Pandey (256) indicated that resveratrol significantly inhibited breast cancer cell viability and mammosphere formation, subsequently promoting apoptosis in cancer stem-like cells ($CD24^-/CD44^+/ESA^+$). The authors found that resveratrol induced apoptosis in CSCs through the alteration of FAS (fatty acid synthase) mediated cell survival signalling. Importantly, resveratrol was able to significantly decrease the growth of CSCs in a xenograft model of breast cancer without any indication of toxicity (256). The authors concluded that resveratrol can induce apoptosis in CSCs through lipogenesis blocking via alteration of FAS expression, showing a novel anti-tumour effect mechanism for resveratrol (256).

Resveratrol was shown to inhibit the self-renewal ability of PCSCs obtained from human primary tumours and KrasG12D mice *in vitro* (254). Furthermore, resveratrol induced apoptosis via capase-3/7 activation and repressing XIAP

activity and Bcl-2 in PCSCs. This resulted in less primary and secondary sphere formation in PCSCs obtained from KrasG12D mice. Activity of the multidrug resistance gene, ABCG2, which research indicates is overexpressed in PCSCs, was inhibited by resveratrol. Concurrently, resveratrol reduced expression of slug, vimentin and snail, and also slowed the rate of PCSC migration and invasion. Nanog and other transcription factors that have a role in pluripotency maintenance, including Sox2 and Oct4a, were also downregulated by resveratrol (254).

In nasopharyngeal carcinoma (NPC), resveratrol causes an increase in ROS levels, leading to depolarization of mitochondrial membranes, concurrent with inhibition of CSC self-renewal ability, resistance to therapy, tumour initiation ability and metastatic capability (257). The molecular mechanism by which resveratrol induced its effects was via the activation of p53. This therefore suggests that resveratrol may act via the p53 pathway in regulating stemness, EMT, and metabolic reprogramming (257).

1.11. Combining diet-derived compounds for cancer prevention and treatment

There is a limited amount of research exploring the interactions of drugs with natural products, despite estimates that 50% of oncology patients utilise such natural products, often without the knowledge of their doctors (258, 259). There is an inadequate volume of data available on such interactions and there is a significant gap in the literature in this regard, particularly with respect to the clinical consequences of using untested combinations.

The primary aims of using drugs in combination are to reduce toxicity and dosage, to minimise or delay the onset of drug resistance and to achieve improved results of therapy. Recently, a few novel *in vitro* combinations of dietary-derived agents and chemotherapeutics have been reported including curcumin and resveratrol (260) or celecoxib and curcumin (190) for colon

cancer, curcumin and omega-3 fatty acids for prevention and treatment of pancreatic cancer (261), curcumin and Garcinol for apoptotic and antiproliferative effects in pancreatic cancer cells (262).

1.11.1. Combining diet-derived compounds for pancreatic cancer prevention and treatment

Beneficial effects for combination of different compounds have been shown to enhance chemopreventive abilities when compared with using the compounds alone. Research has been done regarding the beneficial effects of differing combinations of natural products for therapeutic purposes in the pancreatic cancer treatment and prevention. Parasramka and Gupta (262) used a combination of garcinol and curcumin on human pancreatic cells (BxPC-3 and Panc-1) and assessed their antiproliferative and apoptotic activity. There was a significant reduction in the viability of both cell lines in a dose-dependent manner as compared to the controls. It was observed that there was a 2 to 5-fold lower concentration of garcinol and curcumin needed to show similar effects to those observed in combination compared to use of the single agents. There was also an increase in apoptosis observed, and a significant increase in caspase-3 and -9 activity, with induction apparent at lower concentrations when garcinol and curcumin were used in combination (262). Wang *et al* (2008) studied the effects of a combination of curcumin and isoflavone, a flavonoid found in soy products, to determine whether there was a synergistic antiproliferative effect in the BxPC-3 and Colo-357 pancreatic cancer cell lines. It was reported that compared to either treatment alone, and to the controls, there was a greater increase in the inhibition of cell growth following exposures ranging from 24 to 72 h. In BxPC-3 cells at 24 h, the combination of 10 µM isoflavone and 2.5 µM curcumin caused a 65% growth inhibition, with an even greater effect at 72 h (80% reduction). Even when treated with 10 µM isoflavone plus 1 µM curcumin for 72 h, proliferation was decreased by 35%. Observations of Colo-357 cells with 10 µM isoflavone and 5 µM curcumin, similarly, resulted in a reduction of cell number by 60% at 24 h. This cell growth inhibition was found, in part, to be because of the higher rate of apoptosis, where the

combination treatments (10 µM isoflavone and 2.5 µM curcumin) caused an 8-fold increase in apoptosis at compared to each treatment individually (263). A combination of curcumin, resveratrol, EGCG and isoflavone was also studied with a 40% cell growth inhibition observed when compared to the solvent controls (263). When Pancreatic cancer BxPC-3 cells were dosed with a combination of curcumin and do-cosahexanenoic acid (DHA), a six-fold increase in the induction of apoptosis was observed with the combination treatment and there was a 70% cell proliferation inhibition when exposed to 5 µM curcumin and 25 µM DHA (261) the combination being more effective than either treatment alone. Overall, these studies demonstrate that different compounds in combination can enhance the effects of treatment, with a lower concentration being required for a significant increase in antiproliferative and apoptotic activity.

A combination of curcumin and resveratrol has been used in various cancers, with enhanced effect observed when compared to curcumin and resveratrol alone. Curcumin and resveratrol in combination results in the inhibition of colon cancer cell growth by causing a reduction in cell proliferation and apoptosis induction. This occurred in both p53-positive (wt) and p53-negative colon cancer HCT-116 cells *in vitro* and in a mouse xenograft derived from the same cells. When curcumin (500 mg/kg body wt) and resveratrol (150 mg/kg body wt) were given individually, they inhibited the growth of HCT-116 tumours by 40% and 28%, respectively. When given in combination, tumour growth was inhibited by >50%, relative to the control group of mice, which represents a greater effect than either monotherapy (260). A combination of liposomally encapsulated curcumin and resveratrol has been used in the prostate-specific PTEN knockout mouse model. Here, the combination inhibited prostatic adenocarcinoma development *in vivo* more potently than either of the single agents and induced apoptosis *in vitro* (264) which might contribute to reduced incidence of prostate cancer because of the loss of PTEN, the tumour suppressor gene. In another study, when the combination treatment was used on Hepa1-6 hepatocellular carcinoma cells, there was a dose and time dependent effect on the inhibition of cellular proliferation, and an increase in apoptosis alongside caspase-3, -8 and -9 activation. There was also an up

regulation of intracellular reactive oxygen species (ROS) levels and a down regulation of XIAP and survivin expression (265).

Curcumin and resveratrol individually have been used in models of pancreatic cancer and have been shown to have chemopreventive potential. However, these two compounds have yet to be used in combination to assess whether these preventive effects can be enhanced. This research aims to identify whether low clinically achievable concentrations of these agents could have potential utility in the treatment or prevention of pancreatic cancer, either alone or in combination, and to examine their effects on PCSCs.

Aims:

This project is focused on pancreatic cancer; on the prevention of it, and attempts to find a better treatment. In recent years the cancer stem cell theory has increased in popularity among developmental biologists and cancer biologists, and is based on the theory that a small set of cells is responsible for the initiation of pancreatic cancer, as well as its maintenance and reoccurrence. Therefore, a rational approach for cancer prevention and better treatment would be targeting this small subset of cells for elimination. Consequently, the overall goal of this project is to evaluate the ability of two phytochemicals, curcumin and resveratrol alone or in combination, to target the tumour-initiating cells within pancreatic cancer using a panel of cell lines, and with an emphasis on using clinically relevant concentrations. Given the importance of stroma in pancreatic cancer, these two compounds have been tested for activity against pancreatic stromal cells, significant activity in this context could allow more effective drug delivery to the tumour site and may be a valuable target in both chemopreventive or treatment strategies for pancreatic cancer. In addition, the activity of curcumin metabolites has also been investigated to ascertain whether they might contribute to efficacy in humans.

To achieve the overall aims the following specific objectives have been completed:

1- Assessment of the growth inhibitory effects of curcumin and resveratrol alone and in combination on pancreatic cancer cell lines using relevant concentrations that are achievable in human plasma and potentially pancreatic tissues (Chapter 3).
2- Determination of whether curcumin and resveratrol alone and in combination inhibit the growth of pancreatic stellate cells, which can contribute up to 90% of the pancreatic tumour mass (Chapter 3).
3- Exploration of whether curcumin and resveratrol singularly and in combination inhibit the growth of various cancer stem like cell populations in pancreatic cancer cell lines, defined using the cell surface markers CD44, CD24, ESA, CD133, ALDH-1 activity or sphere forming ability (Chapter 4).

4- Establish the CSC profile for tumours obtained from pancreatic cancer patients and comparison with the CSC profile in pancreatic cancer cell lines (Chapter 4).
5- Investigation of the processes underlying the growth inhibition of CSCs in pancreatic cancer cell lines, including effects on Nanog (Chapter 4).
6- Evaluation of curcumin mono-sulfate activity, a major metabolite of curcumin, in pancreatic cancer cell lines (Chapter 5).
7- Elucidation of the intracellular uptake and metabolism of curcumin in pancreatic cancer cell lines in an attempt to explain the differential activity of curcumin and its metabolites (Chapter 5).

Overall, it is hoped that this project will improve understanding of the effects of curcumin and resveratrol on PCSs, and provide information to assess the potential clinical utility of these agents alone and in combination for the prevention or treatment in pancreatic cancer.

Chapter Two: Materials and Methods

2.1. Materials

All cell culture and laboratory plastic ware was obtained from Appleton Woods (UK) unless stated otherwise. Heparin, DMEM high glucose media, curcumin (≥94% curcuminoid content) and all general laboratory reagents were obtained from Sigma (Dorset, UK). Curcumin mono-sulfates (185) and curcumin mono-glucuronide (266) were synthesised in house by Dr Robert Britton and Mr Jagdish Mahale (Unpublished data). Cell strainers (40, 100 µm) were obtained from (VWR International). Phosphate buffered saline (PBS), 50% Neurobasal Medium, DMEM/F12 Medium (1:1) hyclone, N-2 Supplement, B-27 Supplement, Antibiotic-Antimycotic, FGF-2, EGF and all solvents were obtained from Fisher Scientific unless stated otherwise. Tissue culture supplements, including foetal bovine serum (FBS), media, glutamax and trypsin were obtained from Invitrogen. Accudrop, CST beads and FACS flow sheath fluid were obtained from BD Biosciences (Oxford, UK) and the aldefluor kit was supplied by Stem Cell technologies (Cambridge, UK). Antibodies were obtained from multiple suppliers; the Anti-Sox2 antibody was purchased from Merck Millipore (Hertfordshire, UK). EpCam (ESA) and CD133 were obtained from Miltenyi Biotech (Surrey, UK). CD24-FITC and CD44-APC supplied by BD Pharmingen (Oxford, UK) and ALDH1A1 was from BD Biosciences. IgG negative control for immunohistochemistry was from Dako (Cambridge, UK). Antibodies for western blot included Cleaved Caspase-3 (Asp175) from Cell Signaling Technology (Hitchin, UK), Nanog and Oct4 from Novus Biologicals (Abingdon, UK), ALDH-1 BD Pharmingen (Oxford, UK) and actin was sourced from Santa Cruz (Middlesex, UK). Resveratrol (99.9% purity) was obtained from Shanghai Novanet Co. Ltd. (Shanghai, China). HPLC (high performance liquid chromatography) supplies were from obtained from Waters (Hertfordshire, UK). These included Deactivated Clear Glass 12 x 32mm Screw Neck Total Recovery Vials, Atlantis dc18 Sentry Guard Cartridge, 100Å, 3 µm, 4.6 mm X 20 mm, Atlantis dc18 100Å, 3 µm, 4.6 mm X 150 mm column.

Immunohistochemistry materials included Distyrene Plasticizer Xylene (DPX) supplied by Sigma (Dorset, UK) and xylene and Industrial Methylated Spirits

(IMS) obtained from Geneta Medical (York, UK). Novolink TM Polymer Detection Kit was purchased from Novocastra TM and polysine adhesion slides from Thermo Scientific (Paisley, UK).

2.2. Preparation of buffers

2.2.1. Western blotting

Running buffer comprised a 1 to 10 dilution of 0.25M Tris/1.92 M glycine/1 % SDS (10X) running buffer from Geneflow (Lichfield, UK) by adding 100 mL of stock buffer to 900 mL water. Transfer buffer was prepared by adding 100 mL of stock buffer of (0.25 M Tris/1.92 M glycine Geneflow (Lichfield, UK)), to 700 mL water and 200 mL methanol. Ammonium Persulphate (AMPS) (Sigma) was prepared at 10% w/v in dH_2O, aliquoted and stored at -20°C. PBST (phosphate buffered saline – tween-20) was prepared by addition of 10 PBS tablets (Sigma) and 1 mL tween-20 (Sigma) to 1000 mL of water.

Five % or 3% Marvel milk (Spalding, UK) or 5% of BSA (Life Technologies (Paisley, UK)) in PBST were used for blocking purposes, with 3% milk (in PBST) or 3% BSA used for diluting both primary and secondary antibodies. All cell lysis was undertaken using RIPA buffer (Sigma) with the addition of one tablet each of Phospho-stop (Invitrogen (Paisley, UK)) and Complete Mini protease inhibitor (Invitrogen) added per 10 mL of RIPA buffer.

2.2.2. Preparation of Antigen retrieval buffer (Immunohistochemistry)

For all antigen retrieval, freshly-made citrate buffer was used (10 mM citric acid at pH 6).

2.2.3. Preparation of Antibody diluent (Immunohistochemistry)

One % (w/v) BSA in PBS was used for both primary and secondary antibody dilution, and was freshly prepared prior to each use.

2.2.4. Preparation of Freezing Mix

Cells were re-suspended in 10% dimethyl sulfoxide (DMSO) and 90% foetal calf serum, frozen overnight at -80°C and transferred to liquid nitrogen for long-term storage.

2.2.5. Stem cell media composition

Stem cell media (sphere media) was prepared in 100 mL aliquots and stored at 2-8°C. Each aliquot comprised the following: 50% Neurobasal Medium (50 mL); 1% N-2 Supplement (1 mL); 2% B-27 Supplement (2 mL); 2% Antibiotic-Antimycotic (2 mL); 2 µg/mL Heparin (100 µL); 20 ng/mL FGF-2 (20 µL); 20 ng/mL EGF (20 µL); DMEM/F12 Medium (1:1) hyclone (45 mL). Media was filtered-sterilised prior to use.

2.3. Cell lines

2.3.1. Cell line suppliers

All pancreatic cancer cell lines were supplied by ATCC (UK). Panc-1 is an epithelioid carcinoma adherent cell line, derived from pancreatic cells of a 56 year old Caucasian male. Capan-1 is an epithelial adenocarcinoma adherent cell line of pancreatic origin, derived from a metastatic site in the liver of a 40 year old Caucasian male. AsPC-1 is an epithelial adenocarcinoma adherent cell line of a pancreatic origin, derived from a metastatic site in the ascites of a 62 year old Caucasian female.

Table 2.1 Pancreatic cell lines and media requirements.

Cell line	Supplier	Media	Supplier
Panc-1	ATCC (CRL-1469)	Dulbecco's Modified Eagle's Medium	Sigma
Capan-1	ATCC (HTB-79)	Iscove's Modified Dulbecco's Medium	Invitrogen
AsPC-1	ATCC (CRL-1682)	RPMI 1640	Sigma
RLT-PSC	Dr. Jesenofsky and Prof. M. Löhr, University of Heidelberg, Department of Medicine	Minimal Essential Medium	Sigma

Immortalized pancreatic stellate cells (RLT-PSC) derived from a patient with chronic pancreatitis. They were immortalized by transfection with SV40 large T antigen and human telomerase (Jesenowski et al., 2005) (Table 2.1). For genetic data regarding the cell lines see (Table 2.2).

Table 2.2 Genetic mutations in the pancreatic cell lines and frequencce mutations found in pancreatic cancer patients. WT= Wild type, HD= Homozygous deletion. (Deer EL et al (2010)				
Cell line	***KRAS (>90)***	***TP53 (75)***	***CDKN2A/p16(>95)***	***SMAD4(50)***
Panc-1	Mutated	Mutated	HD	WT
Capan-1	Mutated	Mutated	HD	Mutated
AsPC-1	Mutated	Mutated	WT	WT

Note: The numbers represent frequency of the mutations found in pancreatic patients.

2.4. Methods

2.4.1. Maintenance of cell lines

Panc-1 cells were cultured in high glucose (4500 mg/mL) Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS) and 1X Glutamax. Capan-1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM)/GlutaMAX, supplemented with 20% foetal calf serum (FCS). The AsPC-1 cells were cultured in RPMI 1640 media with L-Glutamine and 10% FCS. The stellate cells (RLT-PSC) were cultured in Minimal Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS) and 1X Glutamax.

All four cell lines were kept in a humidified incubator at 37°C, supplemented with 5% CO_2 and grown to 70–80% confluency prior to passage/harvest. Media was aspirated and the cells washed with sterile PBS before addition of 1-2X trypsin/EDTA (T/E) for 5 min (for Panc-1, AsPC-1 and Stellate cells) or 5-10 min (for Capan-1), at 37°C. Following cell detachment, complete medium was added to neutralise the trypsin. Cell suspensions were then centrifuged at 207 × g for 5 min, the supernatant discarded and cells plated into new flasks at the necessary density. The maximum cell passage number did not exceed 20. Cells were harvested and re-suspended in an appropriate medium and counted using

a Z2 particle count and size analyser from Beckman Coulter (High Wycombe, UK).

2.4.2. Sphere growth and maintenance

To investigate whether Capan-1, Panc-1 and AsPC-1 cells can grow into spheres, single cell suspensions of Panc-1 were plated in ultra-low attachment plates in sphere growth media. Cells were fed weekly with 0.5 mL of fresh sphere media and passaged after two or three weeks, depending on the rate of sphere growth. For passaging, spheres were filtered through 100 µm filters to remove debris and the supernatant discarded. The filter was washed with DMEM supplemented with 10% FCS and spheres collected in the flow through. The sphere solution was centrifuged at 200 x g for 10 min, and the supernatant discarded. The sphere pellet was re-suspended in 1x TE and incubated for 10 min at 37°C to make a single cell suspension. Trypsin was neutralised by adding DMEM media containing 10% FCS, cells pelleted and washed in PBS, counted and plated in sphere growth media at the required seeding densities. Freshly prepared single cell suspensions of Capan-1, Panc-1 and AsPC-1 cells were plated at 5,000 cells per well in 6-well ultra-low attachment plates for sphere growth, and cells supplemented with sphere growth medium. Plates were kept in a humidified incubator at 37°C, supplemented with 10% CO_2.

One to two weeks were required for sphere formation. Curcumin and resveratrol were dissolved in DMSO and added to the cells to final concentrations of 0.01, 0.1, 1 and 5 µM. Cells were exposed twice a week for a period of two weeks. During each exposure of either curcumin or resveratrol, the appropriate volume was added on top of the existing incubation volume. After two weeks of sphere growth, sphere numbers were counted and sizes were measured. All treatments and control incubations contained an equivalent concentration of DMSO (diluted in the media), which did not exceed 0.1%. All treatments were carried out in triplicate and three biological replicates for each cell type. Spheres from each well were collected in 15 mL centrifugation tubes and centrifuged at 190 x g for 10 min. The supernatant was discarded leaving a residual volume

of 30-50 µL with the sphere pellet. Gridded slides were circled around the grid with wax to define an area within which the spheres were counted and measured. Sphere pellets were re-suspended in the residual volume and plated into the circled area of the gridded slide for counting and size measurements using an inverted light microscope (Nikon EclipseTE2000U) at 10X optical zoom. The sphere size was determined using Eclipse software that measured an average diameter of two measurements for each sphere.

2.4.3. Checking cell lines for ALDH activity

All the necessary reagents were supplied in the Aldefluor assay kit (Stem Cell Technologies). Inactivated Aldefluor substrate was dissolved and activated in compliance with the instructions given by the manufacturer. Twenty five µL of DMSO were added to Aldefluor substrate (BiodipyTM – aminoacetaldehyde) for a period of 1 min. Subsequently, 25 µL of 2N hydrochloric acid was added and incubated at room temperature for 15 min. Aldefluor assay buffer (360 µL) was then added to the solution and the activated substrate aliquoted and stored at -20°C.

Prior to use, all reagents were equilibrated to room temperature. Approximately, $1x10^6$ cells in a single cell suspension were used for staining. Cell pellets were re-suspended in 1 mL aldeflour buffer (supplied with the kit) and split into two tubes; one labelled 'control' and the other ‘test’, with each sample to be tested for ALDH1 activity.

The ALDH1 inhibitor, DEAB (diethylaminobenzaldehyde) (10 µL), was added to each control tube and mixed with the cell suspension. Immediately, 2.5 µL of activated aldefluor substrate was added to each of the 'control' and 'test' tubes. Following this, all of the tubes were incubated at 37°C for 40 min. The activated substrate was converted by intracellular ALDH into a negatively charged fluorescent compound (BIODIPY^{TM}-aminoacetate). The negative charge of this reaction product prevents diffusion and retains it inside the cell. However this

can be easily effluxed from cells by the ATP-binding cassette (ABC) transporter system. This active efflux is prevented by the use Aldeflour assay buffer which contains inhibitors of the ABC transporter system. After the incubation period, all tubes were washed with 500 µL of adleflour buffer and centrifuged at 207 x g for 3 min. The supernatant was discarded and the pellet re-suspended in 250 µL of Aldefluor buffer for FACS analysis.

2.4.4. Co-staining for CD44 and CD24 and single staining for CD-133

Approximately $1x10^6$ cells were used for staining Panc-1, AsPC-1, and Capan-1 cells with fluorophore-conjugated antibodies raised against CD44 and CD24. Prior to staining, cells were harvested using trypsin, washed with PBS and re-suspended in complete medium, ensuring that a single cell suspension was obtained. Cells were aliquoted (100 µL at 1 x 10^7 cells/mL) into pre-chilled tubes as follows: unstained cells; CD44 only; CD24 only and co-stained CD24/CD44. The cells were washed in PBS (3 min, 277 x g), the supernatant discarded and the cells re-suspended in Aldefluor buffer. Appropriate antibodies (10 µL, 1:10 dilution) were added to the 'test' samples only. Cells were incubated with the primary antibody for 30 min on ice, and then washed twice with PBS (3 min, 277 x g). Following centrifugation, the supernatant was discarded and cells re-suspended in 500 µL of Aldefluor buffer. Analysis of the stained populations was subsequently undertaken by flow cytometry using a BD FACS Aria II with Diva 6 analysis software (Becton Dickinson). For CD133 single staining the same procedures outlined above were followed.

2.4.5. FACS analysis and sorting conditions

All FACS analysis and sorting was carried out using the BD FACS Aria II SORP machine. The instrument was QC tested prior to any sample analysis. Single cell analysis was achieved by exclusion of doublets through different gating strategies based on the forward and side scatter profiles of each sample analysed. All the events were recorded and saved for further analysis. The minimum number of events recorded for any analysis was 10,000 provided that there were enough cells for analysis. Laser delay was set up using Accudrop beads when sorting cells using an 85 µm nozzle. Sorted cells were collected in DMEM media supplemented with 10% FCS.

2.4.6. Sorting Capan-1 for ALDH-1 activity by FACS for Western blotting

Capan-1 cells were sorted after being exposed to curcumin at two different concentrations (1 µM and 2.5 µM) for 6 days, with exposure on a daily basis. Sorted cells were lysed and the expression of embryonic pluripotent transcription factors assessed using antibodies raised against Nanog, Oct4 and Sox2.

2.4.7. Evaluating effects of curcumin and resveratrol on cell proliferation in Panc-1, Capan-1, AsPC-1 and RLT-PSC

Five different concentrations (0 µM, 0.01 µM, 0.1 µM, 1 µM and 5 µM) of curcumin and resveratrol were used. Cells were seeded at densities of $2x10^4$, $1x10^4$, $8x10^3$ and $5x10^3$ cells per well in a 24-well plate, for Panc-1, AsPC-1, Capan-1 and RLT-PSC, respectively. Cells were allowed to adhere for 24 h before commencing daily dosing treatments. ALDH activity and co-staining with

CD24 and CD44 was performed on days 3 and 6. Daily dosing was also undertaken using a combination of curcumin and resveratrol at the following respective concentrations: 0.1 µM curcumin + 0.1 µM resveratrol, 5 µM curcumin + 0.1 µM resveratrol, 5 µM curcumin + 5 µM resveratrol, 5 µM resveratrol + 0.1 µM curcumin. Cells were harvested and re-suspended in 10 mL of isotone and counted on days 1-7 using a Z2 particle count and size analyser from Beckman Coulter (UK).

2.4.8. Determination of protein concentration using Pierce BCA Assay

The protein assay was conducted in accordance with the manufacturer's instructions. BSA (2 mg/mL stock) was diluted with distilled water to prepare a standard curve (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) and the cell lysates diluted at ratios of 1:10, 1:50 and/or 1:100. Ten µL of each sample was aliquoted into 96 well plates (Corning, UK) and 200 µL of BCA (Bicinchoninic Acid) reagent was mixed with each and incubated for 30 min at 37°C. The intensity of colour change was measured at 595 nm using a Fluostar Optima plate reader (BMG Labtech) and the concentration of the lysates determined from the standard curve.

2.5 Western Blot

2.5.1. Preparation of the gels

Gels were prepared using gel casting apparatus (Bio-Rad, mini gel apparatus). Based upon the molecular weight of the protein, a Sodium Dodecyl Sulfate (SDS)-polyacrylamide (8-15%) resolving gel was prepared, a stacking gel (5%) was poured on top, appropriate combs placed in and the gel allowed to set.

Gels were then placed in a gel tank with running buffer and the gels loaded with one aliquot of appropriate molecular weight marker.

2.5.2. Sample preparation and running of the gel

Protein samples were thawed and the required volumes of the protein lysate diluted in water to give samples of equivalent concentration in a final volume of 20 µL. Added to this was an equal volume (10 µL) of sample loading buffer (2X Laemmli, Sigma, UK). Samples were heated (5 min, at 100°C) to denature the proteins, followed by a quick vortex and a pulse centrifuge. Samples were then loaded into the wells and run at 100 V for 60-90 min at room temperature.

Proteins were transferred from the gel to a nitro cellulose membrane by first placing them onto a transfer cassette consisting of a sponge, blotting paper (Whatman, UK), the gel, the nitrocellulose membrane (Geneflow Ltd UK), blotting paper and a final sponge. The transfer cassette was placed into a mini gel holder which was colour coded to ensure the transfer cassette was correctly orientated. All blotting paper, nitrocellulose membranes, and sponges were pre-soaked in 1X transfer buffer before being placed in the transfer cassette. Subsequently, the transfer cassette was inserted into a blotter assembly in a tank that was filled with transfer buffer. Protein transfer was conducted at 120 V for 1.5h at room temperature.

2.5.3. Primary and secondary antibodies

Nitrocellulose membranes were stained using Ponceaus S solution (Sigma Life Science), to ensure that protein transfer was achieved. Subsequently, it was washed twice with PBST for a period of 5 min on a shaking platform so that the stain was removed. The membrane was then blocked using a 5% blocking solution overnight at 4°C, or for 2 h at room temperature, on a shaking platform. Following the blocking step, the membrane was washed once for 10 min and twice for 5 min with PBS on a shaking platform. Primary antibodies were

prepared in the appropriate antibody dilution solution and at the correct concentration (Table 2.3) and were applied to the membranes overnight at 4°C, or for 2 h at room temperature, on a shaking platform. The membrane was then washed as above, and a secondary antibody, labelled with a detectable enzyme or fluorophore was added for 1 h at RT on a shaking platform. The membrane was then washed as previously described, prior to developing the signal.

Table 2.3 list of anti-body used for FACS, Western and IHC.

Antibody	Size kDa	Dilution	Incubation	Antibody Supplier	Method
CD133 (APC)	n/a	1/10	30 min on ice	Miltenyi Biotech	FACS
EpCam (PE)	n/a	1/10	30 min on ice	Miltenyi Biotech	FACS
CD24-FITC	n/a	1/10	30 min on ice	BD Pharmingen	FACS
CD44-APC	n/a	1/10	30 min on ice	BD Pharmingen	FACS
Sox2	37	1/1000	2 h RT	Merck Millipore	Western
Cleaved Caspase-3	17	1/1000	2 h RT	Cell Signaling	Western
Nanog	36	1/2000	Overnight in cold room	Novus Europe/UK	Western
Oct4	38	1/1000	2 h RT	Novus Europe/UK	Western
Actin	43	1/20000	1 h RT	Santa Cruz	Western
ALDH-1		1/2000	2 h RT	BD BIOSCIENCES	IHC

2.5.4. Developing the intracellular membrane

The membrane was put upon a flat surface protein side up. As the secondary antibodies used were conjugated to horseradish peroxidsae (HRP), an Enhanced Chemiluminescence (ECL) (Geneflow, Lichfield, UK) detection system was used for protein visualisation. The luminol and stabilised peroxidase solution were mixed in a 1:1 ratio and applied to the membrane for 2 min. The membrane was placed into an autoradiographic cassette with film (AGFA Gevaert N.V., Mortsel, Belgium) and developed in a dark room. The developed film will show bands, band intensity was quantified using Image J (1.49E).

2.5.5. Equal Loading

For the purposes of confirming that equal loading has taken place and to normalise the proteins, the membrane were re-probed to detect α-tubulin or β-actin proteins, which are expressed by house-keeping genes.

2.6. Immunohistochemistry procedures for the detection of ALDH expression in pancreatic cancer patient samples

Slides were heated for 10 min at 100°C allowing the paraffin to melt, then were immediately immersed in xylene. Slides were then hydrated as follows: xylene 2 x 3 min, industrial methylated sprit (IMS) 99% 2 x 3 min, IMS 95% 2 x 3 min and under running water for 5 min. Antigen retrieval was carried out in citric acid at pH 6 for 18.46 min in a microwave at maximum temperature. Slides were allowed to cool for 10 min and incubated in PBS for 5 min. Slides were developed using the Novolink TM Polymer Detection Kit, following the manufacture's protocol. Endogenous peroxidase was neutralised using one drop of hydrogen peroxidise block for 5 min, then the slides were washed in PBS twice for 5 min, followed by incubation in protein block for 5 min to prevent nonspecific binding. Slides were then washed in PBS twice for 5 min and incubated in optimally diluted primary antibody (1/2000) for the required time and at the appropriate temperature for a period of 2 h (ALDH). Slides were then washed twice in PBS for 5 min before incubation with post primary block for 30 min; again slides were washed twice in PBS for 5 min. Subsequently, slides were incubated with NovoLink Polymer for 30 min; next they were washed in PBS for 5 min x 2, while rocking gently. In order to develop the peroxidise activity, DAB working solution (50 µL DAB chromogen to 1 mL of NovoLink Dab substrate buffer) was added for 5 min then slides were rinsed in water and counterstained with Mayer's Haematoxylink for 30 sec. Finally, slides were washed under tap water and dehydrated back up through graded alcohols and

xylene, then mounted using DPX mounting solution. Picture taken by light microscopy (LeitZ V2.8.8).

2.7. Double Liquid Phase Extraction (LPE) method for curcumin and curcumin metabolites from cell pellets and media

The Double Liquid-Phase Extraction method has been previously validated within the laboratory for plasma analysis.

2.7.1. Standard curve preparation

Standard curves were obtained for cell pellets and media. Thirty 175 cm^3 flasks were seeded with 5 million Capan-1 cells per flask and allowed to adhere overnight. Media was collected in 1 mL aliquot and the cells were harvested and stored at -80°C until required. To the cell pellet, PBS was added in the ratio of 1:2 (e.g. 200µL PBS for 100 mg cell pellet). Cell pellets were homogenised with a magnetic homogeniser for 10-15 seconds. Stock solutions of curcumin, mono-glucuronide, mono-sulfate and β-estradiol (internal standard) were prepared at a concentration of 1 mg/mL in DMSO. The stock solutions were further diluted with DMSO to give final concentrations ranging from 10-1000 µg/mL for all the analytes and 600 µg/mL for β-estradiol. One µL from stock solution of each analyte and internal standard was spiked into the 100 µL of cell homogenate or 100 µL media. Extraction of the compounds was then undertaken by adding 200 µL of 9:1 Acetone: Formic Acid to cell homogenate/media. The samples were vigorously vortexed for 10 seconds and subsequently incubated at -20°C for 30 min. Following a further vortex and centrifugation at 13200 x g for 20 min at 4°C, the supernatants were transferred into new Eppendorfs and kept at 4°C. This step was repeated by adding a further 200 µL of 9:1 Acetone: Formic Acid to ensure all compounds were extracted properly. All samples were then dried using a SpeedVac with no heat, for approximately 2.35 h. The sample residues were then re-suspended in 100

µL of mobile phase A (50:50 Ammonium Acetate: Acetonitrile). Following a final vigorous vortex for 10 seconds and centrifugation at 13200 x g for 3 min, the supernatant was transferred into HPLC vials and 50 µL was injected. All samples were analysed on the same day.

The retention times for mono-glucuronide, mono-sulfate and curcumin using the HPLC-UV assay established in our laboratory was around 15.77, 20.1 and 23.53 min respectively. The limit of detection (LOD) and limit of detection quantification (LOQ) were calculated using signal:noise ratio which were 3:1 and 10:1 respectively. LOD was at 0.1 µg/mL on LOQ was 0.4 µg/mL for the three analytes. Calibration curves for all the analytes in both the matrices were linear with an average correlation coefficient (r^2) value of more than 0.99 for all the three analytes. Concentration of mono-glucuronide, mono-sulfate and curcumin in media with cells/without cells and cell homogenate (Capan-1 and Panc-1) were quantified using these respective standard curves.

2.7.2. Assessment of curcuminoid concentrations in Capan-1 and Panc-1 cell pellets and media over time

Cells (Capan-1 and Panc-1) were seeded under adherent conditions in 175cm^3 flasks at approximately 5 million cells/flask and allowed to adhere overnight. Following exposure to 5 µM curcumin, 5 µM cur-glucuronide or 5 µM cur-sulfate, cells were harvested at time points of 0, 15, 30, min and 1, 6, 24 and 48 h. One ml aliquots of media and cell pellets removed from flasks for each time point and were immediately stored at -80°C. The cell pellets were snap thawed in liquid nitrogen and water bath at 37°C three times to facilitate extraction of intracellular content of cells. The cell pellet and media were then extracted as described in section 2.7.1.

2.7.3. High performance liquid chromatography (HPLC) reversed phase (Waters HPLC-UV System)

The equipment utilised was a 2695 Water Alliance HPLC system with a series separation module with column heater, refrigerated autosampler, inline degasser, a UV visibility detector (Waters) and Empower software for data analysis. The method developed by our lab used the following approach for HPLC. Atlantis dc18 Column, 100Å, 3 µm, 4.6 mm X 150 mm, 1/pKg was used, which is connected through the guard column (Atlantis 4.6 x 20mm) at a flow rate of 1 mL per min, keeping the column temperature at 25°C. The mobile phases were: mobile phase A, 10 mM Ammonium acetate at pH 4.5; mobile phase B, Acetonitrile. The procedure was carried out by injecting all samples prepared daily onto the Waters Atlantis 3 µm C_{18} column that joined to the Waters Atlantis 3 µm C_{18} guard column which allows prolonging the column lifetime. Temperatures were maintained for column and auto-sampler at 25°C and 4°C, respectively. The gradient elution states for each injection are shown in table 2.3.

Time	% of mobile phase A	% of mobile phase B
0	90	10
15	60	40
25	15	85
30	0	100
30.10	90	10
40	90	10

Table 2.4 Mobile phase gradient for the Waters HPLC-UV system applied for curcumin, Cur-Glucuronide and mono-Sulfate separation. The mobile phases were: mobile phase A, 10 mM Ammonium acetate at pH 4.5; mobile phase B, Acetonitrile.

2.8. Cell cycle analysis

Cells were seeded into 175cm^3 flasks for Panc-1, AsPC-1 and Capan-1 200,000, 300,000 and 350,000 cells respectively. After seeding, cells were treated with curcumin/resveratrol for 24, 48 and 72 h. Cells were harvested by trypsinisation then cell pellets were washed once with PBS then ice-cold ethanol (2 mL, 70%) added whilst vigorously vortexing the cells. Samples were kept for maximum 7 days at 4 °C before analysis. All samples were centrifuged at 207 x g for 10 min at RT then the supernatants discarded. Each cell pellet was suspended in 800 µL of PBS then RNase added (10 mg/mL) and the cells incubated overnight at 4 °C. Finally, 100 µL propidium iodide at final concentration of 50 µg/mL was added for 45 min prior for analysis on the Flow Cytometer (FACS Aria11, BD Bioscience). The results were analysed using ModFit LT software version (3.1). A sample DNA histogram is presented in (Figure 2.1).

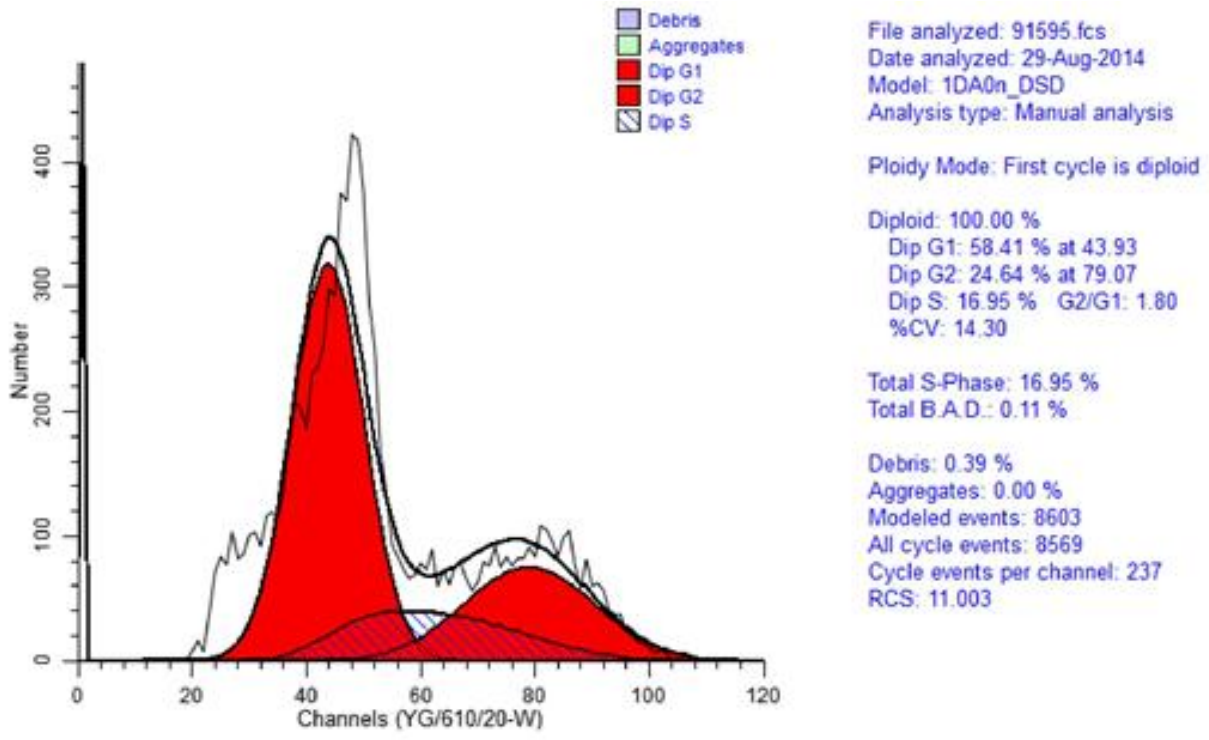


Figure 2.1 A DNA histogram showing cell cycle analysis. Cells were labelled with PI were gated first by FSC-W and FSC-H then gated by YG/610/20-H via YG/610/20-W signals.

Chapter Three: Assessment of cell proliferation in response to curcumin and resveratrol

3.1. Introduction

Within chemoprevention strategies, two of the most extensively investigated compounds to date are resveratrol and curcumin. Limited information is available as to whether the anti-carcinogenic effects of these compounds individually may be enhanced when in combination with one another. For pancreatic cancer, as one of the most aggressive solid tumours, there has been little advance in terms of five year patient survival rate over the last three decades but better a good progression in 1 year survival rate (267). Therefore, the aim of the work described in this thesis is to investigate the potential for efficacy of both resveratrol and curcumin alone and in combination in *in vitro* models of pancreatic cancer. Whilst curcumin has been tested individually in small pancreatic cancer clinical trials (187, 213) and resveratrol in various pancreatic cancer preclinical trials (193). Furthermore, it has been shown that chemo-radiotherapies can be enhanced by resveratrol and curcumin individually as a result of increasing sensitivity of malignant cells to the treatments (182, 188, 193, 215, 235, 255), there is still a lack of evidence at a molecular level alluding to how pancreatic cancer may be targeted by these diet-derived agents. Furthermore, the nature of pancreatic cancer, which exhibits a dense stromal/desmoplastic environment, results in poor drug delivery (268). Thus, administration of a low toxicity agent with potential to target the stroma could have utility in both a prevention and therapeutic setting.

This chapter aims to investigate sensitivity to both curcumin and resveratrol alone and in combination, in a panel of human pancreatic cancer cell lines and a pancreatic stellate cell line (RLT). Endpoint mechanisms of anti-proliferative effect will be assessed, and cell lines profiled to determine whether they are phenotypically representative of cancer stem-like cells.

3.2. Growth inhibition by single dose exposures of curcumin or resveratrol

Effects of both resveratrol and curcumin alone as single agents were first determined for all PDAC cell lines (Panc-1and Capan-1) and for the pancreatic stellate cell line (RLT) in comparison to the vehicle exposure alone (DMSO).

3.2.1. Growth inhibition by curcumin and resveratrol in Capan-1 cells

The concentration dependent change in Capan-1 cell numbers following curcumin and resveratrol exposure is demonstrated in Figure 3.1. In general, for curcumin, the higher the concentration and longer the exposure, the greater the reduction in cell numbers (Figure 3.1B). In Capan-1 cells, curcumin significantly inhibited proliferation from day 1 by 36±3%, at concentrations of 1 µM and above. By day 6, significant growth inhibition of 16±3.4% was observed from 0.1 µM curcumin, rising to 86±2.5% at 5 µM. In contrast, the Capan-1 cells appeared much less sensitive to resveratrol, which only caused a significant decrease in cell numbers from 3 days of treatment with the highest concentration (5 µM) (18±4.1%). After 6 days, this inhibition rose to 43±7.1%, but again, effects were only apparent at the highest resveratrol concentration.
IC_{50} values were calculated from the plot of cell number as a percentage of DMSO control versus agent log concentration at 6 days, at which time cells were still in linear growth phase for curcumin and resveratrol (269). The number generated from the equation was converted back from the log10 concentration to give the actual IC_{50} value. The IC_{50} for curcumin in Capan-1 cells was calculated to be 1.76±0.21 µM. This value is the mean of 3 separate experiments (Appendix 7.1-7.6), whereas the IC_{50} value for resveratrol was predicted to be beyond the concentration used in this study (Figure 3.1B). IC_{50} is the concentration of an inhibitor where the response is reduced by half, and this value could be used to test the concentration of a compound required to

achieve half maximal inhibition as a parameter that is indicative of antiproliferative potency (270).

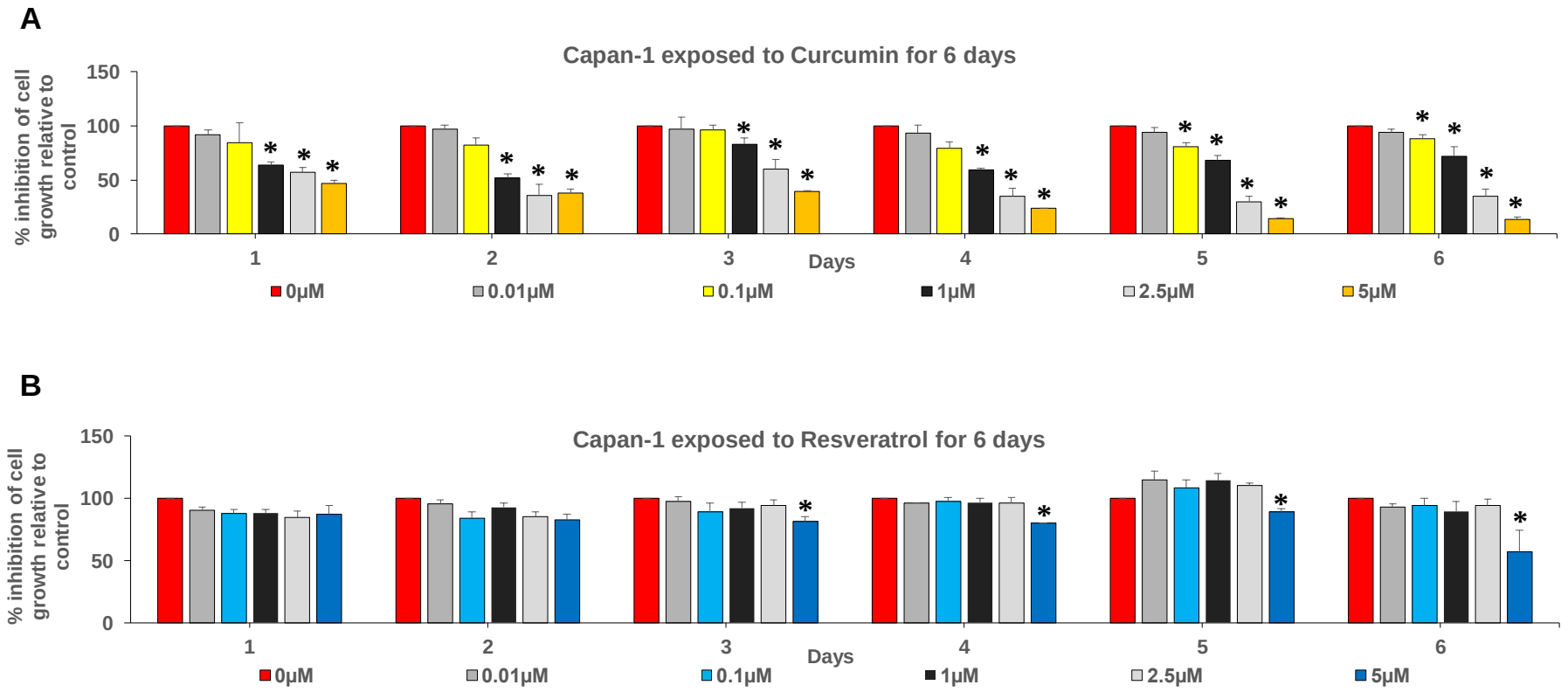


Figure 3.1 Capan-1 cells exposed to curcumin (A) and resveratrol (B) at varying concentrations for 6 days, with daily dosing. Bar charts show the dose response for cell numbers over time after repeated daily exposure at the following concentrations: 0.01, 0.1, 1, 2.5 or 5 μM curcumin or resveratrol. Data are expressed relative to the DMSO solvent control, which is set at 100% and represent the average +SEM of three independent experiments, each performed in triplicate, Significant differences compared to the control were determined using a student's T-test and are shown, where * = p≤0.05.

3.2.2. Growth inhibition by curcumin and resveratrol in Panc-1 cells

Growth inhibition of Panc-1 cells after exposure to curcumin and resveratrol was assessed in a similar way as described in Section 3.2.1. This cell line appeared relatively refractory to both curcumin and resveratrol (Figure 3.2), with significant inhibition observed for both agents only at 5 µM. At the highest exposure to curcumin, significant growth inhibition was observed from as early as day one at 5 µM (41±7% reduction compared to control); the effect remained relatively consistent over time, increasing to 57±3% on day 6 (Figure 3.2A). Similarly, resveratrol (5 µM) significantly inhibited growth from day 1, by 35±7% compared to the control. As with curcumin, the effect was constant over the dosing period, with a slightly greater reduction (40±5%) on day 6 (Figure 3.2B).

For both compounds, given that only a single concentration out of the four tested had a significant effect on cell numbers, it is not possible to reliably calculate an IC_{50} value based on these data. However, since 5 µM curcumin reduced cell numbers by over half on day 6, the IC_{50} may be predicted to be close to this value, whereas for resveratrol it is likely to be >5 µM using this dosing protocol.

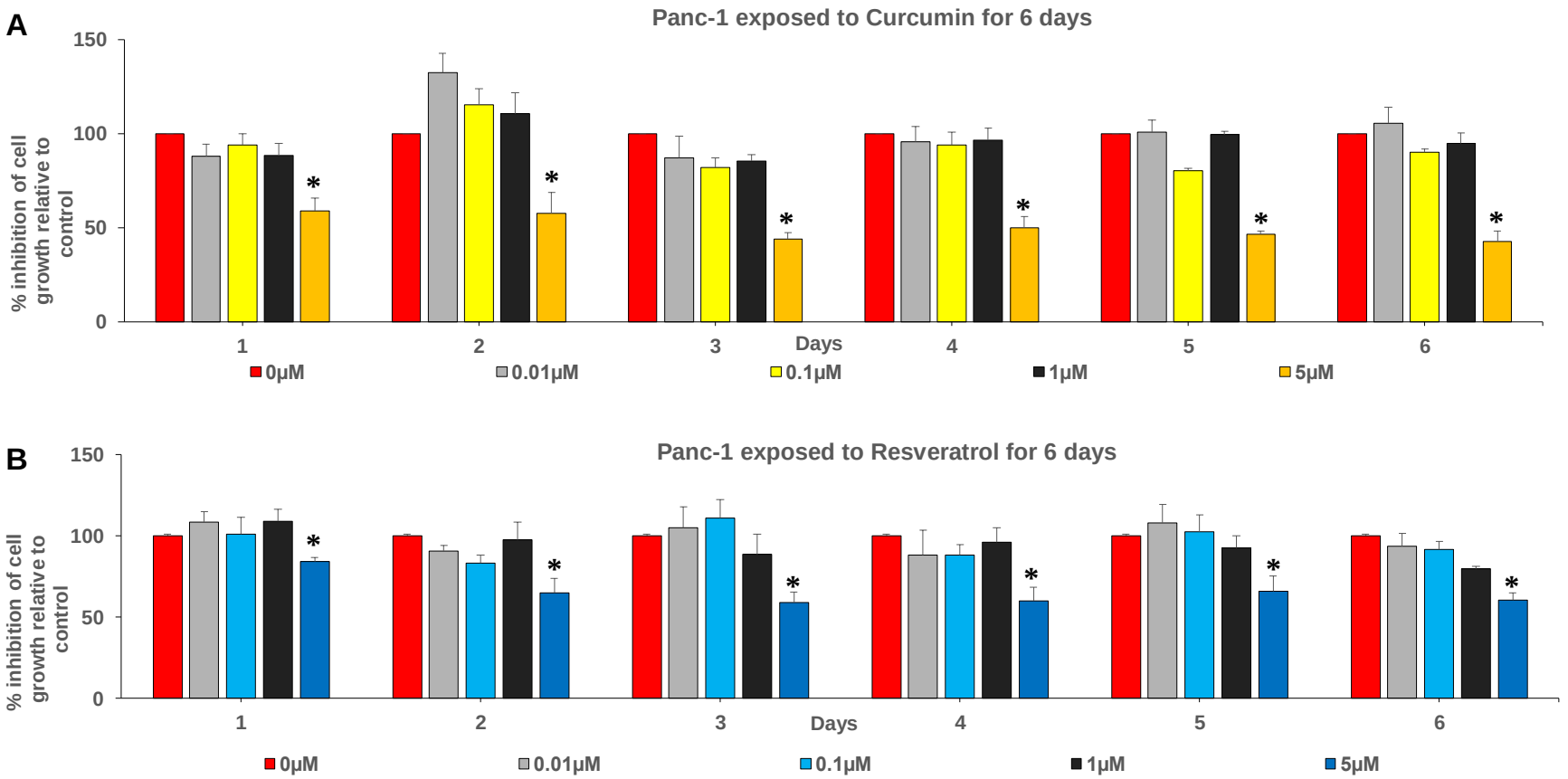


Figure 3.2 Panc-1 exposed to curcumin (A) and resveratrol (B) at varying concentrations for 6 days, with daily dosing. Bar charts show the dose response for cell numbers over time after repeated daily exposures at the following concentrations: 0.01, 0.1, 1, 2.5 or 5 μM curcumin or resveratrol. Data are expressed relative to the DMSO solvent control, which is set at 100% and represent the average +SEM of three independent experiments, each performed in triplicate. Significant differences compared to the control were determined using a student's T-test and are shown, where * = p≤0.05.

3.2.3. Growth inhibition by curcumin and resveratrol in RLT-PSC stellate cells

The bulk of a tumours' mass consists of stellate cells which can prevent access of drugs to the cancer cells. Moreover, PSCs not only survive, but actually thrive and proliferate allowing the tumour microenvironment to take over up to 90% of the tumour total volume (268). It was therefore considered pertinent to determine whether single use or a combination of curcumin and resveratrol could bring about growth inhibition in stellate cells. To date, little attention has been given to the environment surrounding and influencing PDAC cells as a target for therapy, with most research only focusing on pancreatic cancer cells. So far, the role of stellate cells in resistance to treatment or prevention has not been fully considered in preclinical studies; this could explain the failure in translating effective treatments identified from *in vitro* and animal studies to the clinical setting, since the experimental models do not accurately replicate the interactions that occur in this microenvironment in humans.

The growth inhibition of RLT-PSC cells after exposure to curcumin and resveratrol was assessed in a similar way as described in Section 3.1.1. At the highest concentration of curcumin (5 µM), a significant and pronounced growth inhibition relative to the control was observed from day one (39±17%) and the effect increased over time, rising to 95±24% by day 6 (Figure 3.3A). At days 3, 4, 5 and 6, 2.5 µM curcumin also significantly inhibited growth, but to a lesser extent than the highest exposure (39±6%; 51±15%; 46±4% and 39±2%, respectively).

A similar pattern was observed when RLT-PSC cells were exposed to resveratrol, although as seen with the pancreatic cancer cell, they were less sensitive to resveratrol than to curcumin. Resveratrol at 5 µM significantly decreased cell number on day 1 with 30±12% inhibition compared to the control. Cell growth inhibition was relatively stable over time increasing slightly to 36±9% by day 6 (Figure 3.3B). Significant reductions in cell number were

also observed from day 3 onwards at 2.5 µM resveratrol, which decreased the percentage of viable cells by 18-33%.

The IC_{50} value for curcumin in RLT-PSC cells was calculated to be 2.25±0.05 µM on day 6 as described in section 3.1.1. Again, due to the lack of activity over the concentration range employed, it was not possible to determine the IC_{50} for resveratrol in RLT-PSC cells, since it is predicted to be greater than 5 µM.

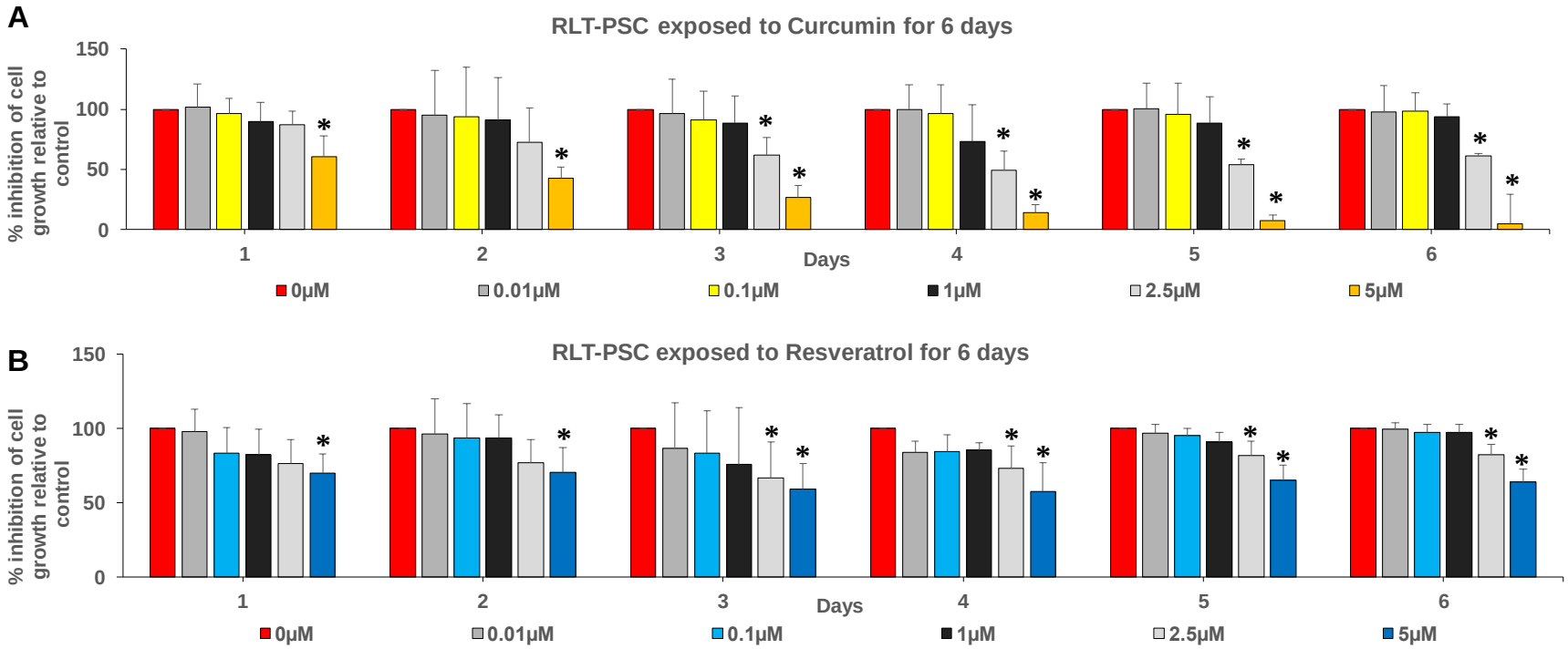


Figure 3.3 RLT-PSC exposed to curcumin (A) and resveratrol (B) at varying concentrations for 6 days, with daily dosing. Bar charts show the dose response for cell numbers over time after repeated daily exposure at the following concentrations: 0.01, 0.1, 1, 2.5 or 5 μM curcumin or resveratrol. Data are expressed relative to the DMSO solvent control, which is set at 100 % and represent the average +SEM of three independent experiments each performed in triplicate. Significant differences compared to the control were determined using a student's T-test and are shown, where * = p≤0.05.

3.3. Growth inhibition by combination exposures of curcumin and resveratrol

To assess the anti-proliferation characteristics of the combination of curcumin and resveratrol, further dose-response growth curves were performed in triplicate for the three cell lines used previously plus another cancer cell line, AsPC-1. For combination two concentrations were chosen (0.1 µM and 5 µM); as 5 µM was the only concentration that consistently had an effect, but this concentration is high and difficult to achieve in human plasma. Regarding 0.1 µM which is a more clinically achievable concentration even though it was difficult to see its activity in any of the cell lines alone, but examined for whether the combination might have activity. For simplicity purposes when describing the treatments, abbreviations have been used, such that C stands for curcumin and R stands for resveratrol. The following combinations were employed in these studies: (0.1 µM C, 0.1 µM R), (5 µM C, 0.1 µM R), (5 µM C, 5 µM R) and (5 µM R, 0.1 µM C), and cells were exposed for six days with repeated dosing every day. Cell numbers were counted daily.

3.3.1. Growth inhibition by combined exposure of curcumin and resveratrol in Capan-1 cells

On day 1 when Capan-1 cells were exposed to 5 µM C, a significant 61±4.9% growth inhibition was recorded but the lower concentration had no effect (Figure 3.4). Resveratrol alone failed to significantly reduce cell number at either concentration following 24 h exposure. When 0.1 µM R was added (5 µM C and 0.1 µM R), there was a significant cell growth inhibition of similar magnitude, (64±1.2%), suggesting no further benefit. Furthermore, when both compounds were used at the higher concentration (5 µM C, 5 µM R) resveratrol did not seem to improve the antiproliferative effect observed with curcumin alone after 24 h. The effectiveness of all three treatments containing 5 µM C increased with time. By day 6, when exposed to 5 µM C alone there was 98±5.2% inhibition of

cell growth, which is comparable to the effects achieved with the combinations (98±0.1% decrease with 5 µM C, 0.1 µM R; and 99±0.1% reduction with 5 µM C and 5 µM R). In addition, with repeated exposure the high concentration of resveratrol began to cause significant growth inhibition, with a ~30% reduction by day 6 and when combined with 0.1 µM C the mixture (0.1 µM C and 5 µM R) decreased cell numbers by 71±5.3%, which represents a considerable improvement on either agent alone at the same concentration; 0.1 µM C gave a 10±2.1% reduction and 5 µM R resulted in a 30±3.2% decrease, as noted above. Another interesting point from this study is that when Capan-1 cells were treated with 0.1 µM C and 0.1 µM R, a significant, albeit relatively small reduction 14±0.8% was observed from day 3 onwards, which could be valuable in clinical applications.

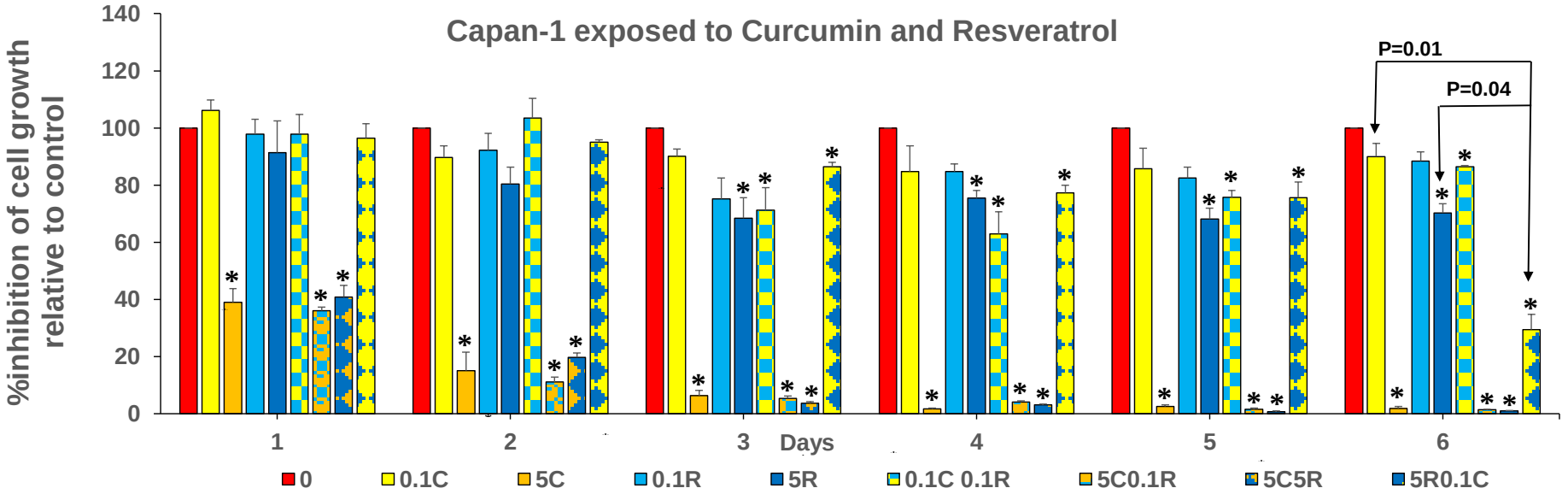


Figure 3.4 Capan-1 cells exposed to curcumin and resveratrol alone and in combination at varying concentrations for 6 days, with daily dosing. Bar chart shows the dose response for cell numbers over time after repeated daily exposure at the following concentrations: 0, 0.1C, 5C, 0.1R, 5R μM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) μM. Data are expressed relative to the DMSO solvent control and represent the average +SEM of three independent experiments each performed in triplicate. Significant differences between treatments and the solvent control are designated, where,* = p≤0.05, determined using a student's T-test.

3.3.2. Growth inhibition by combination exposure of curcumin and resveratrol in Panc-1 cells

As illustrated in figure 3.5, significant inhibition of Panc-1 cell proliferation was observed from day 1 following treatment with 5 µM C (31±4.2%), 5 µM R (22±4.1%), 5 µM C+0.1 µM R (41±8.8%), 5 µM C+5 µM R (43±8.6%) and 5 µM R+0.1 µM C (30±6.4%). These effects were enhanced over 6 days to (46±1.8%, 27±1.8%, 63±4.6%, 61±2.1% and 30±4.6%, respectively) but Panc-1 cells remained relatively refractory to these interventions compared to the Capan-1 cells (Figure 3.5). An interesting observation was that the combination of 5 µM C+0.1 µM R inhibited cell growth more than either compound alone, even though this mixture contained the low concentration of resveratrol which itself lacked activity in this assay. In addition, the combination of 5 µM C + 5 µM R also induced growth inhibition more than either compound alone, although this was more expected given that both compounds individually had activity at these concentrations. Overall, the results from this study indicate that combination treatments did enhance activity in Panc-1 cells compared to either compound alone as single exposure produced 5 µM C (31±4.2%), 5 µM R (22±4.1%) inhibition while combination 5 µM C+0.1 µM R (41±8.8%), 5 µM C+5 µM R (43±8.6%) and 5 µM R+0.1 µM C (30±6.4%). Similar patterns were observed for other days and P value only shown for day 6 (Figure 3.5).

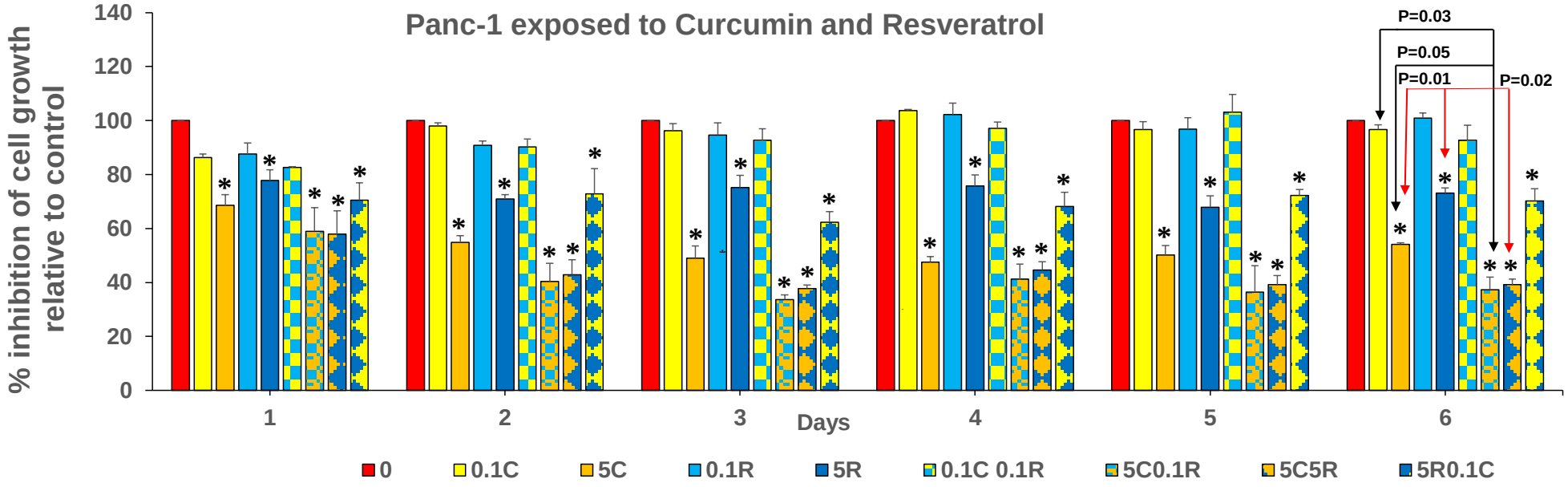


Figure 3.5 Panc-1 cells exposed to curcumin and resveratrol alone and in combination at varying concentrations for 6 days, with daily dosing. Bar chart shows the dose response for cell numbers over time after repeated daily exposure at the following concentrations: 0, 0.1C, 5C, 0.1R, 5R µM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) µM. Data are expressed relative to the DMSO solvent control and represent the average +SEM of three independent experiments, each performed in triplicate. Significant differences between treatments and the solvent control are designated, where * = p≤0.05, determined using a student's T-test.

3.3.3. Growth inhibition by combination exposure of curcumin and resveratrol in AsPC-1 cells

Results of the assessment for anti-proliferation in AsPC-1 cells were shown (Figure 3.6). Curcumin 5 μM alone gave a significant growth inhibition from day 1 (22±4%), as did 5 μM resveratrol alone from day 2 (27±2%). The combination exposure of 5 μM C and 5 μM R resulted in significant growth inhibition from day 1 (27%±4.1), and the magnitude of effect for this mixture remained greater than that produced by 5 μM C or 5 μM R alone at days 2, 3, 4 5 and 6. In addition, the combination of 5 μM R and 0.1 μM C at day 6 inhibited growth by 72±2%, which was considerably greater than either treatment alone, since 0.1 μM C caused only a small, non-significant reduction (8±4%) and 5 μM R decreased the number of cells by one third at this time point. At day 6, the treatment that caused the greatest cell reduction was the mixture of high concentrations (5 μM C and 5 μM R); this resulted in significant inhibition of cell growth by 88±1.8%, which was greater than either compound alone but only significantly different from the effect observed with 5 μM R.

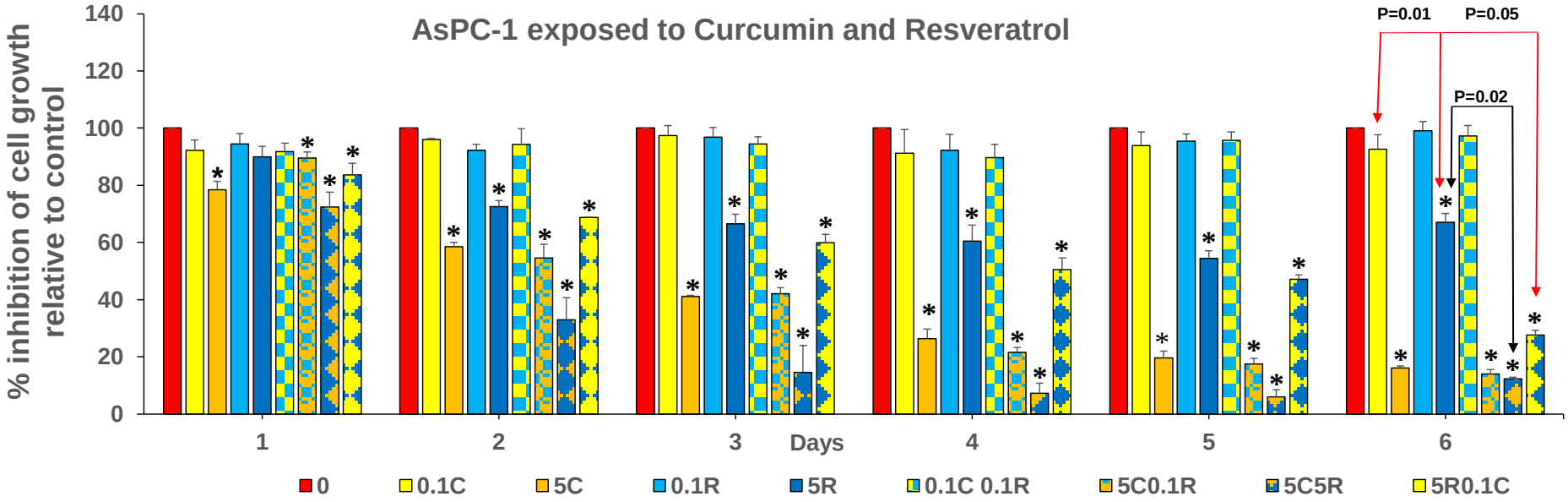


Figure 3.6 AsPC-1 cells exposed to curcumin and resveratrol alone and in combination at varying concentrations for 6 days, with daily dosing. Bar chart shows the dose response for cell numbers over time after repeated daily exposure at the following concentrations: 0, 0.1C, 5C, 0.1R, 5R µM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) µM. Data are expressed relative to the DMSO solvent control and represent the average +SEM of three independent experiments, each performed in triplicate. Significant differences between treatments and the solvent control are designated, where * = p≤0.05, determined using a student's T-test.

3.3.4. Growth inhibition by combination exposure of curcumin and resveratrol in RLT-PSC stellate cells

Figure 3.7 reveals that significant growth inhibition of RLT-PSC cells was observed from day 1 following treatment with 5 µM C (24±6%), 5 µM R (30±7%), 0.1 µM C + 0.1 µM R (37±2.8%), 5 µM C + 0.1 µM R (61±1.7%), 5 µM C + 5 µM R (40±9%) and 5 µM R + 0.1 µM C (27±3.9%) (Figure 3.7). These effects were enhanced over time, such that by 6 days there were hardly any cells remaining (<3%) for three of the treatments, namely, 5 µM C alone, and the combinations of 5 µM C plus both concentrations of resveratrol. This finding is consistent with RLT-PSC cells being highly sensitive to curcumin at the higher concentration, as was previously noted for Capan-1 cells. Resveratrol (5 µM) alone also had a marked effect, decreasing cell numbers by 67% after 6 days. Although the strong potency of curcumin at the high concentration makes it difficult to assess whether adding in resveratrol enhances activity, it is interesting to note that the combination of 0.1 µM C and 0.1 µM R showed a significant but small antiproliferative effect from day one onwards, even though neither of these two compounds alone showed any significant effects at the low concentration. However, it is recognised that the difference between the combination and individual treatments did not reach significance.

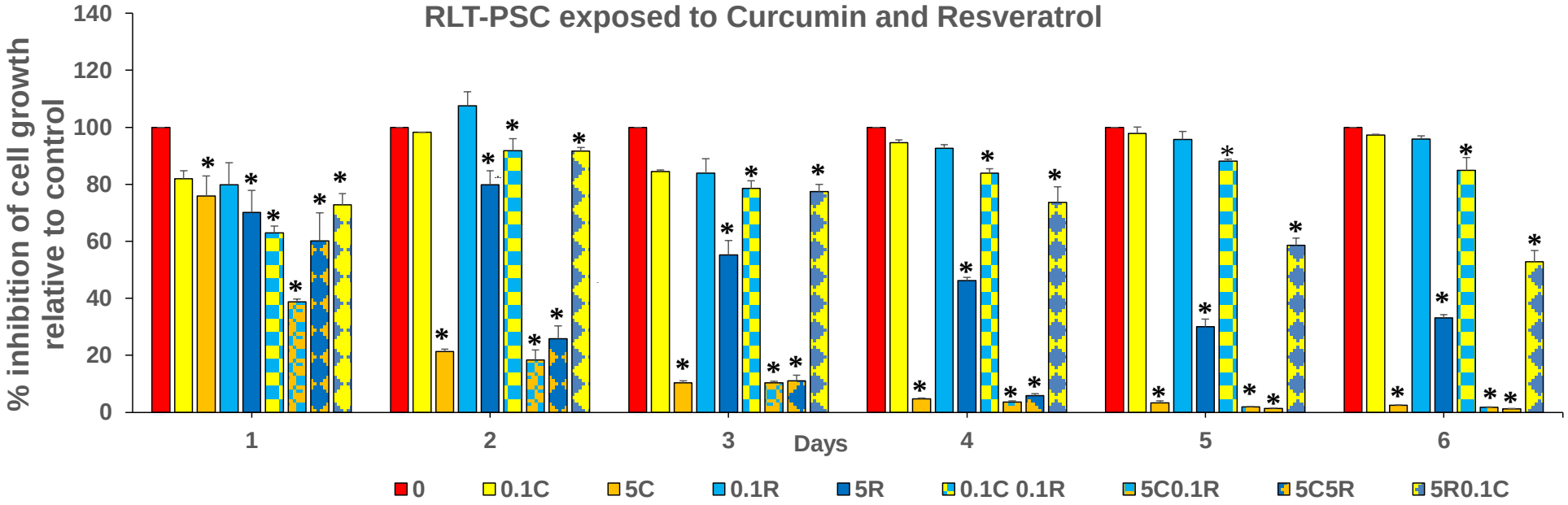


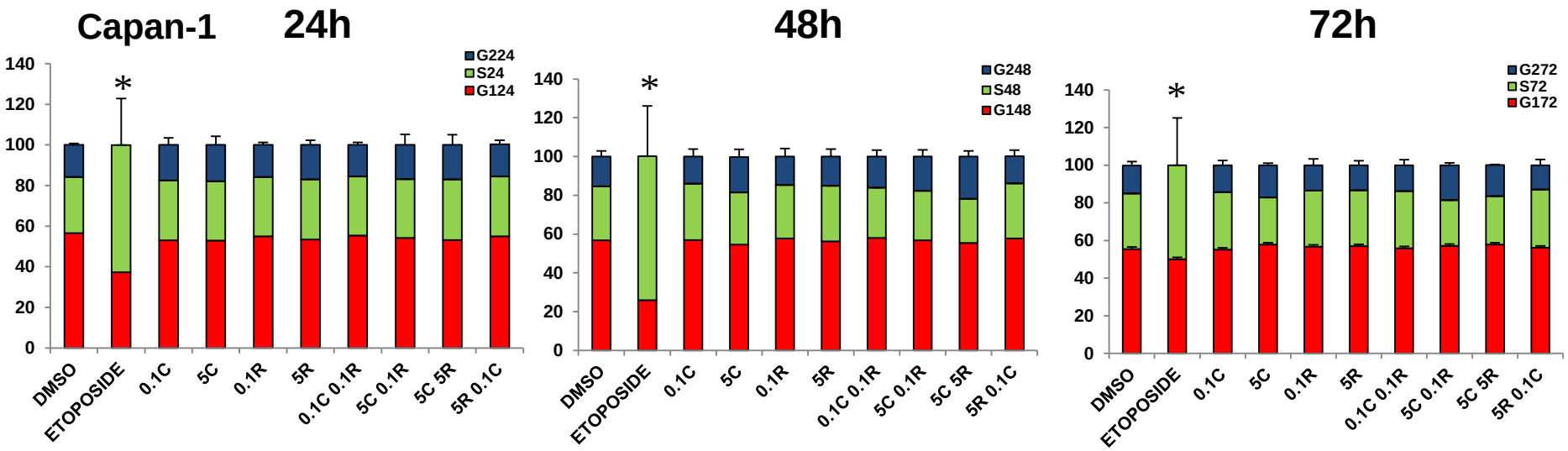
Figure 3.7 RLT-PSC cells exposed to curcumin and resveratrol alone and in combination at varying concentrations for 6 days, with daily dosing. Bar chart shows the dose response for cell number over time after repeated daily exposure at the following concentrations: 0, 0.1C, 5C, 0.1R, 5R µM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) µM. Data are expressed relative to the DMSO solvent control and represent the average +SEM of three independent experiments, each performed in triplicate. Significant differences between treatments and the solvent control are designated, where * = p≤0.05, determined using a student's T-test.

3.4. Cell Cycle Analysis following combined exposure of Capan-1, Panc-1 and AsPC-1 cells to curcumin and resveratrol

It was important to determine the processes underlying the anti-proliferative activity caused by the combination of curcumin and resveratrol. Therefore, the effects of the single agents and combinations on cell cycle progression and the induction of apoptosis were subsequently investigated to assess whether the anti-proliferation observed was due to cytostatic or cytotoxic effects.

3.4.1. Cell cycle analysis following combined exposure of Capan-1 cells to curcumin and resveratrol

When Capan-1 cells were exposed to different concentrations of single compounds and combinations by daily dosing for up to 72 hr, no cell cycle arrest was observed in any phase, as illustrated by a lack of significant difference between the solvent control and various treatments (Figure 3.8). Cells were also incubated with etoposide (50 μM) to provide a positive control for the analysis. Importantly, etoposide arrested cells primarily in late S/G2 as reported in the literature (271, 272).



Treatments	G124	S24	G224
DMSO	56±6	27±4	15±3.5
ETOPOSIDE	37±8	62±8	
0.1C	52±3	29±4	17±2.2
5C	52±4	29±4.7	17±0.6
0.1R	55±2	29±4	15±2.8
5R	53±2.5	29±5	17±2.1
0.1C 0.1R	55±1.6	29±4	15±0.6
5C 0.1R	54±5	28±8	16±2.9
5C 5R	53±5.5	29±4	16±1.2
5R 0.1C	54±1.9	29±3	15±1.9

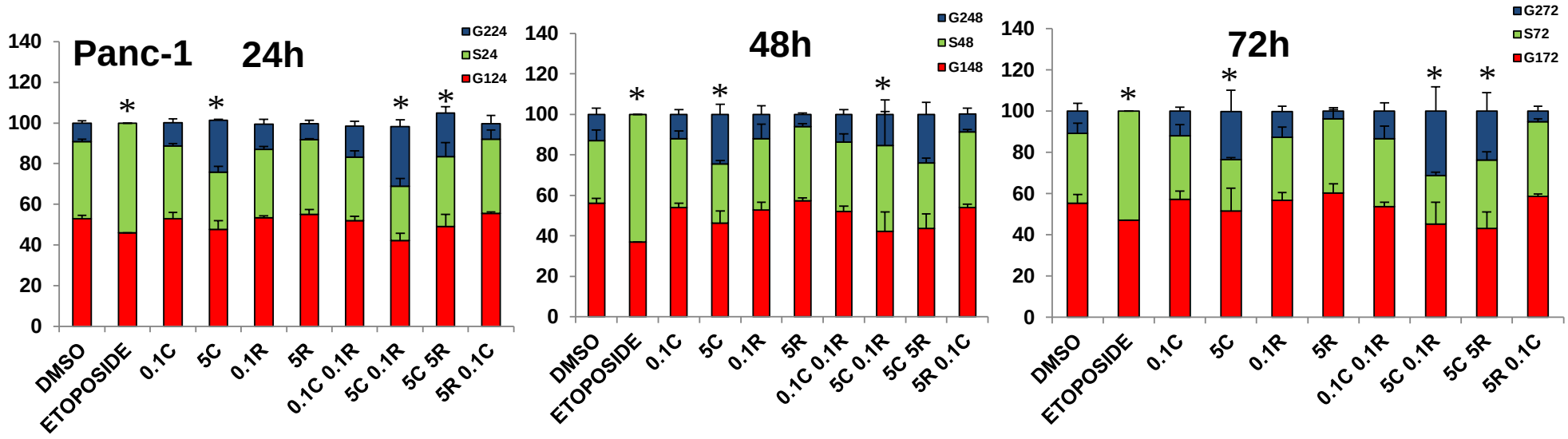
Treatments	G148	S48	G248
DMSO	56±3	27±2.6	15±0.8
ETOPOSIDE	25±8	74±10	
0.1C	56±4	29±2.7	14±2
5C	54±3.9	26±12	18±0.9
0.1R	57±4.1	27±2.9	14±1.2
5R	56±3.8	28±2.4	15±1.7
0.1C 0.1R	58±3.2	25±4.2	15±0.9
5C 0.1R	56±3.4	25±4.7	17±1.6
5C 5R	55±2.8	22±4.5	21±4.3
5R 0.1C	57±3	28±2.3	13±1

Treatments	G172	S72	G272
DMSO	55±2	29±3	14±0.4
ETOPOSIDE	50±11	49±2	_ _
0.1C	55±2.5	30±3	14±0.8
5C	57±1.1	24±3.7	17±4.7
0.1R	56±3.6	29±2.5	13±0.8
5R	56±2.6	29±3.6	13±1.6
0.1C 0.1R	55±3	30±2.7	13±0.59
5C 0.1R	57±1.2	24±5.5	14±4.5
5C 5R	57±6	25±2.9	16±3
5R 0.1C	56±3	30±.1.4	12±1.8

Figure 3.8 Capan-1 cell cycle analysis at 24, 48 and 72 h following treatment with combinations of curcumin plus resveratrol and the individual compounds. (**A**) Bar charts show the proportion of cells in each phase of the cell cycle (G1, S and G2) after repeated daily exposure to the following concentrations: 0, 0.1C, 5C, 0.1R, 5R μM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) μM (**B**) Tables are also included for easy comparison of the data. Data represent the average (+SEM) percentage of cells in each phase of the cell cycle, for three experiments, each conducted in triplicate Significant differences between treatments and the solvent control are designated, * = p≤0.05, as determined using a student's T-test

3.4.2. Cell cycle analysis following combined exposure of Panc-1 cells to curcumin and resveratrol

When Panc-1 cells were exposed for 24 h, significant G2 arrest was observed following treatments with 5 µM C when compared with DMSO and the combinations that also contained the high concentration of curcumin, 5 µM C+0.1 µM R and 5 µM C+5 µM R (Figure 3.9). Interestingly, at this time point the greatest increase in G2 cells compared to the solvent control was evident in the combination with the lower resveratrol concentration. However, the effect was no longer apparent at 48 h, as significant G2 arrest was only observed following treatments with 5 µM C and 5 µM C+5 µM R . The same pattern was observed at 72 h, as significant G2 arrest was observed following treatments with 5 µM C (23.37%±10), 5 µM C+0.1 µM R again (31.33%±12) and also 5 µM C+5 µM R (23.65%±9) when compared with DMSO only (10.91%±4). Of note, no significant difference between curcumin alone and curcumin plus either concentration of resveratrol, suggested that resveratrol may not be contributing greatly to the arrest and this is supported by the lack of G2 arrest in cells treated with resveratrol alone. On the other phases of cell cycle such as G1 or S, no significant different was observed as compared with the vehicle (DMSO).



Treatments	G124	S24	G224
DMSO	52.81±2	37.97±1	9.22±1
ETOPOSIDE	46±0	54±0	0±0
0.1C	52.87±3	35.82±1	11.38±2
5C	47.62±4	28.23±3	25.57±0
0.1R	53.28±1	33.72±1	12.34±2
5R	55.10±2	36.58±0	8.06±2
0.1C 0.1R	51.96±2	31.23±3	15.22±2
5C 0.1R	42.13±4	26.80±4	29.36±3
5C 5R	49.02±6	34.31±7	21.56±3
5R 0.1R	55.51±1	36.51±5	7.74±4

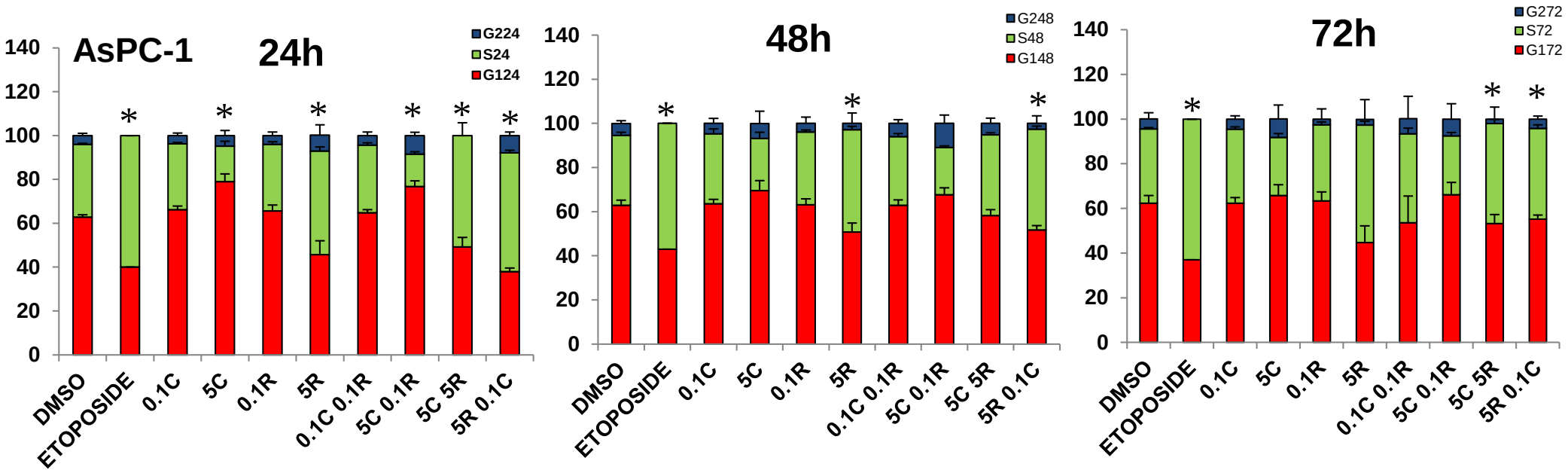
Treatments	G148	S48	G248
DMSO	56.09±2	30.86±5	13.06±3
ETOPOSIDE	37±0	63±0	0±0
0.1C	53.88±2	34.07±4	12.05±2
5C	46.17±6	29.32±2	24.51±5
0.1R	52.77±4	35.2±7	12.09±4
5R	57.35±1	36.61±1	6.05±1
0.1C 0.1R	52.04±3	34.29±4	13.67±2
5C 0.1R	42.09±10	42.55±17	15.36±7
5C 5R	43.60±2	32.48±7	23.92±6
5R 0.1R	53.96±2	37.24±1	9±3

Treatments	G172	S72	G272
DMSO	55.39±4	33.7±5	10.91±4
ETOPOSIDE	47±0	53±0	0±0
0.1C	57.06±4	30.88±5	12.06±2
5C	51.53±11	24.95±1	23.37±10
0.1R	56.73±4	30.56±5	12.59±2
5R	60.32±4	35.95±4	3.73±2
0.1C 0.1R	53.55±2	33.06±6	13.39±4
5C 0.1R	45.11±11	23.56±2	31.33±12
5C 5R	42.97±8	33.38±4	23.65±9
5R 0.1R	58.68±1	36.12±1	5.20±2

Figure 3.9 Panc-1 cell cycle analysis at 24, 48 and 72 h following treatment with combinations of curcumin plus resveratrol and the individual compounds. (A) Bar charts show the proportion of cells in each phase of the cell cycle (G1, S and G2) after repeated daily exposure to the following concentrations: 0, 0.1C, 5C, 0.1R, 5R μM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) μM (**B**) Tables are also included for easy comparison of the data. Data represent the average (+SEM) percentage of cells in each phase of the cell cycle, for three experiments, each conducted in triplicate Significant differences between treatments and the solvent control are designated, where * = p≤0.05, as determined using a student's T-test.

3.4.3. Cell cycle analysis following combined exposure of AsPC-1 cells to curcumin and resveratrol

For this cell line the responses in terms of cell cycle arrest were different for curcumin and resveratrol exposures (Figure 3.10). Curcumin caused cell cycle arrest at G1 but on the other hand resveratrol caused S-phase arrest. At 24 h there was a significant G1 arrest following treatment with 5 µM C (79% versus 63% in control cells) and 5 µM C+0.1 µM R (77%) but the addition of resveratrol did not seem to enhance the level of arrest caused by curcumin. This G1 arrest was not sustained at the longer time points particularly at 72 h where the proportion of cells in this phase were similar to the control. Resveratrol treatments caused significant S-phase accumulation at the 5 µM concentration alone and both combinations with curcumin, 5 µM R+0.1 µM C and 5 µM C+5 µM R, (47% versus 33% in the control), (54%) and (51%) at 24 h, and this effect was sustained at 72 h. Combination of curcumin with resveratrol marginally enhanced G1 and S-phase arrest but not significantly (Figure 3.10).



Treatments	G124	S24	G224
DMSO	62.75±0	33.17±0	4.07±0
ETOPOSIDE	40±2	60±1	
0.1C	66.23± 2	30±1	3.78±1
5C	78.99±3	16.15±2	4.86±2
0.1R	65.67±3	30.34±1	4±2
5R	45.63±6	47.33±2	7.28±5
0.1C 0.1R	64.76±1	30.88±1	4.36±2
5C 0.1R	76.75±3	14.76±1	8.48±2
5C 5R	49.16±4	50.86±0	0±6
5R 0.1R	37.90±2	54.19±1	7.85±2

Treatments	G148	S48	G248
DMSO	62.93±2	31.60±1	5.43±1
ETOPOSIDE	43±0	57±0	0±0
0.1C	63.62±2	31.67±2	4.71±2
5C	69.5±5	23.73±3	6.76±5
0.1R	63.09±3	32.98±1	3.93±3
5R	50.82±4	46.28±2	2.90±5
0.1C 0.1R	62.87±2	31.14±1	5.98±2
5C 0.1R	67.63±3	21.5±1	10.87±4
5C 5R	58.26±3	36.59±1	5.16±2
5R 0.1R	51.66±2	45.69±1	2.65±3

Treatments	G172	S72	G272
DMSO	62.30±3	33.26±1	4.45±3
ETOPOSIDE	37±0	63±0	0±0
0.1C	62.34±2	33.07±1	4.59±1
5C	65.73±5	26.05±2	8.22±6
0.1R	63.28±4	34.09±1	2.63±4
5R	44.66±8	52.68±2	2.46±9
0.1C 0.1R	53.61±12	39.77±3	6.78±10
5C 0.1R	66.12±6	26.34±2	7.54±7
5C 5R	53.21±4	44.82±2	1.96±5
5R 0.1R	55.22±2	40.55±2	4.22±1

Figure 3.10 AsPC-1 cell cycle analysis at 24, 48 and 72 h following treatment with combinations of curcumin plus resveratrol and the individual compounds. (A) Bar charts show the proportion of cells in each phase of the cell cycle (G1, S and G2) after repeated daily exposure to the following concentrations: 0, 0.1C, 5C, 0.1R, 5R µM of single compounds and combinations of (0.1C, 0.1R), (5C, 0.1R), (5C, 5R) and (5R, 0.1C) µM (**B**) Tables are also included for easy comparison of the data. Data represent the average (+SEM) percentage of cells in each phase of the cell cycle, for three experiments, each conducted in triplicate Significant differences between treatments and the solvent control are designated, where * = p≤0.05, as determined using a student's T-test.

3.5. Induction of apoptosis in Capan-1, Panc-1 and AsPC-1 cells by curcumin and resveratrol exposure:

Apoptosis was assessed by Western blotting, utilizing an antibody directed against cleaved capase-3 (19kDa and 17kDa are large fragments of full length caspase 3) as a marker of apoptosis. Etoposide was used as a positive control in cell lines to show that the cells were responding to treatments and for comparison in the Western blot analysis.

3.5.1. Capan-1 cells

Out of the three cell lines used Capan-1 cells were the most sensitive to induction of apoptosis by curcumin and resveratrol. A significant induction of apoptosis was observed with 24 h exposure to 5 µM C, which resulted in a 3.8 -fold increase compared to the vehicle control (DMSO); this was further increased to a ~5-fold induction following treatment with 5 µM C+ 0.1 µM R. A combination of the high concentrations (5 µM C+5 µM R) also caused apoptosis, but the effect was slightly reduced (3.4-fold induction) compared to the mixture containing just 0.1 µM R (Figure 3.11A & B).

At 48 h, a significant induction of apoptosis in Capan-1 cells was observed at 0.1 µM curcumin (2.7±1.1-fold increase), 5 µM curcumin (6.7±2.1-fold increase), 0.1 µM resveratrol (3.1±1.2-fold increase) and 5 µM resveratrol When Capan-1 cells were exposed to the combination of 5 µM C+0.1 µM R, this induced a 4.7±1.8-fold increase relative to the control, but 5 µM C+5 µM R caused only 2.9±1.1-fold increase in apoptosis (Figure 3.11A & B).

At 72 h, significant increases in apoptosis were also observed in response to curcumin at 0.1 µM (1.3±0.03-fold increase) and 5 µM (1.9±0.09-fold increase), but the magnitude of effect was smaller than at the earlier 48 h time point. The combinations of 5 µM C+0.1 µM R and 5 µM C+5 µM R also elevated apoptosis

by 1.4±0.15 and 2±0.57-fold, respectively. However, overall, the addition of resveratrol did not significantly enhance apoptosis over that caused by curcumin alone (Figure 3.11A & B).

3.5.2 Apoptosis induction by curcumin and resveratrol exposure in Panc-1 cells

After three days exposure to curcumin and resveratrol as a single compound or in combination, no consistent indication of apoptosis was observed at any time point in Panc-1 cells, over the background level in control incubations, where this was detectable (Figure 3.11C).

3.5.3 Apoptosis induction by curcumin and resveratrol exposure in AsPC-1 cells

After three days exposure to curcumin and resveratrol as a single compound or in combination, there was no indication of apoptosis observed at any time point in AsPC-1 cells (Figure 3.11D).There seemed to be a very low basal level of apoptosis in the control cells but treatment with etoposide caused a strong apoptotic response, as measured by cleaved caspase-3.

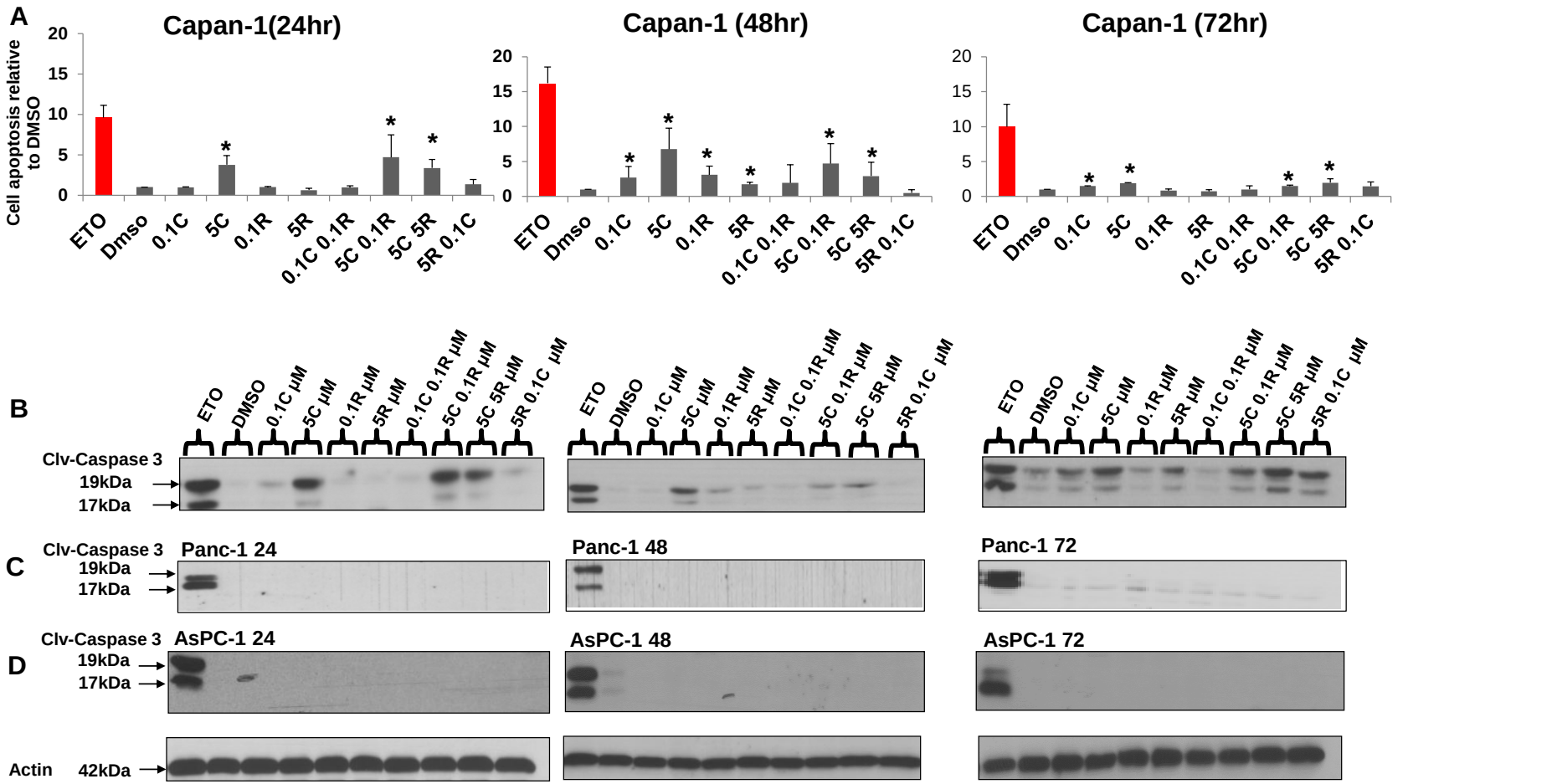
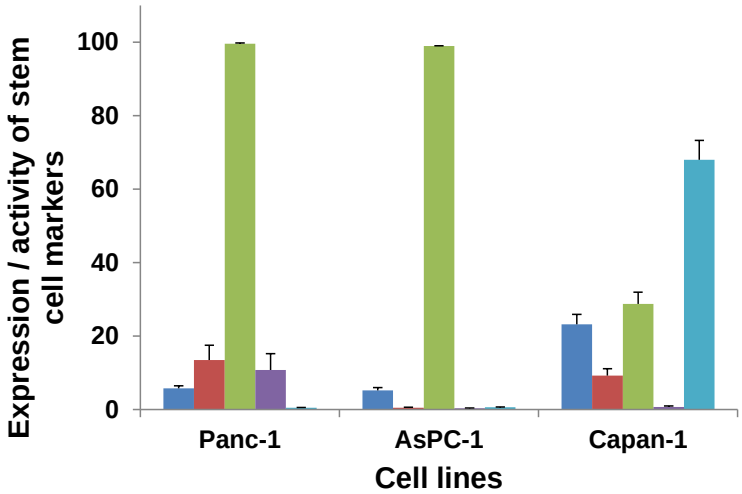


Figure 3.11 Expression of cleaved-caspase 3 (19kDa and 17kDa) in Capan-1, Panc-1 and AsPC-1 cells repeatedly exposed to curcumin and resveratrol, analysed by Western blot. (A) Quantification of cleaved caspase-3 protein levels in Capan-1 cells after exposure to combinations of resveratol (R) or curcumin (C) at 0.1 µM or 5 µM for 24, 48 and 72 h. Data are the average (+SEM) of three independent experiments. Significant differences between treatments and the solvent control are indicated , where * = p≤0.05, determined using a student's T-test. Statistical analysis between the treatments carried out using T-test, no significant result was observed **(B-D)** Representative Western blot analysis for the expression of cleaved caspase-3 in **(B)** Capan-1, **(C)** Panc-1 and **(D)** AsPC-1 cells exposed to combinations of curcumin and resveratrol for 24, 48 and 72 h. β-actin (42kDa) was used as a control for protein loading. Etoposide represents a positive control, which is known to induce apoptosis and was incubated with each cell line.

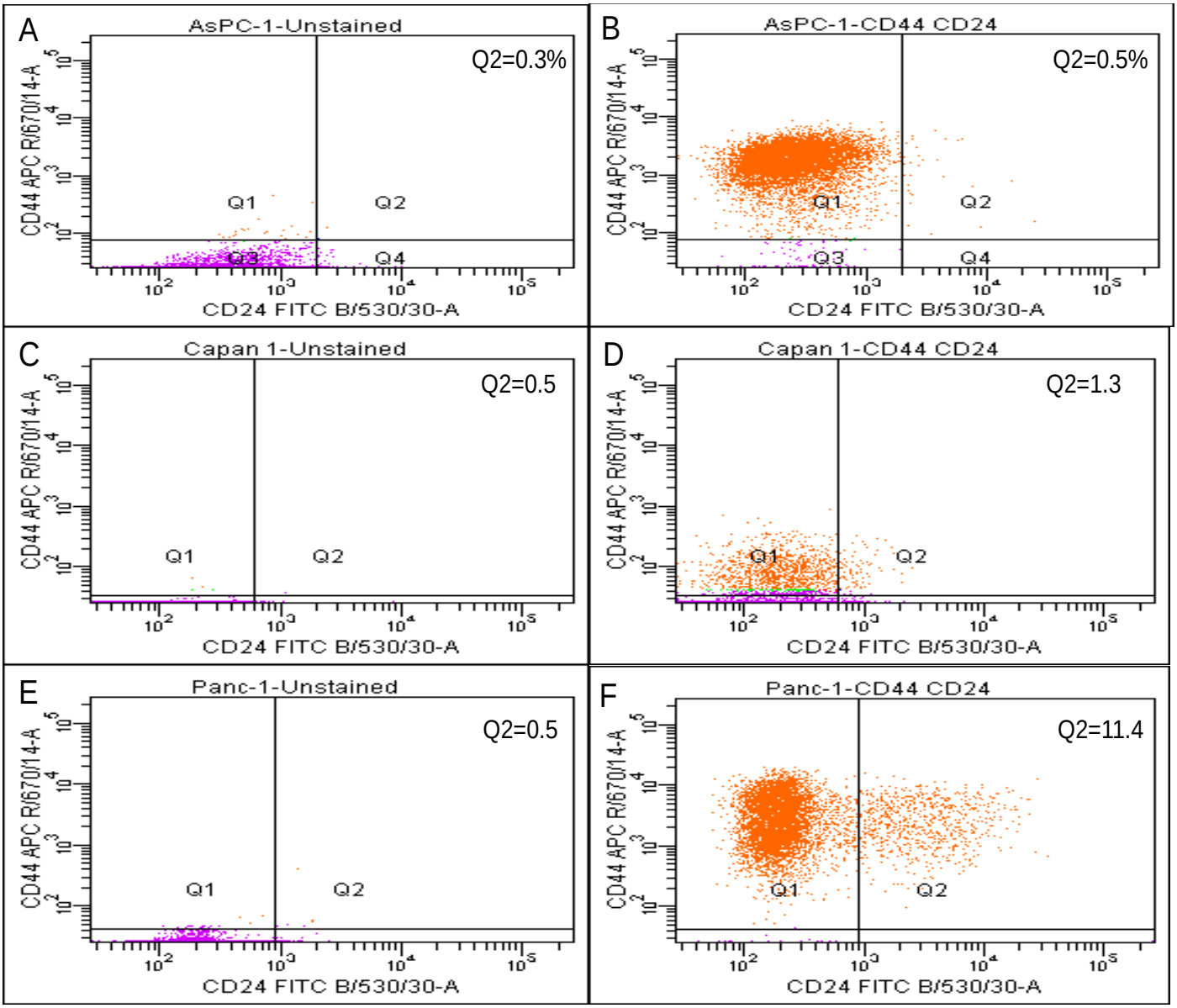
3.6. Stem cell profile in Capan-1, Panc-1 and AsPC-1 cells

Having established that curcumin and resveratrol as single compound exposures or in combination, affect proliferative capacity of the chosen cell lines, the next goal was to assess their effects on the cancer stem cell populations using flow cytometric analysis. Baseline properties of the cell lines were first assessed. In Panc-1 cells, $CD44^+$ cells constituted 99.6%, $CD24^+$ cells 13.4% and $ALDH^{high}$ constituted 5.8% of the total population. AsPC-1 also had a high percentage of cells expressing $CD44^+$ (98.97%), with very low expression of all the other markers. Capan-1 had low levels of $CD44^+$ cells (28.7%) and (9.2%) $CD24^+$ cells but they had the highest expression of $CD133^+$ (68%) and the largest fraction of cells with ALDH-1^{high} activity (23.2%). Panc-1 cells exhibited the highest co-expression of $CD44^+/CD24^+$, with the other cell lines exhibiting <1% of this expression pattern (Figures 3.12, 3.13 and 3.14).

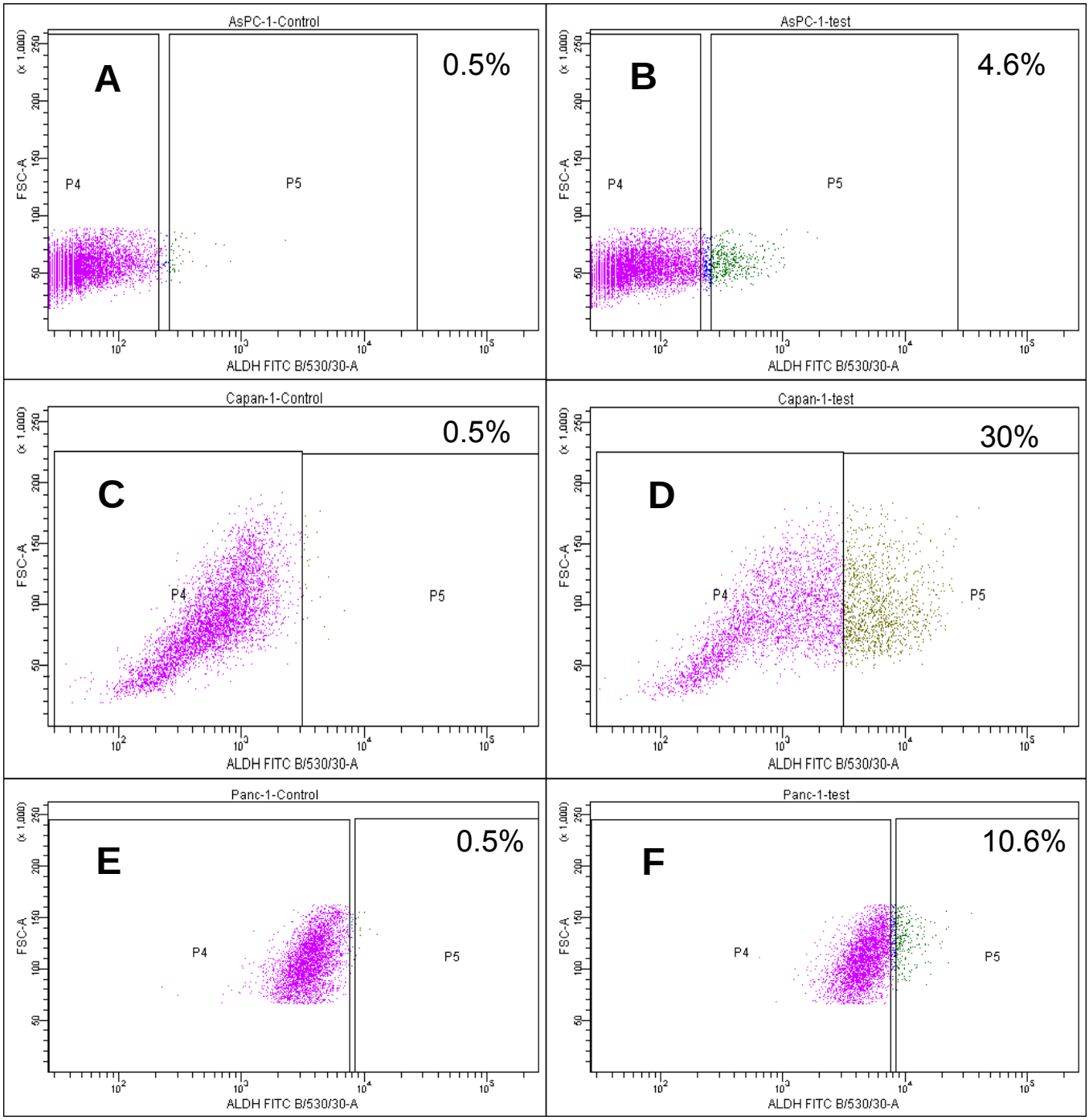


Cell lines	ALDH-1	CD24	CD44	CD24 and CD44	CD133
Panc-1	5.7±0.6	13.4±4	99.6±1.7	10.7±3	0.5±0.1
AsPC-1	5.2±0.7	0.5±0.1	98.9±1.3	0.3±0.2	0.6±0.12
Capan-1	23.2±2.7	9.2±1.8	28.7±3.7	0.6±0.18	68±5

Figure 3.12 Stem cell profile for Capan-1, AsPC-1 and Panc-1 cells using cell surface markers and intracellular activity of ALDH-1. Bar Chart shows cellular expression and activity. Cells were stained for $CD24^+$, $CD44^+$ and $CD133^+$ expression and ALDH-1 activity with appropriate antibodies then analysed by FACS. The Table reports the overall percentage of cells expressing each phenotype. A minimum of 10,000 cells were analysed. Data are the average (+SEM) of three independent experiments.



3.13 Example of gating strategies used to determine CD24⁺ and CD44⁺ expression on the surface of AsPc-1, Panc-1 and Capan-1 cells. X-axis shows Forward side scatter (FSC-A) CD44⁺ APC R/670/14-A, Y-axis shows CD24⁺ FITC B/530/30A. The left panel represents no staining which are gated at 0.5% and the right panels represent cells stained for CD24⁺ and CD44⁺. Q1= Stained for CD44⁺, Q2= Stained for both CD24⁺ and CD44⁺, Q3= Unstained cells and Q4= Stained for CD24. (**A** and **B** for AsPC-1), (**C** and **D** for Capan-1) and (**E** and **F** for Panc-1).



3.14 Example of gating strategies used to determine ALDH activity in AsPc-1, Panc-1 and Capan-1 cells. X-axis shows Forward side scatter (FSC-A), Y-axis ALDH FITC B/530/30A. The left panel represents the controls (DEAB added) which are gated at 0.5% and the right panels represent ALDH activity above the control level. (**A** and **B** for AsPC-1), (**C** and **D** for Capan-1) and (**E** and **F** for Panc-1).

3.7. Stem cell profile for Capan-1, Panc-1 and AsPC-1 using embryonic stem cell markers:

The major regulatory roles of Nanog, Oct4 and Sox2 in pancreatic CSCs have not been fully interpreted, but when they are overexpressed together or individually they correlate with tumour metastasis, transformation, tumourigenicity, distant recurrence, poor prognosis and poorly differentiated tumours. This causes crucial genes for pluripotency to be triggered while genes accountable for differentiation are deactivated and switched off. This is a common feature between ESCs and CSCs, suggesting that Oct4, Nanog, and Sox2 could be interrelated and cooperate to regulate pluripotency and self-renewal in tumours. The expression of Oct4, Sox2 and Nanog is highly heterogeneous in different tumours and there may also be differences within the same tumour (120).

Recently, there have been a number of specific publications analysing the role of Sox2, Oct4 and Nanog in pancreatic cancer. Embryonic transcription factors might have an association with stem cell markers that confer the development, therapeutic resistance and reoccurrence of pancreatic cancer. Therefore, the baseline cellular expression of Sox2, Oct4 and Nanog was determined in the panel of three pancreatic cancer cells employed in this project. Their expression was compared under adherent culture conditions as well as when they were incubated under sphere forming conditions. Nanog, Oct4 and Sox2 baseline expression were measured in the Panc-1, Capan-1 and AsPC-1.

Nanog, Oct4 and Sox2 baseline expression was measured in the Panc-1, Capan-1 and AsPC-1 cells, under both adherent (Figure 3.15) and sphere culture conditions (Figure 3.16). Nanog expression in Capan-1 cells was similar in both systems at and for adherent and sphere cultures. Nanog expression in Capan-1 cells slightly increased from 1.6±0.3 in adherent cultures to 1.9±0.1 in spheroids (Figure 3.17A) but the difference was not significant, unlike Sox2 expression, which was significantly enhanced in spheres (Figure 3.17C). In spheroid cultures Nanog, Oct4 and Sox2 were significantly increased in Panc-

1 cells and nearly doubled compared to adherent conditions. The lowest expression of Nanog was observed in AsPC-1 cells (data not shown).

The Oct4 baseline expression in adherent Panc-1 cells was 1.4±0.15 and increased significantly to 2.2±0.18 spheroid cultures. In adherent conditions, the Oct4 baseline expression in Capan-1 cells was 1.4±0.07 while significantly increased in sphere condition into 1.9±0.1 (Figure 3.17B). The lowest expression of Oct4 was observed in AsPC-1 (Data is not shown).
In adherent conditions, the Sox2 baseline expression in Capan-1 cells was 0.4±0.05 while significantly increased in sphere condition into 0.7±0.03 (Figure 3.17C). The lowest expression of Oct4 was observed in AsPC-1 (Data is not shown).

The Sox2 baseline expression in adherent Panc-1 cells was 0.4±0.09 and increased significantly to 0.8±0.04 in Panc-1 spheres. In adherent conditions, the Sox2 baseline expression in Capan-1 cells was 0.4±0.05 while significantly increased in sphere condition into 0.7±0.03 (Figure 3.17C).

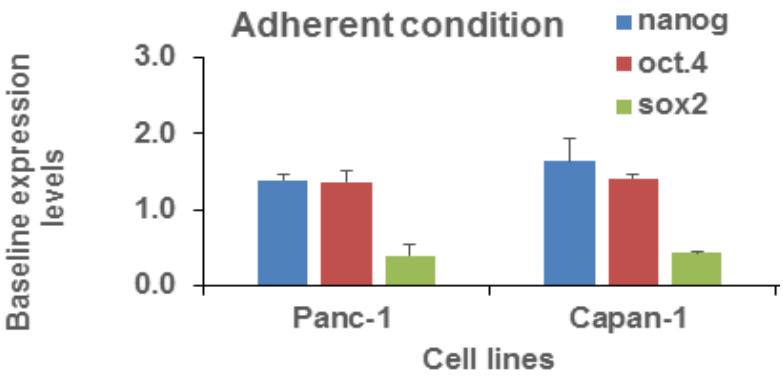


Figure 3.15 Baseline protein expression levels in Adherent condition. Baseline protein expression for Nanog, Oct4 and Sox2 in adherent condition for Panc-1 and Capan-1. X-axis= cell lines and Y-axis= level of protein expression. (+SEM) of three independent experiments.

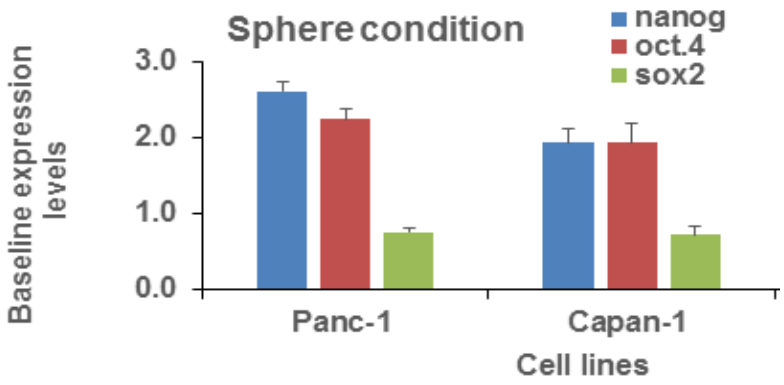
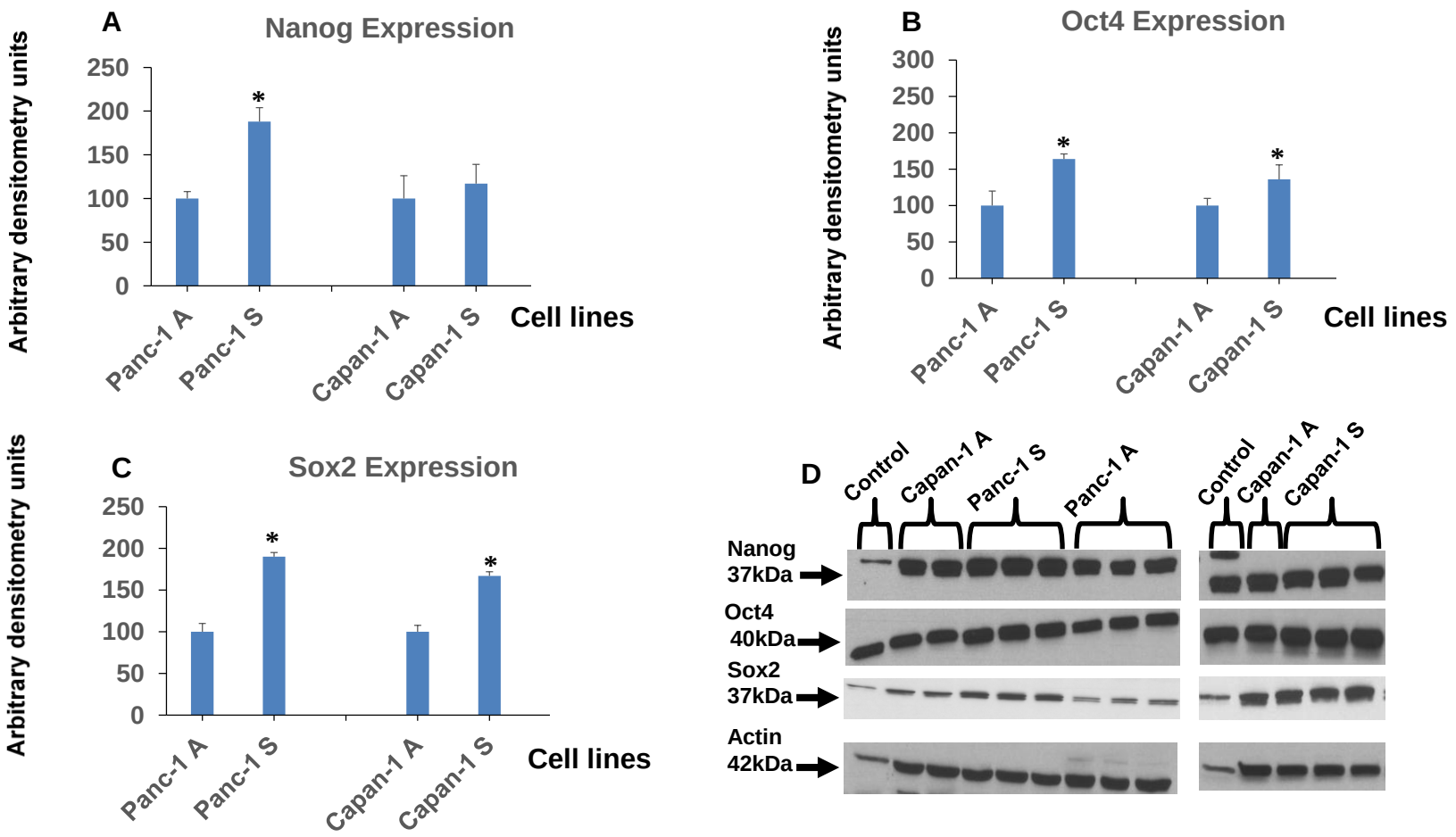


Figure 3.16 Baseline protein expression levels in Sphere condition. Baseline protein expression for Nanog, Oct4 and Sox2 in sphere condition for Panc-1 and Capan-1. X-axis= cell lines and Y-axis= level of protein expression. (+SEM) of three independent experiments.



3.17 (Nanog 37kDa, Oct4 40kDa and Sox2 37kDa) analysed by Western blot. (A-C) The charts shows quantification of the expression level for **(A)** Nanog, **(B)** Oct4 and **(C)** Sox2, comparing cells grown under adherent conditions (designated by A), with those under sphere forming conditions (designated by S). **(1-D)** Representative Western blot for all three proteins. Data are the average (+SEM) of three independent experiments.

3.8. Discussion

Three pancreatic cancer cell lines (Capan-1, Panc-1 and AsPC-1) were assessed for their sensitivity to curcumin and resveratrol. These cell lines were representative of both primary adenocarcinoma (The most common one; represents 85-90% of all pancreatic cancer types) (46, 273) and metastatic deposits found in the liver and lymph node (as pancreatic cancer frequently presents at late stage, when the tumour has disseminated into other organs). Whilst it is acknowledged that cell lines often give poor representation of clinical disease, a number of publications have suggested that the genomic status of cell lines remains representative of the primary tumour, exhibiting many similar features (274, 275). Furthermore, primary pancreatic cell lines are difficult to derive, due to limited tissue availability following surgical resection, and the need for much of the resected tissue to be used clinically in histological diagnosis. Recently, 80 pharmacological drugs were analysed in 16 pancreatic cancer cell lines with Capan-1 cells shown to be highly resistant to many chemotherapeutics, including docetaxel (276). Capan-1 represent a well differentiated cell type despite being metastatic in origin. It is now well documented that Capan-1 cells are a highly resistant cell line, with increasing resistance observed in response to repeat administration of both 5-FU and gemcitabine (277, 278). This enhanced resistance was thought to be due, in part, to a highly elevated expression of the export pump multidrug resistant (MDR) protein 5 (MRP5), which facilitates faster drug detoxification through enhanced excretion (277). Additionally, the highly resistant phenotype of Capan-1 may be due to overexpression of MUC1 and MUC4, which regulate the MDR genes (275, 279). For these reasons, Capan-1 cells were of particular interest, as there is evidence that putative chemopreventive agents may have the ability to target genes associated with chemoresistance (277, 278)

Anti-proliferative effects of curcumin have previously been shown in a wide variety of tumour cells, including colon carcinoma, breast carcinoma, hepatocellular carcinoma, renal cell carcinoma, T cell leukaemia, basal cell carcinoma, B cell lymphoma, acute myelogenous leukaemia, melanoma and

prostate carcinoma (178, 181, 183, 280). The range of pharmacologically relevant curcumin concentrations that elicit effects on biomarkers in tumours and induce outcome such as apoptosis and growth inhibition *in vitro* for various cancer cell lines has been previously reported to be in the range of 1 to 12 µM (174).

Similarly, resveratrol has been shown to regulate multiple cellular pathways related to carcinogenesis via anti-proliferative (235-238, 241, 242). In pancreatic cancer cell lines, its potential anti-tumour activities include induction of apoptosis, inhibition of cell proliferation and inhibition of angiogenesis (172, 173, 193, 254, 281). In addition to this, resveratrol in combination with gemcitabine potentiates anti-tumour activity *in vitro* and in an orthotopic mouse model of human pancreatic cancer (193). Furthermore, it has been shown that chemo-radiotherapies can be enhanced by resveratrol as a result of increasing sensitivity of malignant cells to the treatments (235, 254).

Exposure of the panel of pancreatic cell lines to curcumin and resveratrol revealed Capan-1 cells to be the most sensitive to curcumin, with a significant decrease in proliferation observed at 0.1 µM, a concentration at which little efficacy has been observed in other cancer cell lines. Following repeat daily dosing, this sensitivity to curcumin was maintained, which is in contrast to observations for chemotherapeutic interventions in this cell line (278). This leads to the intriguing possibility that there is perhaps potential for curcumin to be used as a co-therapeutic to enhance sensitivity and decrease the rate at which chemotherapeutic resistance occurs, however longer term exposures would be required to test this. In all cell lines, curcumin appeared to elicit anti-proliferative effects at lower concentrations than that observed for resveratrol.

When IC_{50}s were calculated only for cell lines were 50% of cell reduction observed. The IC_{50} of curcumin in Capan-1 and RLT-PSC were 1.7 and 2.25 µM respectively. Of note however, most other studies report effects from a single dose rather than the daily dosing strategy employed here, which would better mimic any future clinical regimen. It is therefore likely that the daily dosing regimen may give greater efficacy at lower doses.

In addition to the pancreatic cancer cell lines, pancreatic stellate cells (RLT-PSC) were also exposed to curcumin and resveratrol. These type of cells play a very important role in pancreatic cancer, such as promoting an inflammatory environment that will promote progression of tumourigenesis, or be responsible for production of dense desmoplasia to form barriers preventing chemotherapeutic drugs getting to the cancer cells. Known inhibitors of PSC activity in pancreatic cancer include drugs such as halofuginine, a smad3-phosphorylation-inhibitor, which decreases PSC activation and inhibits pancreatic xenograft tumour development (145). Retinoic acid can also prevent pro-carcinogenic PSC activity and decreases wnt-β-catenin signalling in cancer cells, reducing their invasive capability. Key signalling interactions between PSCs and cancer cells have been proposed, including sonic hedgehog, galectins, endothelins and platelet-derived growth factor, which could be targeted by potential therapeutic drugs (146, 147).

In a study by Masamune *et al*, (2006) curcumin blocked pancreatic stellate cell activation (140, 282) from a concentration of 5 µM. The anti-proliferative effects of curcumin on PSCs was also reported in a mouse model and was mediated by induction of HO-1 gene expression (283). This is consistent with the results found here, which showed that RLT-PSC cells are very sensitive to curcumin exposure, with significant growth inhibition observed from 2.5 µM. The PSCs were also sensitive to low concentrations of resveratrol, and this is the first time such an effect has been described for this compound. The identification and use of agents to inhibit proliferation of PSCs might decrease the barrier effect of the desmoplasia, thus allowing chemotherapy drugs to reach their target cells. Evidence for success of this approach has been observed via targeting of the hedgehog pathway in stromal cells, which resulted in a 10-fold improvement of drug delivery (125). Furthermore, inhibition of PSC proliferation may result in decreased pro-oncogenic signalling, invasion and metastatic spread.

An important aim of the work presented here was to use concentrations of both curcumin and resveratrol that are within a clinically achievable range, but when used as a single treatment, significant changes to proliferation were not observed across all cell types. It was hypothesized that combination of the two

agents may reduce the concentration required to elicit pharmacologic effects. Recently, additive and synergistic effects of various combinations of natural products have been studied for their properties and usefulness in the prevention and treatment of pancreatic cancer.

Evidence of enhanced efficacy when combining curcumin with resveratrol has been observed in various cancers, with enhanced anti-proliferative and pro-apoptotic effects observed in colon cancer (260) (10 µM curcumin combined with 10 µM of resveratrol), and in prostate cancer (5 µM each of curcumin and resveratrol) (264). However, it has also been observed that concentrations used to achieve this enhanced effect were very high in some combination studies. For example, when the combination (curcumin and resveratrol) treatment was used on Hepa1-6 hepatocellular carcinoma cells, inhibition of cellular proliferation and an increase in apoptosis were reported with 10 µM curcumin combined with 40 µM of resveratrol (265).

In this thesis the results from combination exposure after 6 days daily dosing showed enhanced anti-proliferative effects for the first time in pancreatic cancer cell lines. The main outcome observed was that across all cell lines, the addition of very low dose curcumin (0.1 µM) to 5 µM resveratrol, resulted in an enhancement of the anti-proliferative activity of resveratrol at this concentration. The growth inhibition data also indicated that combination of resveratrol and curcumin both at clinically achievable concentrations of 0.1 µM induced significant growth inhibition in 2 out of 4 cell lines. Here it has been demonstrated that the combination of curcumin and resveratrol in pancreatic cancer cell lines is more effective at lower concentrations of each compound than has been previously shown for other cancer cell lines derived from colon, prostate and hepatocellular carcinoma, as reported in the literature.

In order to try and identify the main mechanisms by which growth inhibition occurred, the cancer cell lines were assessed for apoptotic cell death. It was only possible to detect induction of apoptosis in Capan-1 cells, where it was observed after 24 h exposure to a combination of 5 µM curcumin and 0.1 µM resveratrol at almost 5-fold above the basal level in the DMSO control. In

addition, the greatest induction of apoptosis with any treatment was apparent at 48 h in cells exposed to 5 µM curcumin (6.7-fold increase). It was impossible to see any trace of apoptosis in the other two cell lines (Panc-1 and AsPC-1), even though the positive control compound, etoposide has the desired effect.

For the cell lines where induction of apoptosis could not be observed, it was important to ascertain whether the cells underwent cell cycle arrest, as it has previously been reported that curcumin and resveratrol both cause arrest in other cancer cell lines.

It has previously been shown that activation of ATM/Chk1 by curcumin caused cell cycle arrest at G2/M in pancreatic cancer cells (BxPC-3) and led to apoptosis at 2.5 µM (284). It was also suggested that curcumin treatment inhibits Wnt signaling and cell-cell adhesion pathways leading to a G2/M phase cell cycle arrest in HCT-116 cells (285). In pituitary tumour cell lines and adenomas, curcumin caused cell cycle arrest at G2/M and apoptosis at 20 µM (286). In terms of cell cycle arrest by curcumin and resveratrol in pancreatic cancer cell lines various concentrations have been shown to induce arrest at different stages of cell cycle arrest (Table 7.7 and 7.8 in Appendices).

When Capan-1, Panc-1 and AsPC-1 cells were exposed to a single compound and combinations for 24, 48 and 72 h, the following observations were similar to those previously reported by other investigators, with the exception that there is no cell cycle data relating to Capan-1 cells in the literature to the best of my knowledge. No cell cycle arrest was observed in Capan-1 cells, but in this cell line there was marked apoptosis induced at the earliest time point. Cell cycle arrest occurred in both the Panc-1 and AsPC-1 cell lines. Panc-1 cells underwent G2 arrest in response to 5 µM curcumin and any combination containing 5 µM of curcumin. No significant increase in arrest when combinations were used compared to either compound alone. Aspc-1 cells underwent significant S phase arrest in response to 5 µM resveratrol and curcumin caused cell cycle arrest at G1.

In order to explain the lack of induction of apoptosis in Panc-1 and AsPC-1 cells, despite observation of significant anti-proliferative effects, other mechanisms of cytotoxicity or cytostasis may have been induced by resveratrol and curcumin. Other potential mechanism that may have been invoked by both resveratrol and curcumin include necrotic cell death, autophagyic cell death and pyroptosis (287). In addition, Mosieniak *et al.* (2015) (288) recently investigated curcumin for its ability to cause senescence in cancer cells and the existence of a functional link between senescence and autophagy in HCT-116 cells, whilst Patel *et al.* (2013) showed that resveratrol exposure caused senescence and autophagy in colorectal cancer cells (289). The genetic makeup of the cell lines has a dominant role in cell death pathways, and the way in which they respond to drug insults. Interestingly, the cells with wild-type p21 or SMAD4 (Panc-1 and AsPC-1) underwent a cell cycle growth arrest when exposed to a combination of curcumin and resveratrol, whereas cells lacking p21 or SMAD4 (Capan-1), did not and furthermore proceeded to apoptosis. Similar types of response have been demonstrated in two colon cancer cell lines that were genotypically similar; apart from their p21 status. Wild-type p21 cells, when irradiated with γ-radiation, experienced cell cycle growth arrest, but cells with no activity of p21, did not experience a cell cycle growth arrest when irradiated and instead progressed to apoptosis (290).

In general, data reported in the literature have used higher drug concentrations in pancreatic cancer cell lines than in the present study, with the exception of Sahu *et al.* (2009) (284). This group applied 2.5 μM curcumin to BxPC-3 cells, and observed curcumin-induced G2 cell cycle arrest. It seems BxPC-3 and Capan-1, are both very sensitive to curcumin as they have many similar genetic and molecular pathways (291).

It is clear that the ability of curcumin and resveratrol to either invoke a cell cycle arrest or induce cell death is likely to be cell line dependant. It is also likely that both curcumin and resveratrol have effects on multiple signalling pathways linked to proliferation and cell death. It was reported that more than 90 alternations of cancer-linked cell-signalling pathways occurred following

treatment with curcumin (280, 292) in different cell lines, and a similar pleiotropic effect is likely in response to resveratrol.

To sum up this chapter, it is clear that both curcumin and resveratrol, following individual exposure, induce anti-proliferative effects, and that the cell lines are more sensitive to curcumin than resveratrol. Combinations of these two compounds could have beneficial effects on growth inhibition at lower concentrations which is clinically achievable in 2 out of 4 cell lines. In addition, it was observed that these two phytochemical substances can induce their effects through multiple signalling pathways which rely on the cell lines to bring about growth inhibition either by cell cycle arrest or apoptosis. Last but not least, these two compounds have a great potential for de-bulking the pancreatic cancer cell lines by inhibition of cell growth particularly for stellate like cells which allows the barrier for drug delivery into the site of cancer cells to be overcome, as well as de-bulking the whole tumour mass.

Chapter Four: Targeting of cancer stem-like cells by curcumin and resveratrol

4.1. Introduction

Pancreatic cancer is a very heterogeneous cancer, known to harbour rare populations of cancer stem cells (CSCs) (118, 293-295). Preclinical studies have previously described a wide array of potential approaches that target CSCs via specific surface antigens and cellular pathways involved in cell survival, adhesion, self-renewal and differentiation (108, 117). Both curcumin and resveratrol may have potential to target the CSC population, which may provide a basis for both chemopreventive and therapeutic effects (231, 254, 295). Current research on pancreatic cancer stem cells has focused on targeting specific and phenotypically defined CSCs, often relying upon single CSC populations which can be targeted. However, the existence of multiple CSC populations representing different levels of potency and exhibiting differential chemo-radio resistance and self-renewal properties, recently been identified in pancreatic carcinoma (106, 294). It is likely that the dismal prognosis for pancreatic cancer may be contributed to by the inability of common chemotherapeutic agents to target these self-renewing populations, and therefore, treatments which are able to target CSCs may offer a promising therapeutic or preventive approach (105, 109).

The aim of the work described within this chapter was to determine the sensitivity of cellular populations expressing characteristics of differing CSC populations, to the investigational agents. This was undertaken using both measures of surface marker expression ($CD24^+/CD44^+$ and $CD133^+$), endogenous activity of ALDH-1, and by assessment of levels of proteins known to be associated with pluripotency (Nanog, Oct4 and Sox2). In addition, functional measures of CSC phenotypes (sphere formation assay) were assessed in response to curcumin and resveratrol.

4.2. Effect of curcumin and resveratrol on sphere formation by Capan-1 and Panc-1 cells

In order to test whether pancreatic adherent cells lines (Capan-1, AsPC-1, and Panc-1) can form spheres in serum free media, the cells were grown in stem cell media. Initially, bulk cell populations for both cell lines were cultured, in order to establish that sphere formation occurred under low adherence conditions. In general, the size of spheres formed by AsPC-1 cells were the smallest, between 40-60 µm, and the biggest spheres were formed by Panc-1 cells, which ranged in size between 40-400 µm. Of note, AsPC-1 cells did not form 'true' spheres, as they had the appearance of small aggregates. The Panc-1 "megaspheres" consisted of many aggregates of small spheres. Only the Capan-1 cells formed true, well-defined spheres. The size of the spheres formed by Capan-1 cells was between 40-230 µm. It is worth highlighting that Panc-1 and AsPC-1 cells did not form what might be considered to be ideal spheres as the AsPC-1 spheres were very small in size and low in number, whilst the Panc-1 spheres were giant and tended to look like aggregates of small spheres rather than one sphere. However, the Capan-1 cells formed similar shaped spheres to those reported for primary pancreatic cancer cell lines (99, 296, 297) (Figure 4.1).

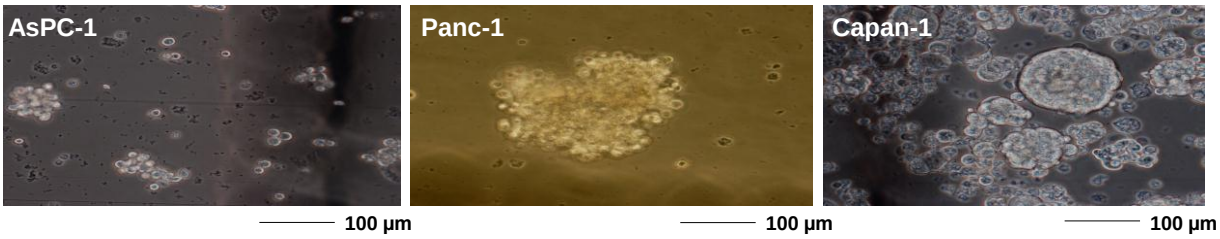


Figure 4.1 Representative light microscopy images showing the three cell lines. (AsPC-1, Panc-1 and Capan-1) forming spheres. Cells were grown in stem cell medium for 2 weeks then pictures were taken (under a 20X objective) using an inverted light microscope and Nikon EclipseTE2000U merging system with built in Eclipse software. The scale bar is 100 µm.

Following this, each cell type was subsequently sorted for two populations, cells with ALDH-1^{high} activity and cells expressing high levels of the two cell surface proteins CD24^{+} and CD44^{+} (CD24^{+}/CD44^{+}, double positive population), and the

sphere forming ability of each isolated population was assessed (Table 4.1). For comparison, the negative populations for each potential CSC marker were also evaluated for sphere formation.

Table 4.1 Pancreatic cancer cells sorted according to various potential stem cell markers to check sphere forming ability in stem cell media across multiple wells per sort. Statistical analysis was performed using a Student's T-test.

Cell lines	Seeding density (cells/well)	Sorted for stem cell markers	Average Number of spheres formed after 14 days across multiple wells
Capan-1	5000	ALDH-1^{High}	500
Capan-1	5000	ALDH-1^{Low}	150
Panc-1	5000	ALDH-1^{High}	180
Panc-1	5000	ALDH-1^{Low}	140
AsPC-1	5000	ALDH-1^{High}	220
AsPC-1	5000	ALDH-1^{Low}	170
Capan-1	5000	CD24^{+}/CD4^{+}	450
Capan-1	5000	CD24^{-}/CD4^{-}	300
Panc-1	5000	CD24^{+}/CD4^{+}	300
Panc-1	5000	CD24^{-}/CD4^{-}	250

For Panc-1, there was no significant difference in sphere formation between the ALDH-1^{High} and ALDH-1^{Low} or CD24^{+}/CD44^{+} and CD24^{-}/CD44^{-} populations. With Capan-1 cells, there was a significant difference in sphere formation between ALDH-1^{High} and ALDH-1^{Low} (P=0.001) but CD24^{+}/CD44^{+} and CD24^{-}/CD44^{-} populations were similar (Table 4.1).

Sphere culture under low serum conditions enriches cell populations for CSCs, and so provides a useful model for testing anti-CSC efficacy of chemopreventive and chemotherapeutic agents. To determine whether curcumin or resveratrol can affect sphere growth, number or size, Capan-1 and Panc-1 cells were cultured under non-adherent conditions using the standard stem cell conditions and exposed to curcumin and resveratrol individually

(Figure 4.2). AsPC-1 was not used for further experiments as they did not form spheres and lacked expression of CSC markers. A significant reduction in sphere number was observed following exposure of Panc-1 cells to curcumin at 5 µM only, with a 45±26.5% reduction in sphere number compared to the control. A significant but small decrease (11±5.03% relative to control) in sphere size was also seen when Panc-1 cells were exposed to curcumin at 5 µM. No significant effects on sphere number or size were observed in response to resveratrol treatment at any concentration (Figure 4.2 C & D).

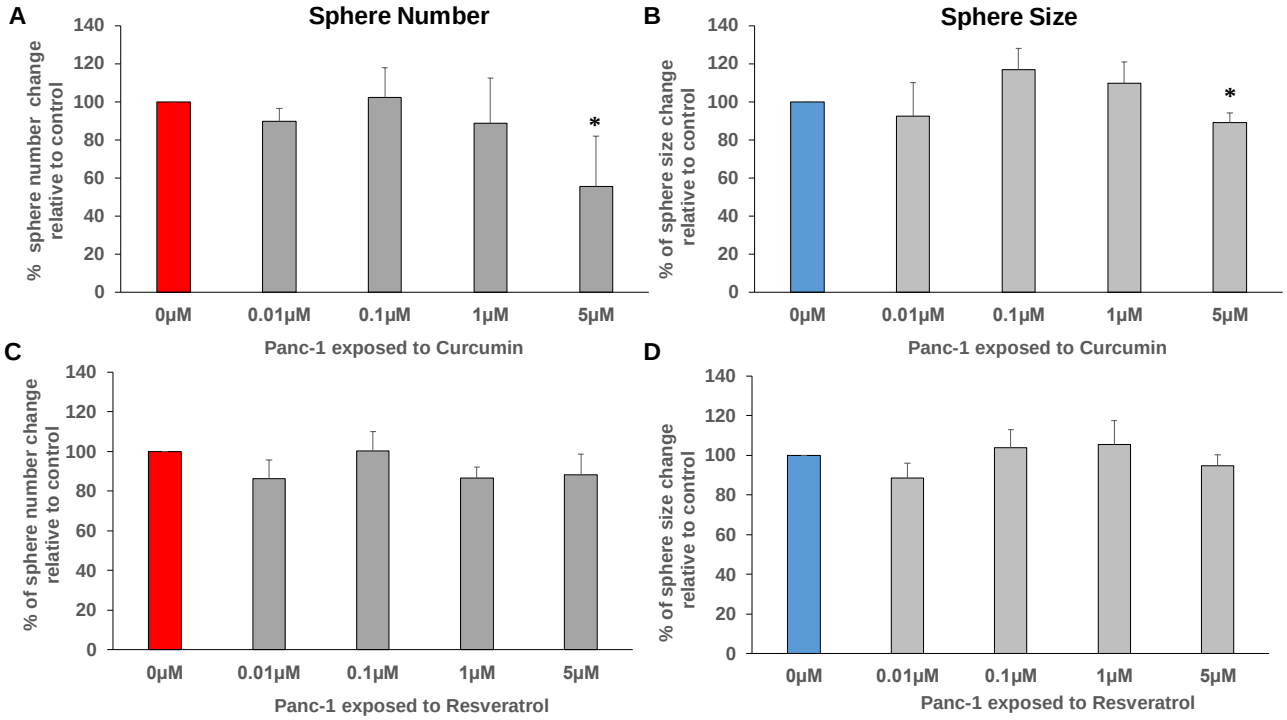


Figure 4.2 Sphere number and size after two weeks exposure of Panc-1 cells to curcumin or resveratrol, relative to solvent control. (A) Sphere number following curcumin treatment. **(B)** Sphere size following curcumin treatment. **(C)** Sphere number following resveratrol treatment. **(D)** Sphere size following resveratrol treatment. Data are expressed as a percentage of the solvent control (red & blue bars) and represent the average (+SEM) of three experiments, each performed in triplicate wells. Significant differences in number and size relative to the control are indicated, where * = p≤0.05. Statistical analysis was performed using a Student's T-test.

In Capan-1 cells, a significant dose-dependent reduction in sphere number was observed following curcumin treatments at 0.1 µM (15±4.5% decrease), 1 µM

(55±4.2%) and 5µM, which dramatically decreased sphere numbers by 96% (Figure 4.3). A significant reduction in sphere size was also detected after exposure to 1 µM (22±4.9%) and 5 µM curcumin (53±7.6%). Resveratrol also reduced sphere number but was less potent than curcumin, causing a significant reduction of ~12% at 1 µM and ~36% at 5 µM. Only the highest concentration of resveratrol had a significant effect on sphere size, but the magnitude of the reduction was very small (6±2.1%).

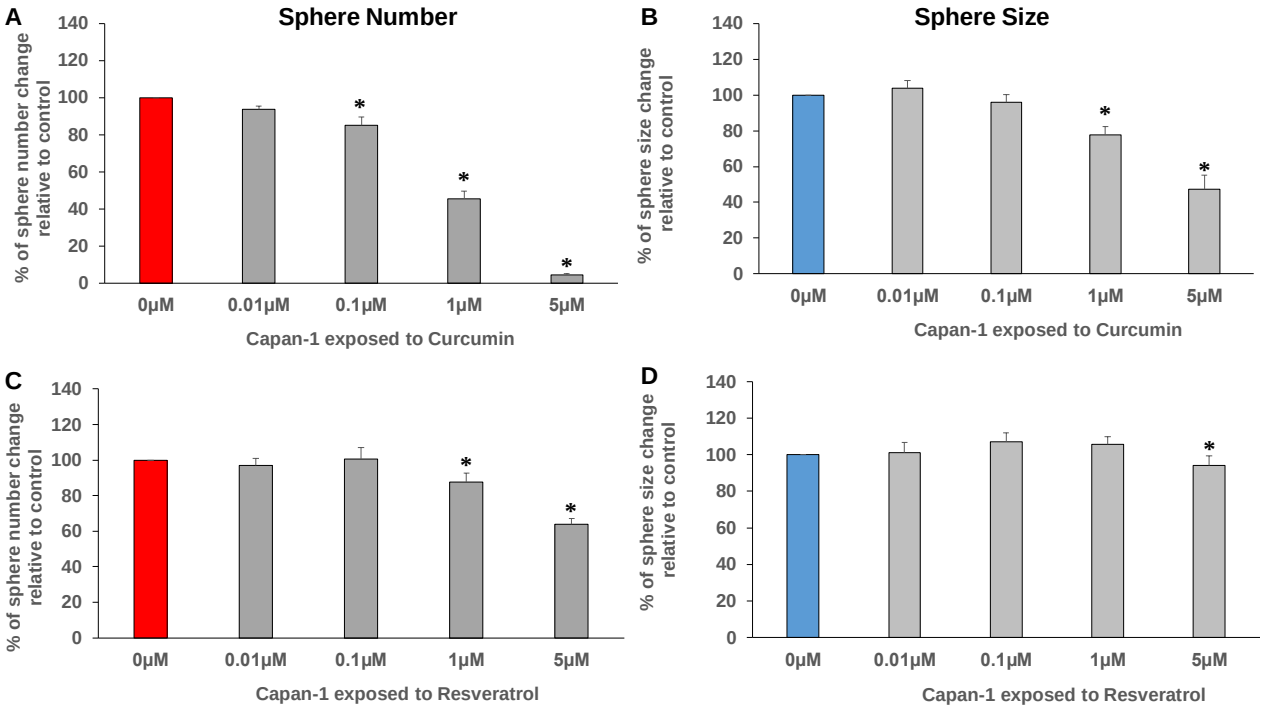


Figure 4.3 Sphere number and size after two weeks exposure of Capan-1 cells to curcumin or resveratrol, relative to solvent control. (A) Sphere number following curcumin treatment. **(B)** Sphere size following curcumin treatment. **(C)** Sphere number following resveratrol treatment. **(D)** Sphere size following resveratrol treatment. Data are expressed as a percentage of the solvent control (red & blue bars) and represent the average (+SEM) of three experiments, each performed in triplicate wells. Significant differences in number and size relative to the control are indicated, where * = p≤0.05. Statistical analysis was performed using a Student's T-test.

4.3. Effect of curcumin and resveratrol on expression and activity of CSC markers in Panc-1 cells

Within cell lines, some CSCs can be identified by their differing cell markers, which are selectively expressed on their surfaces, and conserved throughout the self-renewal process. Panc-1 cells in monolayer culture were exposed to curcumin or resveratrol individually at four different concentrations for six days, with repeated administration of curcumin or resveratrol daily. Stem cell populations were subsequently identified via FACS analysis using CD44 (conjugated to APC fluorochrome) and CD24 (conjugated FITC fluorochrome) antibodies and ALDH1 activity on day 3 and day 6. Following 3 days of curcumin treatment (5 µM) there was a significant reduction (53±7.8%) in cells expressing $CD24^{+}/CD44^{+}$ compared to the control incubations. On day 6, cultures treated with 1 µM of curcumin also had a significantly reduced population of $CD24^{+}/CD44^{+}$ cells (38±10.2%) and the activity of 5 µM curcumin seen after 3 days was retained (54±7.1% reduction) (Figure 4.4 A). on the other hand, there was significant effect on the fraction of Panc-1 cells with high ALDH-1 activity following curcumin exposure at 0.01 and 0.1 µM on day 3; 65±5% and 54±4% respectively (Figure 4.4 B). However, no significant effect was observed when higher concentration of curcumin applied. Resveratrol did not affect $CD24^{+}/CD44^{+}$ expression (Figure 4.4 C), but caused a significant reduction in $ALDH\text{-}1^{High}$ activity following both 3 (35±11.9%) and 6 (61±12.9%) days treatment at 5 µM (Figure 4.4 D).

Over all, both resveratrol and curcumin was successful in affecting either sphere numbers or the expression of cancer stem cell like markers. However this was achieved at different concentrations and time points for resveratrol and curcumin respectively. This suggests that individually resveratrol or curcumin may have different mechanisms of targeting cancer stem-like cells. Hence a combination of curcumin and resveratrol at relevant concentrations might elicit a more effective treatment strategy to target cancer stem-like cells.

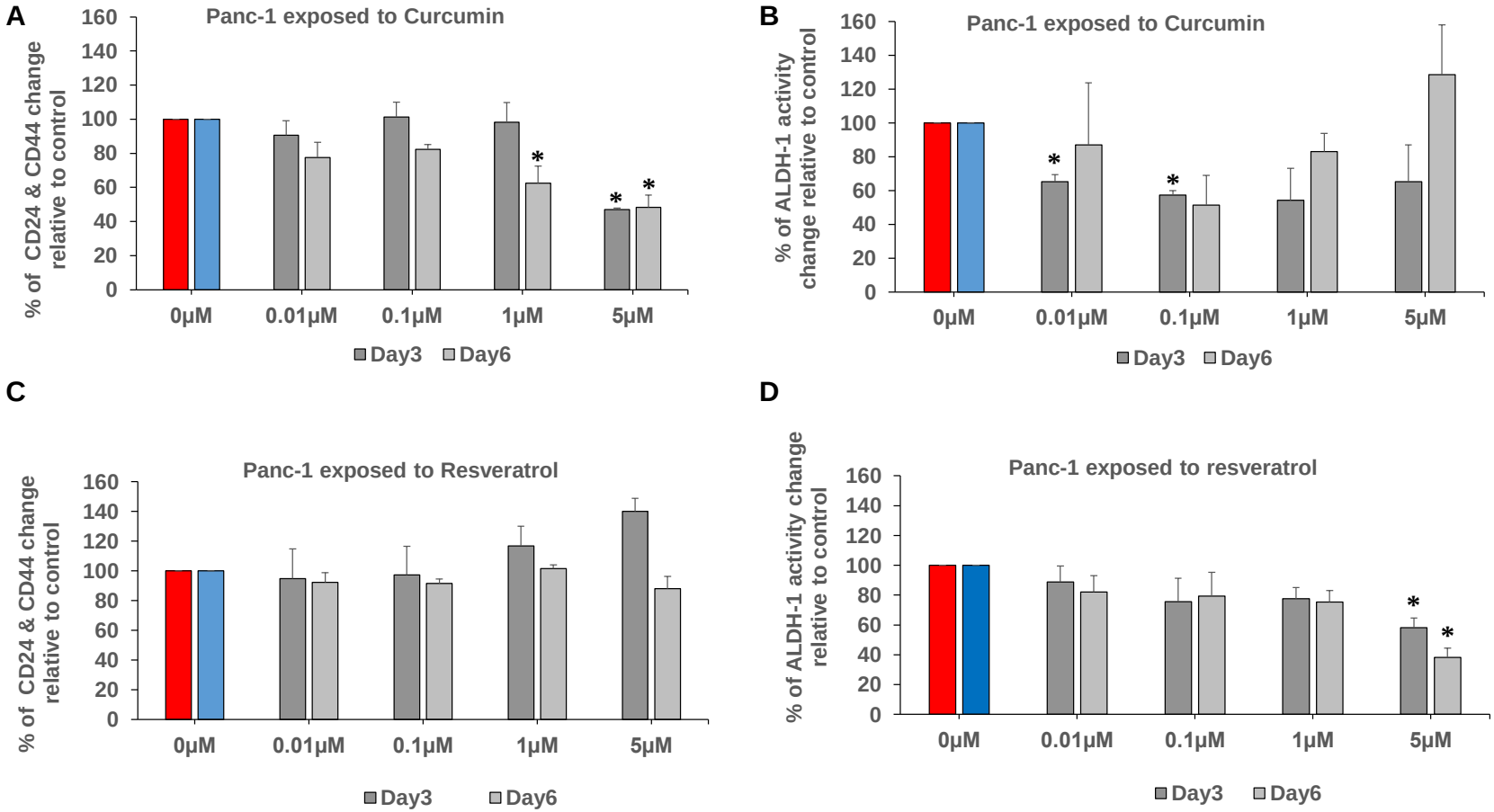


Figure 4.4 The effect of curcumin and resveratrol on the proportion of Panc-1 cells with CD24^{+}/CD44^{+} surface markers and ALDH^{high} activity as determined by FACS analysis. (A) Proportion of cells with double positive staining for CD24^{+}/CD44^{+} relative to DMSO-treated control cells, after 3 & 6 days incubation with curcumin. **(B)** Proportion of cells with ALDH-1^{High} activity relative to the DMSO control after 3 & 6 days incubation with curcumin. **(C)** Proportion of cells with double positive staining for CD24^{+}/CD44^{+} cells relative to DMSO-treated control cells, after 3 & 6 days incubation with resveratrol. **(D)** Proportion of cells with ALDH-1^{High} activity relative to the DMSO control after 3 & 6 days incubation with resveratrol. Data represent the average (+SEM) of three experiments, each performed in triplicate wells. Significant differences relative to the control are indicated, where * = p≤0.05. Statistical analysis was performed using a Student's T-test.

4.4. Combination effects of curcumin and resveratrol on the expression and activity of CSC markers in Panc-1, AsPC-1 and Capan-1 cells.

Adherent cultures of Panc-1, AsPC-1 and Capan-1 cells were exposed to curcumin and resveratrol individually and in the following combinations used in the previous experiments described in Chapter 3: (0.1 µM curcumin 0.1 µM Resveratrol), (5 µM curcumin 0.1 µM resveratrol), (5 µM curcumin 5 µM resveratrol) and (5 µM Resveratrol 0.1 µM curcumin) for 3 and 6 days, with repeated daily administration (Figure 4.5). Staining of surface or intracellular markers was undertaken as previously described. The order of basal expression for the double positive fraction $CD24^+/CD44^+$ was Panc-1>Capan-1>AsPC-1 (Figure 3.11). In Panc-1 cells (Figure 4.5 A), a significant decrease in $CD24^+/CD44^+$ co-expression was observed at days 3 and 6 in response to 5 µM curcumin (30.6±8.2% and 39.9±2.1% reduction, respectively), and also when this treatment was combined with resveratrol at either concentration; 5 µM curcumin plus 0.1 µM resveratrol on day 3 (31.1±5.7%) and day 6 (39.8±4.3%) and 5 µM curcumin plus 5 µM resveratrol on day 3 (25.1±4.5%) and day 6 (24.9±4.9%). However, these decreases were not significantly different from the treatment with curcumin alone, indicating that resveratrol does not enhance the activity of curcumin and has no effect itself on these markers in this cell line. In AsPC-1 cells (Figure 4.5B), significant reductions in $CD44^+/24^+$ co-expression were only observed at day 6 following treatment with 5 µM curcumin (44±4.4%), the combination of 5 µM curcumin plus 5 µM resveratrol (45±2.9%) or the combination of 5 µM resveratrol plus 0.1 µM curcumin (52.5±7.9%). The latter combination reduced $CD44^+/24^+$ co-expression to a greater extent than the single agent treatments, but the decrease was only significant when compared to the control. Capan-1 cells were extremely sensitive to the treatments, meaning that shorter incubation times (1 and 3 days) were chosen (Figure 4.5C). Even at 3 days treatment, the low cell numbers meant that FACS analysis proved problematic. It appeared

that $CD24^+/CD44^+$ expressing cells increased significantly following any treatment containing 5 µM curcumin.

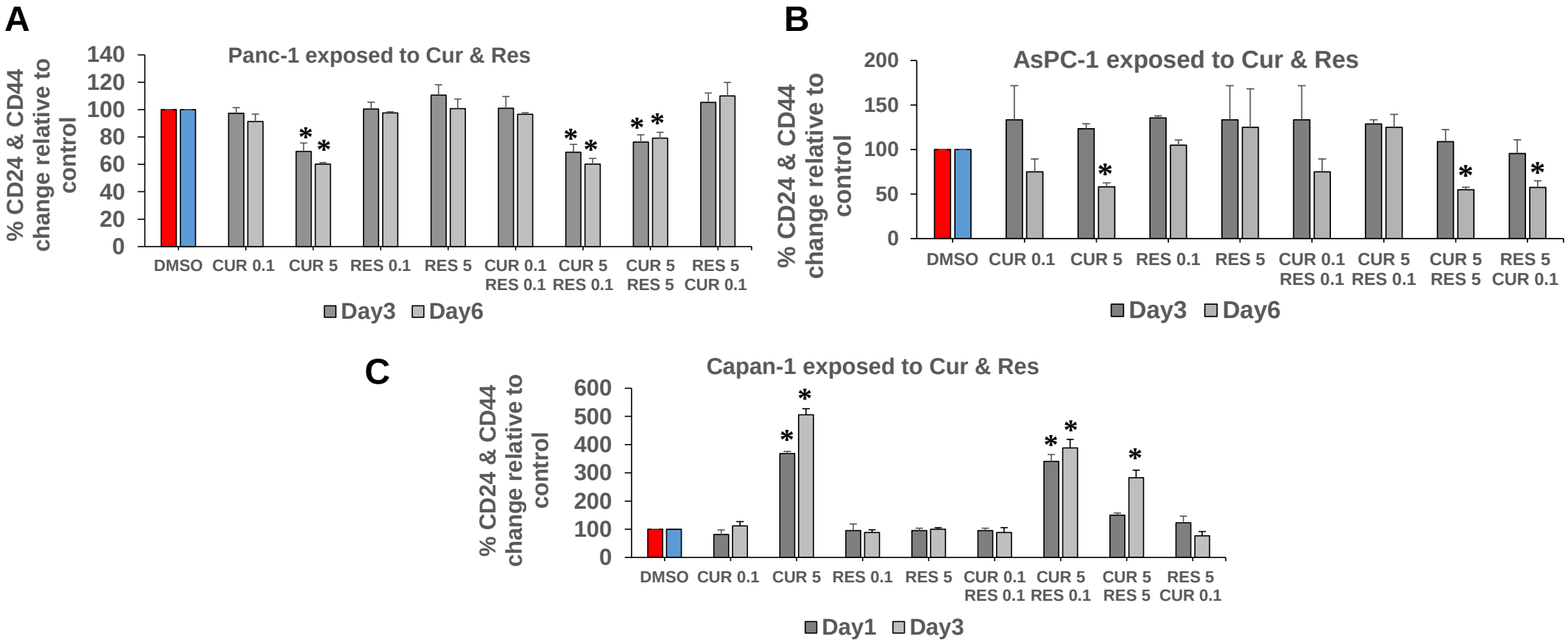


Figure 4.5 The effect of curcumin and resveratrol alone and in combination on the proportion of cells with $CD24^+/CD44^+$ co-expression in the Panc-1, AsPC-1 and Capan-1 cell lines. The charts show relative co-expression of $CD24^+/CD44^+$ following a 3 and 6 day exposure (Panc-1, AsPc-1) or 1 and 3 day exposure (Capan-1) to resveratrol and curcumin, compared to the DMSO solvent control. **(A)** Panc-1 cells. **(B)** AsPC-1 cells. (**C**) Capan-1 cells. Data represent the average (+SEM) of three experiments, each performed in triplicate wells. Significant differences relative to the control are indicated, where * = $p \leq 0.05$. Statistical analysis was performed using a Student's T-test.

Assessment of ALDH1 activity was also undertaken in response to the single and combination treatments (Figure 4.6). The order for the basal levels of ALDH1 activity was Capan-1>Panc-1≈AsPC-1 (Figure 3.12). The effect of resveratrol-containing treatments on Panc-1 cells was more marked than that of curcumin. When Panc-1 cells were exposed to resveratrol alone at 5 µM there was a significant decrease in ALDH-1^{High} activity at both day 3 (71.35±7.95%) and day 6 (66.08±10.29%). A significant reduction in ALDH-1^{High} activity was also observed (day 3 and 6) when 5 µM curcumin was combined with 0.1 µM resveratrol, with the population decreased by 77±8.7% and 54±8.9%, respectively. This is very interesting result as neither compound alone at these particular concentrations reduced the ALDH-1^{High} activity, but it was only significant to the solvent control. When 5 µM curcumin was combined with 5 µM resveratrol, this mixture also showed a significant reduction on both days (44±9.1% and 65±8.2%), but the combination of 0.1 µM curcumin with 0.1 µM resveratrol significantly inhibited ALDH1 activity (day 6) by 64.6±6.6% whereas the individual treatments did not; importantly, this combination is clinically achievable (Figure 4.6 A). In AsPC-1 cells, no significant effects were observed at day 3, with the exception of the combination of 0.1 µM curcumin and 0.1 µM resveratrol where a decrease of 34.2±10.1% was apparent. At day 6, 0.1 µM curcumin resulted in a 23±9.1% reduction, whilst 5 µM curcumin plus 0.1 µM resveratrol had a smaller effect (13±4% decrease) and 5 µM curcumin plus 5 µM resveratrol caused a 42±8% reduction in the ALDH-1^{High} fraction of cells (Figure 4.6 B). The results from combination incubations in AsPC-1 cells suggests that low concentrations of both compounds could have a potential for further investigation in preclinical studies. When Capan-1 cells were exposed to the different treatments, a significant and similar decrease in ALDH-1^{High} activity was observed for all treatments at day 3, the latest time point examined. Exposure to 0.1 µM curcumin caused a 31.3%±3.4% decrease in ALDH-1^{High} activity and 5 µM of curcumin brought about 34.9±11.1% reduction compared to the control. Resveratrol had a comparable effect, with 0.1 µM inducing a 30.6±9.1% reduction in ALDH-1^{High} activity and the 5 µM concentration decreasing this cellular compartment by 31.1±6.9%. For the combination exposures (5 µM curcumin + 0.1 µM resveratrol), (5 µM curcumin + 5 µM resveratrol) and (5 µM resveratrol + 0.1 µM curcumin), significant and

consistent decreases of ~32%, 34%, 40%, and 39%, respectively, were observed (Figure 4.6.C).

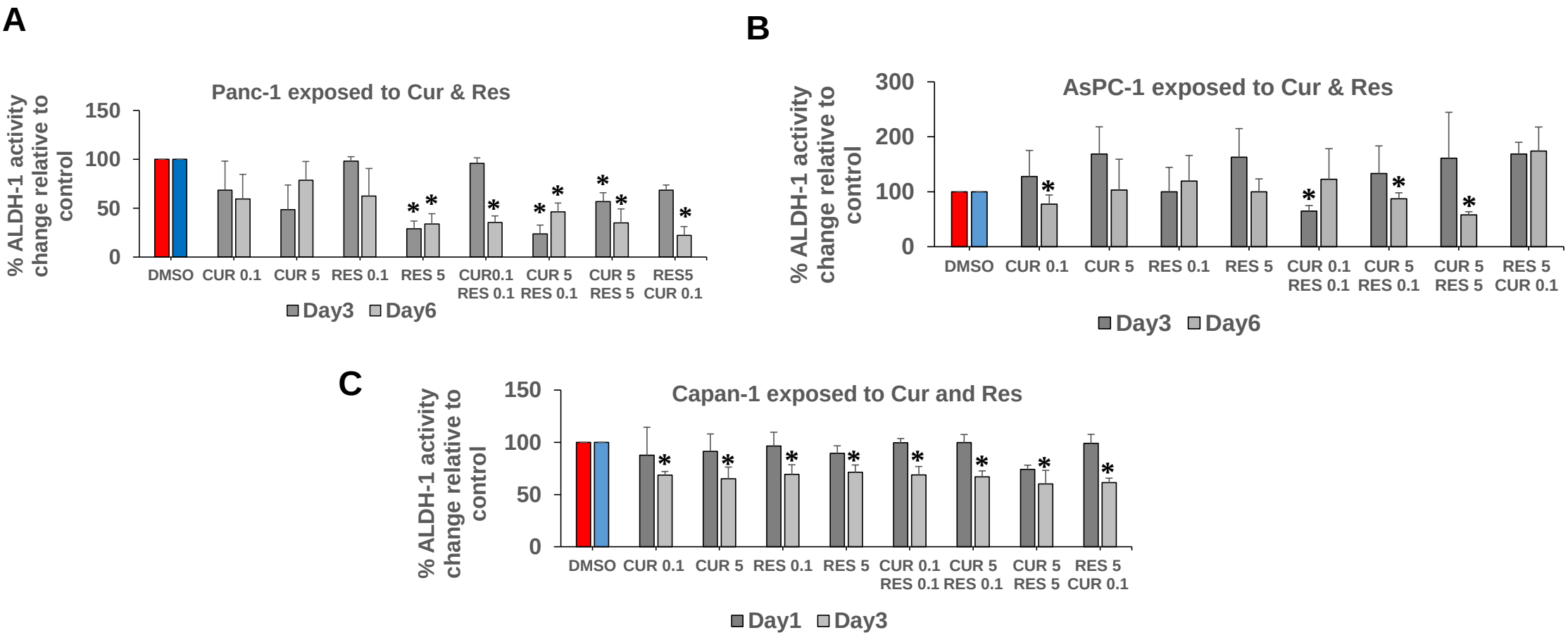


Figure 4.6 The effect of curcumin and resveratrol alone and in combination on the proportion of cells with high ALDH-1 activity in the Panc-1, AsPC-1 and Capan-1 cell lines. The charts show the relative proportion of cells with high ALDH-1 activity following a 3 and 6 day exposure (Panc-1, AsPc-1) or 1 and 3 day exposure (Capan-1) to resveratrol and curcumin, compared to the DMSO solvent control. **(A)** Panc-1 cells. **(B)** AsPC-1 cells. **(C)** Capan-1 cells. Data represent the average (+SEM) of three experiments, each performed in triplicate wells. Significant differences relative to the control are indicated, where * = p≤0.05. Statistical analysis was performed using a Student's T-test.

4.5. Combination effects of curcumin and resveratrol on sphere size and number in Capan-1, AsPC-1 and Panc-1 cells

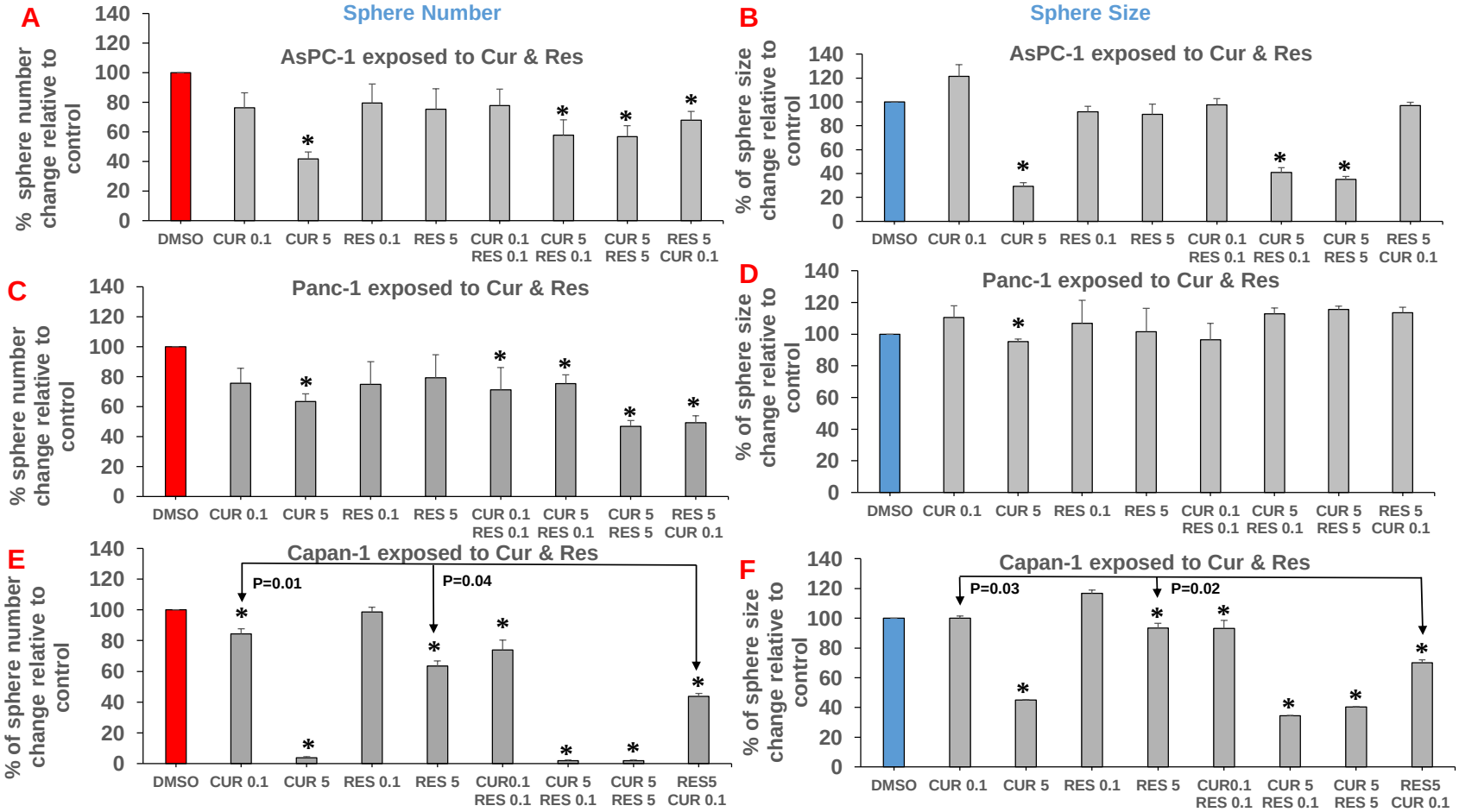
Use of the spheroid model, which enriches for CSCs, provides a more accurate method by which to study the activity of test compounds on CSCs. To determine the effect of curcumin and resveratrol combinations on Panc-1, AsPC-1 and Capan-1 cells, they were exposed to curcumin and resveratrol either alone or in combination for 2 weeks, with repeated administration of the compounds every 3-4 days, without replacing the media; it was not possible to perform daily dosing analogous to that conducted with the 2D-cultures because this would disrupt spheroid formation as they are non-adherent. The capability of these cell lines to form spheres, in terms of increasing numbers, follows the rank order Capan-1>Panc-1> AsPC-1 but for sphere size Panc-1> Capan-1> AsPC-1 (Table 4.1). AsPC-1 sphere number was significantly decreased by almost 60% following exposure to 5 µM Curcumin (4.7A). The significant reduction was maintained when cells were exposed to mixtures containing 5 µM curcumin plus 0.1 µM resveratrol (42.3±10%), and 5 µM curcumin plus 5 µM resveratrol (43.1±7.2%), but the effect was not enhanced by the addition of resveratrol over that achieved with curcumin alone. When AsPC-1 cells were exposed to 5 µM resveratrol plus 0.1 µM curcumin, there was a 32.1±5.8% reduction in sphere number, which was greater than for either agent alone at these same concentrations but not significantly. The average AsPC-1 sphere size was also significantly decreased by ~70% when exposed to 5 µM curcumin. This decrease in sphere size was maintained but not enhanced in the presence of resveratrol.

Exposure of Panc-1 cells to 5 µM curcumin resulted in a 37.5±9.5% reduction in the number of spheres (Figure 4.7 C). Reductions were also observed following exposure to 5 µM curcumin plus 0.1 µM resveratrol (~25%), 5 µM curcumin combined with 5 µM resveratrol (~53%) and to 5 µM resveratrol plus 0.1 µM curcumin (~51%). Interestingly, it was noted that at clinically achievable concentrations, where 0.1 µM curcumin and 0.1 µM resveratrol were combined,

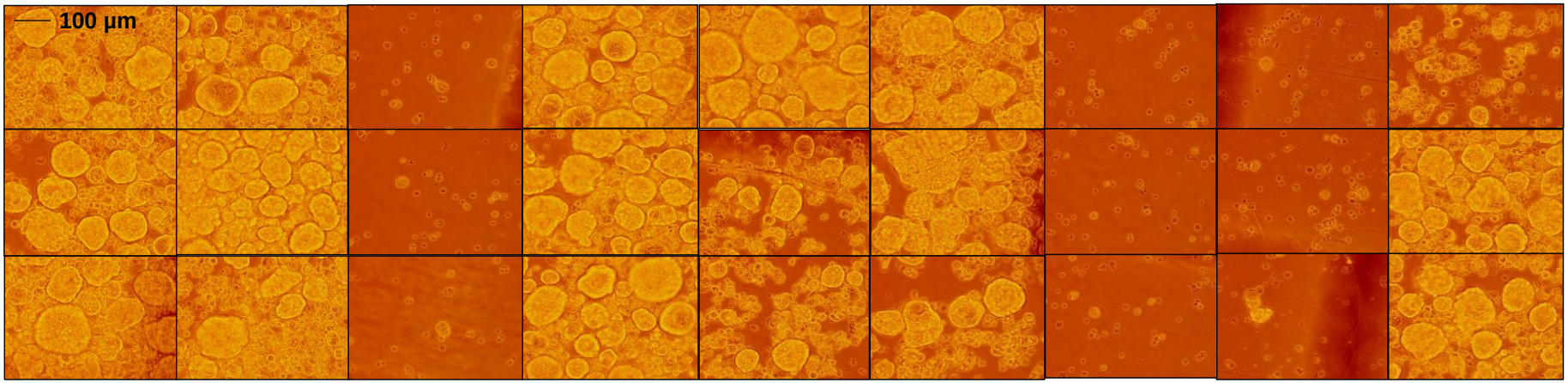
a significant reduction in sphere number occurred (27±4.6%). None of the treatments significantly affected sphere size, except for 5 µM curcumin (5.4%±5 reduction).

In Capan-1 cells, treatment with 5 µM curcumin resulted in a large 96.2±3.1% reduction in the number of spheres, and consequently, no additive effects of the combinations with resveratrol could be observed, although addition of resveratrol did not abrogate this effect. Curcumin at 0.1 µM resulted in a much smaller 15.5±3.1% reduction in sphere number, and the higher concentration of resveratrol (5 µM) was also effective, causing a significant 36.5±3.3% decrease. When Capan-1 cells were exposed to the mixture of 0.1 µM curcumin plus 0.1 µM resveratrol, there was a significant ~26% reduction in sphere number, but this was not significantly different compared to either agent alone at these concentrations. However, when exposed to 5 µM resveratrol in combination with 0.1 µM curcumin there was a 56.3±1.9% reduction in sphere number, and this time the decrease was significantly different compared to either agent alone; this means adding curcumin could be beneficial to resveratrol at lower concentrations. Representative examples of the sphere images observed in this experiment are shown for Capan-1 cells in Figure 4.8.

A significant difference in sphere size was noted when Capan-1 was exposed to 5 µM curcumin which resulted in a 55.05%±2 reduction in the size of spheres. When resveratrol was applied at 5 µM resveratrol, sphere sizes decreased significantly by 6.7%±1.36. When exposed to 5 µM curcumin 0.1 µM resveratrol there was a 65.6%±1.8 reduction in sphere size. When exposed to 5 µM curcumin 5 µM resveratrol there was a 60%±1.7 reduction in the size of spheres (Figure 4.8 A). Again, an additive effect was observed when Capan-1 were exposed to 5 µM resveratrol 0.1 µM curcumin, resulting in a 30.1%±1.94 reduction in sphere sizes compared to 6.7% by resveratrol and 0% by curcumin for either compound alone (Figure 4.8 B) which significantly different from either compound alone.



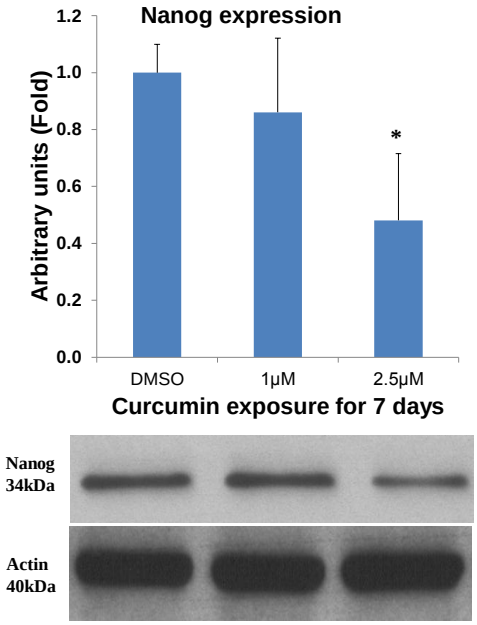
4.7 The effect of curcumin and resveratrol alone and in combination on sphere numbers and size over a period of 2 weeks in AsPC-1, Panc-1 and Capan-1 cells. (A) Sphere numbers and **(B)** sphere size in AspC-1 cells; **(C)** sphere number and **(D)** sphere size in Panc-1 cells; **(E)** sphere numbers and **(F)** sphere size in Capan-1 cells following treatments with curcumin/resveratrol alone and in combination at the concentrations indicated, over a period of 2 weeks. The charts show the number of spheres and sphere size relative to the DMSO solvent control, which is set at 100%. Data represent the average of three experiments, each performed in triplicate wells. Significant differences relative to the control are indicated, where * = p≤0.05. Statistical analysis was performed using a Student's T-test. Comparison between treatments carried out and significant indicated by P values on E and F.



4.8 Sample light microscopy images showing the effect of exposure to curcumin and resveratrol alone and in combination on the number of spheres formed by Capan-1 cells. Capan-1 cells were exposed to curcumin/resveratrol at the concentrations indicated (µM) for 2 weeks (20X objective). After the treatment period, spheres were counted and their sizes measured using a an inverted light microscope (Nikon EclipseTE2000U) at 20X optical zoom. The sphere size was determined using Eclipse software that measured an average diameter from two measurements for each sphere. The scale bar is 100 µm.

4.6. Effect of curcumin exposure on Nanog expression in Capan-1 cells

The aim of the experiment is to examine whether curcumin may be affecting stem cell growth via effects on Nanog protein expression which is an embryonic stem cell transcription factor that regulates the self-renewal of stem cells, as this has been observed by other members of our lab group in colorectal cancer stem cells (A. Karmokar, unpublished data). Adherent cultures of Capan-1 cells were exposed to 1 µM and 2.5 µM curcumin with repeat dose on a daily basis for 7 days. Cells then were harvested and the expression of Nanog was determined in the population with high ALDH-1 activity (ALDH-1^{High} cells) by Western blot. When Capan-1 cells were exposed to 1 µM curcumin there was no significant difference in Nanog expression compared to the DMSO solvent control in the ALDH-1^{High} population, but at 2.5 µM curcumin, Nanog expression was reduced significantly by 48±23 % compared to the control (DMSO) (Figure 4.9).

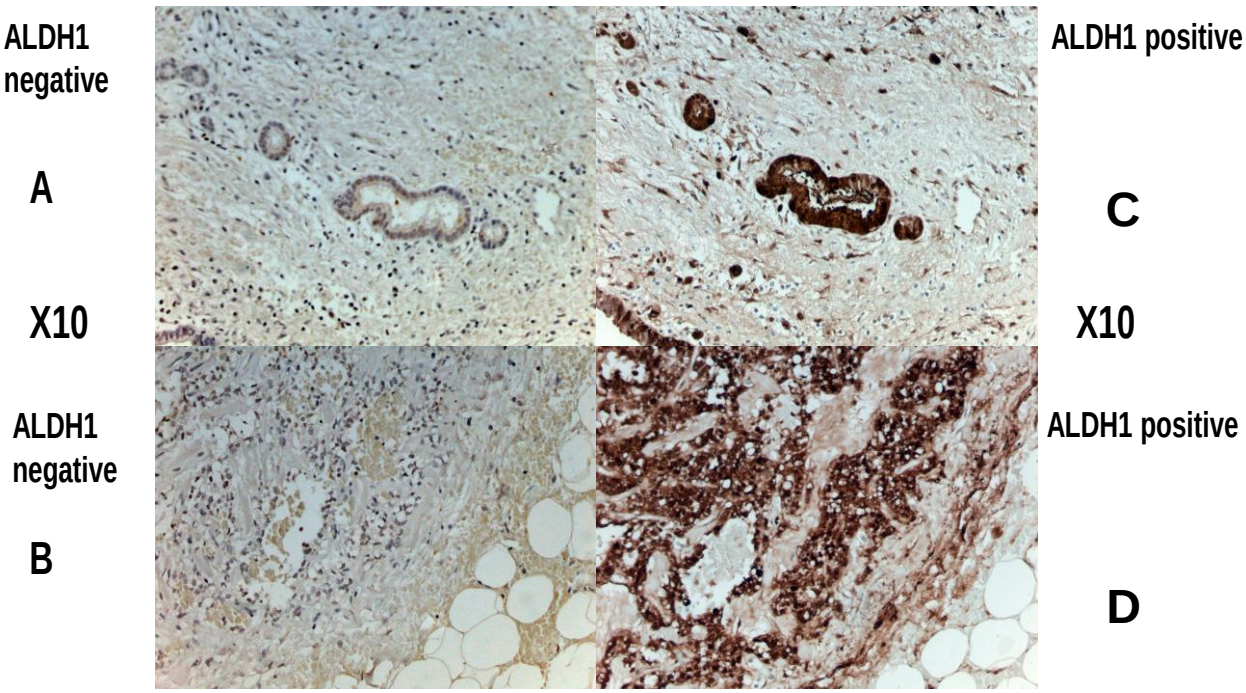


4.9 Effect of curcumin on the expression of the stem cell protein Nanog in the ALDH^{high} population of Capan-1 cells. The level of Nanog was determined in curcumin exposed and control, untreated cells (DMSO). Data represent the average (+SEM) of three independent experiments. All bands are normalised to Actin and expressed as fold change to control DMSO. Significant differences relative to the solvent control are shown, where * indicates $p<0.05$. Statistical analysis was performed using T-test.

4.7. CSC profiles of primary pancreatic cancer tissue obtained from patients

The next aim of the project was to establish CSC profiles in tissue samples from patients who had undergone pancreatectomy following a suspected cancer diagnosis. Previous experiments have profiled only certain markers of pancreatic cancer stem-like cell markers (91, 107, 113, 115). This is first time that we report an extensive CSC marker profile in primary pancreatic cancer cells. Single cell suspensions from each sample were obtained and stained for $CD24^+$, $CD44^+$ and $CD133^+$ expression and ALDH-1^{high} activity and then gated appropriately for the various combinations. In addition, the epithelial cell surface marker (EpCAM) was included in order to differentiate the CSC population arising from the epithelial-derived cancer cells, or those derived from the mesenchyme. When the patient samples were gated to separate the single cell suspension into an epithelial population using EpCAM, a very heterogeneous population was observed, which differed greatly between samples (Table 4.2). Only 8 of 12 samples expressed high levels of $CD133^+$, with high levels of $CD24^+/CD44^+$ co-expression being rare and 8 out of 12 expressed $CD44^+$, $CD24^+$ individually. It was not possible to observe any ALDH ^{high} activity in many samples using FACS analyser for sorting, possibly due to poor sample integrity as ALDH ^{high} activity can only be detected in live cells and all the samples were frozen and stored prior to analysis in batches. In order to try and establish whether ALDH was expressed in these samples, immunohistochemistry was used as an alternative method. After staining all samples were analysed and strong staining for ALDH-1 was detected in 5 out of 9 patient samples (Table 4.2, Figure 4.10). P060 contained only 0.4% stained epithelial cells because it was not a cancer sample. However, it might contain a lot of other cell types or necrotic cells thus it was analysed to observe whether it could be a potential precursor of pancreatic cancer that contains premalignant stem cells. Overall from patient samples after staining for stem cell markers, it was possible to conclude that tumours had $CD24^+$ and $CD24^+/CD44^+$ expression, further investigations are required to define these cancer stem cell markers in pancreatic cancers. FACS data reveal that 8/12 patient samples had

CD133^{+} expression (Table 4.2), which ranged from 1 to 43% of the EpCAM^{+} population.



4.10 Representative images of patient samples stained for ALDH-1 expression and assessed by immunohistochemistry. **(A)** Negative staining (PBS control), **(C)** Positive staining for ALDH-1 in a similar area of the same section of patient sample P059 **(B)** Negative staining (PBS control). **(D)** Positive staining for ALDH-1 in a similar area of the same section of patient sample P060. Pictures were taken by light microscopy (LeitZ V2.8.8) with an X10 objective lens.

Table 4.2 Pancreatic cancer samples from patients analysed for potential CSC markers using FACS and immunohistochemistry. Expression levels for $CD24^+$, $CD44^+$, ($CD24^+/CD44^+$) and $CD133^+$ are shown as percentage of the total EpCAM population, which were correlated to the position of the tumours in pancreas. ALDH expression was assessed by immunohistochemistry and a score has been given (+, ++, +++), to illustrate the strength of expression compared to the background level of staining in normal, non-cancer tissue. Note: x = patient was not analysed. 6 patients were scored for the IHC.

Patient Number	Diagnosis	EpCAM	EpCAM /CD24	EpCAM /CD44	EpCAM /24/44	EpCAM /CD133	ALDH-1/ immuno
P003	Adenocarcinoma	8.8	1.4	24	1.2	0	x
P010	Ductal adenocarcinoma	86.3	53.4	1.3	0.9	32.8	x
P047	Ampullary carcinoma	52.6	0.2	31.7	0.4	0	+
P051	Cancer Pancreas	83.3	34.7	1.1	0.4	43.7	+++
P059	Adenocarcinoma	13.9	5	3.5	3.5	2	+++
P060	Mucinous cyst or inflammatory	0.4	0	0	0	0	x
P090	Pseudopapillary pancreatic tumour	3.8	1.7	79.3	6.1	0	+++
P099	Adenocarcinoma in head of pancreas	68.5	0	0	8.5	0.2	x
P123	Carcinoma Pancreas	18.8	19	29	7.5	3	+++
P124	Ductal adenocarcinoma	21.4	2.4	29.5	1.1	2	+++
P151	Ampullary carcinoma	81.9	x	x	11.6	0.7	x
P170	Ampullary carcinoma	77	x	x	1.7	0.1	x

4.8. Discussion

According to the CSC hypothesis, CSCs have generally been shown to constitute only a small fraction of the cells within a tumour but provide the driving force behind malignancy (87-89, 91). It is now accepted that the majority of tumours have a small subpopulation of cells with stem cell characteristics. Tumours that contain a higher burden of CSC are associated with higher rates of metastasis, poor patient prognosis and increased resistance to chemoradiotherapeutic agents (92, 95, 103, 109, 212) which makes it possible that this small population of cells is also behind the re-occurrence of tumours. The CSC model is expanding from its original definition of a small and distinct subpopulation, and it has been hypothesised that the CSCs may encompass more common and heterogeneous cells. Additionally, CSCs are now thought to be dynamic and reversible entities in cancer, governed by the tumour microenvironment (92, 107, 295).The potential role of pancreatic cancer stem cells in the initiation, progression and recurrence of pancreatic cancer has been explored (91, 113) and identified by surface marker expression profiles of CD44+CD24+ESA+ and $CD133^{+}CXCR4^{+}$. Lately, additional surface markers were suggested, such as ALDH-1^{High} and $CD24^{+}/CD44^{+}$ populations (107, 298) and $CD44^{+}$ c-Met^{+} populations were also deemed highly metastatic (106, 108, 109, 118). Still there is no consensus on which population actually represents the PCSC population. Therefore, in this project the decision was made to investigate three different populations $CD24^{+}/CD44^{+}$ and $CD133^{+}$ using surface markers and ALDH-1^{High} as a marker of internal cellular activity, in the cell lines of interest. In addition to these markers, the functional activity of PCSCs was assessed in terms of their ability to form spheres; a sphere formation assay was used to further identify a sub-population of cells within pancreatic cancer cells that have characteristics of stem cells (114, 115, 118). The expression level of the embryonic transcription factor Nanog was also considered, since its overexpression can correlate with transformation from pre-malignant to malignant conditions, poorly differentiated tumours, recurrence, metastasis, and poor prognosis in various cancers as well as pancreatic cancer (119, 120, 128). It has previously been reported that in cell lines, the side population contains cells that are triple positive for Sox2, Nanog and Oct4, and

these markers correlate with aggressiveness, invasive ability, migratory ability and high resistance to drugs (119-121, 126, 128). There is currently no certain hierarchical structure for PCSCs to develop into mature tumours. A model has been suggested which could be summarized under four headings: the first one would be a linear organization of CSCs, in which a single specific CSC is capable of generating various CSC populations, systematically leading to heterogeneity within a tumour. The second model could mathematically be called a 'one-to-one function', whereby each CSC has its own characteristics, and gives rise to only one mature tumour cell. The third functional model would represent the 'onto' function, where plasticity among different stem cell populations exists and gives rise to mature tumour cells. Another model could be termed a 'one-to-many' relationship, in which one CSC population gives rise to different progeny and each progeny will result in a mature tumour (295). Another layer of complexity is added with the suggestion that some populations of CSCs play a role in initiation while others promote maintenance and chemoresistance. Alternatively, a single population could be responsible for all three functions. Due to the apparent heterogeneity, even within the small CSC subset in pancreatic cancer, an ideal approach would be to target all populations rather than single population within PCSC populations (103, 299).

It is essential to consider all aspects of tumour heterogeneity in order to assess better therapies for pancreatic cancer, and thus far, targeting PCSCs is lagging behind in drug development for pancreatic cancer.

Over the past several years, an incredible amount of effort has been directed to the development of new drugs and therapeutic strategies that specifically target CSCs, with many agents now under evaluation in preclinical and clinical studies (95). Curcumin and resveratrol have already been reported to show significant effects on CSC populations in various cancers (see Table 1.9 and Section 1.10.2) in preclinical models; however, to my knowledge, the combination of these two potential chemopreventive/therapeutic agents has not yet been investigated in PCSC populations. The results described in this chapter demonstrate that both curcumin and resveratrol with their plethora of effects individually and in combination, are able to differentially affect the stem like cell populations in

different pancreatic cancer cell lines. The PCSC heterogeneity was also observed when analysis was undertaken of primary tissue samples from pancreatic cancer patients. The three cell lines were shown to have different sphere forming abilities, decreasing in the rank order Capan1≥Panc-1≥AsPC-1 (Table 4.1). Investigations were conducted to examine the effect of curcumin and resveratrol on the cell lines in terms of effects on sphere number and size reduction. Sphere formation by Capan-1 cells was very sensitive to curcumin and moderately sensitive to resveratrol, whereas Panc-1 cells showed slightly resistance to resveratrol exposure but were sensitive to curcumin (Section 4.1). It has been reported (256) that resveratrol causes sphere reduction in breast cancer cell lines but the concentration required for activity was 10-times higher (50 µM) than the maximum concentration employed in the present study (5 µM). Additionally, resveratrol has previously been found to inhibit the self-renewal ability of PCSCs (identified by the markers $CD133^+$,$CD44^+$, $CD24^+$ ESA^+) obtained from human primary tumours and KrasG12D mice *in vitro,* with significant activity first becoming evident at a concentration of 10 µM (254). In that study resveratrol decreased the formation of primary and secondary spheres from PCSCs obtained from KrasG12D mice. The expression of ABCG2, a multidrug resistance gene which had been shown to be overexpressed in PCSCs, was also inhibited by resveratrol. Nanog and other transcription factors involved in the maintenance of pluripotency, including Sox2 and Oct4, were down-regulated by resveratrol in $Kras^{G12D}$ Transgenic Mice (254, 300).

The different CSC populations in Panc-1 cells had very different responses to curcumin and resveratrol, when exposed to each compound individually. The population identified as being double positive for $CD24^+/CD44^+$ decreased significantly when incubated with 5 µM curcumin, but resveratrol had no significant effect, at any concentration, even though cells were treated for 6 days on a daily basis. However, resveratrol significantly reduced the fraction of cells with $ALDH\text{-}1^{High}$ activity while, curcumin did not have a clear effect. This observation provides a good indication that using a single compound will not eliminate all sources of tumourigenic populations, as the literature suggests that both $CD24^+/CD44^+$ and $ALDH\text{-}1^{High}$ populations are highly tumourigenic. Therefore, it was considered rational to assess the activity of combinations of the

two compounds on these two populations and their effects on sphere formation for the three cell lines.

When Capan-1 cells were exposed to the combination treatments, there was an increase in in $CD24^{+}/CD44^{+}$ population and concurrent decrease in ALDH-1 activity. This might suggest that enrichment of the $CD24^{+}/CD44^{+}$ population is a direct result of targeting the $ALDH^{High}$ population. The effect on the $ALDH^{High}$ population was observed when curcumin and resveratrol were combined at clinically achievable concentrations.

In contrast to the Capan-1 cells, in Panc-1 the combination treatments did not significantly affect the $CD24^{+}/CD44^{+}$ population. However, the ALDH activity was significantly reduced by the combination treatments at a clinically achievable dose. In AsPC-1 cells, the low levels of $CD24^{+}/CD44^{+}$ and $ALDH\text{-}1^{High}$ populations precluded any significant observation being made following the combination treatments. Sphere forming capacity of Panc-1 cells was reduced significantly after combination treatment at clinically achievable concentrations of resveratrol and curcumin.

In Panc-1 cells; the first one at minimum combination brought about a good result which was clinically achievable for the first time and the second one was when the lower concentration of curcumin added for resveratrol. Capan-1 cells formed the most well defined spheres and had the highest formation capability. These spheres were the most sensitive to both the single and combination treatments. AsPC-1 did not form a true spheres hence no real effect of treatments were observed in term of sphere number and sizes.

To investigate how curcumin might be targeting PCSCs, the most sensitive cell line (Capan-1) was exposed to curcumin and the $ALDH\text{-}1^{High}$ population isolated. Western blot analysis revealed that curcumin caused a significant reduction of Nanog expression in this stem cell population, which was consistent with Shankar (254) except our result was clinically achievable. Unpublished work conducted in this laboratory by Dr A. Karmokar, has found similar results in colorectal cancer stem cell models, whereby clinically achievable concentrations of curcumin (~0.1

μM) selectively targets the ALDH-1^{High} population and activity correlates with significant down regulation of Nanog expression, specifically in these cells.

To sum up, various PCSC populations have been identified based on cell surface markers, intracellular enzyme activity and sphere formation *in vitro,* but still, a single CSC population cannot be defined. Therefore, targeting multiple CSC populations within pancreatic cancer using compounds such as curcumin and resveratrol is a sensible and plausible strategy for cancer prevention or as a combined adjuvant in the treatment setting. The results of this chapter have demonstrated that different cell lines have different stem cell populations and varying sensitivities to both compounds individually. The use of combinations was shown in some cases to be capable of targeting PCSCs at the lower, more clinically achievable concentrations. In addition, current drug therapies such as gemcitabine spare these CSC populations, which may then theoretically be eliminated by combinations of curcumin plus resveratrol. These combinations warrant further assessment in pre-clinical models, to determine whether the *in vitro* activity observed translates to *in vivo* models and primary human cultures and to investigate the mechanisms of action, with Nanog as an initial target of interest.

Chapter Five: *In vitro* activity of curcumin metabolites, their cellular uptake and metabolism

5.1. Introduction

Curcumin has been shown to have a plethora of beneficial effects on physiopathological processes, not only in cancer but a variety of other diseases (174, 177-186, 197, 198). It has been the focus of extensive *in vitro*, *in vivo* and pre-clinical/clinical trial investigation over the last 10 years regarding its potential as a cancer chemopreventive agent. Curcumin has poor bioavailability and undergoes rapid metabolism (217). After an oral dose the majority of curcumin ingested is excreted unchanged, and the remaining is biotransformed to produce predominantly glucuronide and sulfate metabolites (198, 223, 301). In a study carried out by Ireson *et al*. (222) humans received 3.6g of curcumin daily for 4 months and curcumin glucuronide and sulfate conjugates were detected in the plasma. Similarly, in two studies carried out in humans when curcumin was given at 2.35 g and 8 g daily dose, curcumin glucuronides and sulfates were the main species detected in taken biopsies and plasma, respectively (197, 225). This suggests that the metabolites may be responsible for some of the chemopreventive effects attributed to curcumin. While there are studies indicating that the metabolites are less active than curcumin (179, 222, 225, 266, 302), there are also studies which come to the opposite conclusion (226, 303, 304), particularly, in regard to tetrahydrocurcumin (THC). Curcumin metabolites could themselves have useful chemopreventive characteristics which need to be explored, particularly in pancreatic cancer cell lines.

In this chapter, the focus was firstly to investigate the growth inhibitory effects of curcumin mono-sulfate in Capan-1 and Panc-1 cell lines, under adherent and sphere forming culture conditions. Secondly, cellular uptake and metabolism of curcumin and its conjugates were investigated in these cells.

5.2. Effect of curcumin mono-sulfate on cell proliferation

5.2.1. Effects of low concentrations

Repeated daily exposure of Panc-1 cells to curcumin mono-sulfate for up to 6 days failed to cause any significant growth inhibition at concentrations ranging from 0.01-5 µM (Figure 5.1A). Significant growth inhibition was only observed in the Capan-1 cells following 6 days exposure to curcumin mono-sulfate at the highest concentration of 5 µM, which caused a small (10.5±4%) reduction in cell numbers (Figure 5.1B).

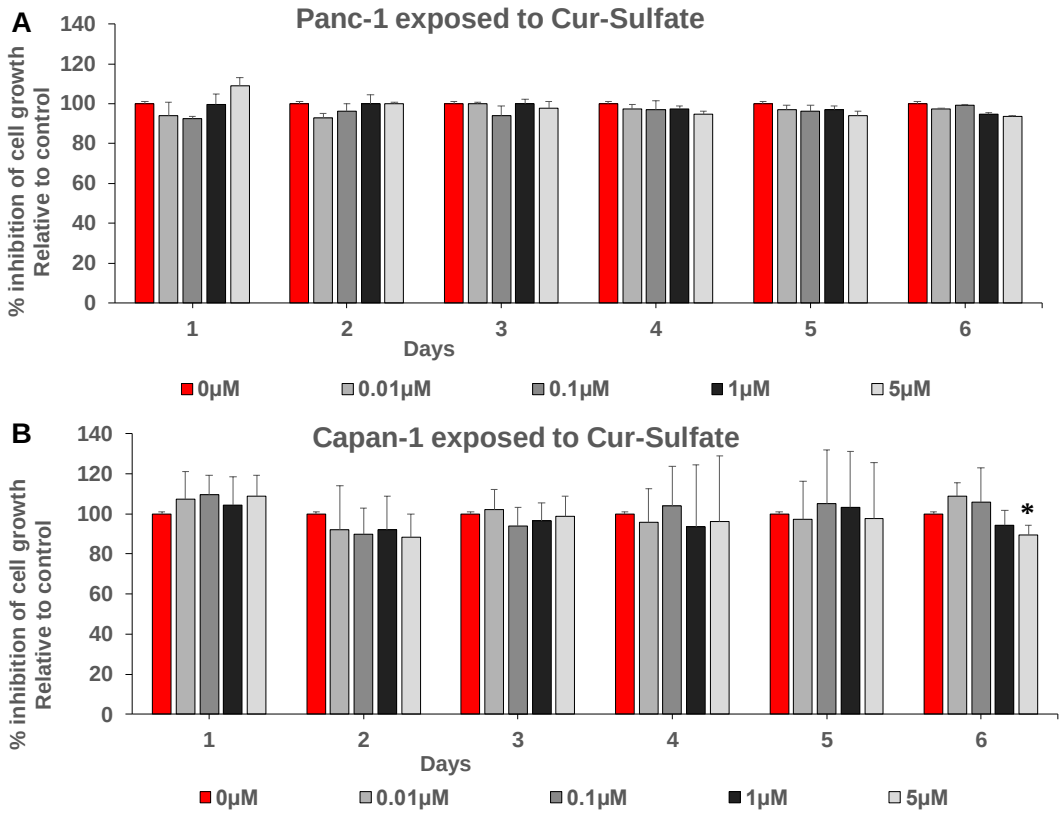


Figure 5.1 Effect of curcumin mono-sulfate (low concentrations) on the proliferation of Panc-1 and Capan-1 cells over 6 days, with repeated daily exposure. (A) Panc-1 and **(B)** Capan-1 cells were exposed to 0, 0.01, 0.1, 1 and 5 µM of curcumin mono-sulfate for 6 days with daily dosing. Data are expressed relative to the DMSO solvent control, which is set at 100% and represent the average +SEM of three independent experiments, each performed in triplicate. Significant differences compared to the control were determined using a student's T-test and are indicated, where * = $p \leq 0.05$.

5.2.2. Effects of high concentrations

As the low concentrations were largely ineffective, the cell lines were exposed to higher concentrations to determine whether they exhibited any sensitivity to the curcumin mono-sulfate. Significant growth inhibition of 27±6% was observed in the Panc-1 cells at day 2 with 100 μM, and the percentage inhibition increased to 54±3% by day 6 (Figure 5.2A). In contrast, the lower concentration had no effect in this cell line. Significant growth inhibition was also observed in Capan-1 cells from day 2 for both concentrations; maximum inhibition occurred at day 6, with a reduction of 76±4% at 100 μM (Figure 5.2B).

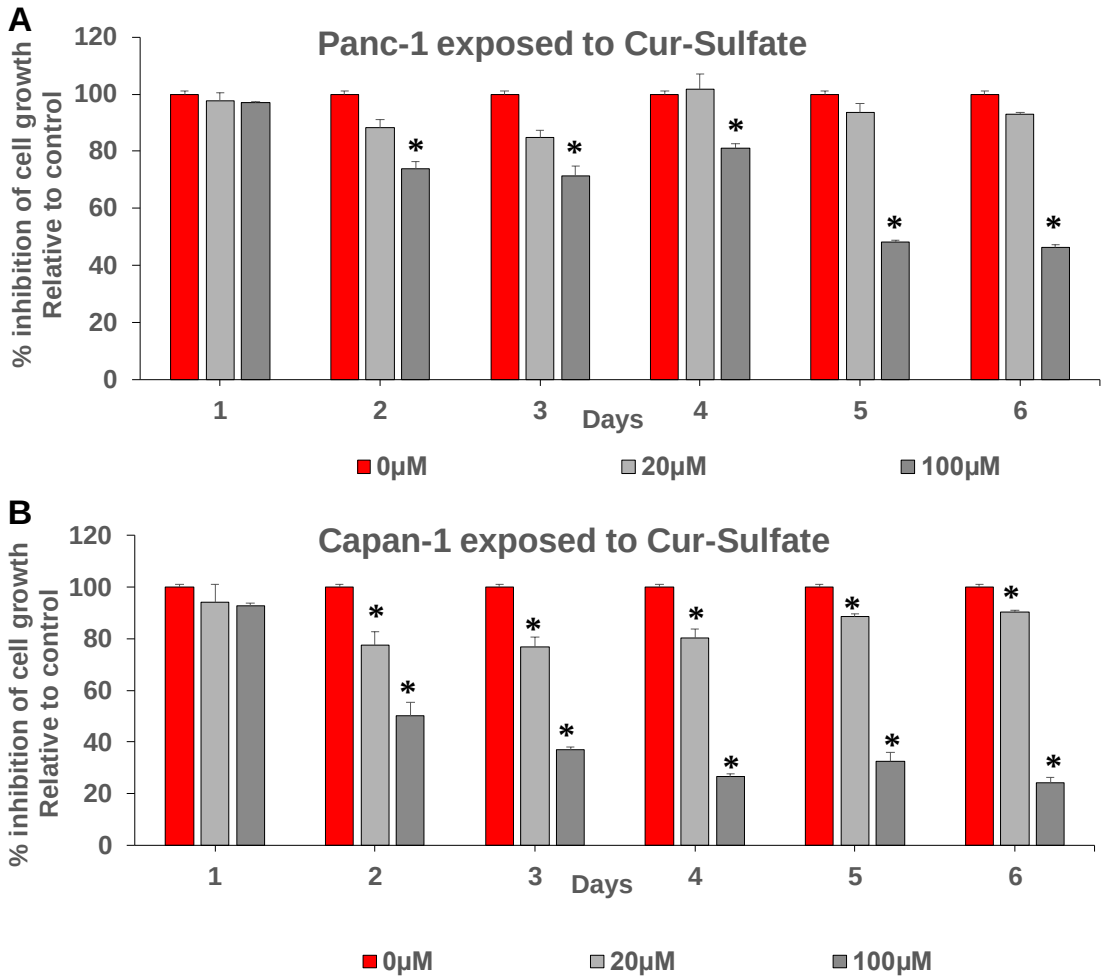


Figure 5.2 Effect of curcumin mono-sulfate (high concentration) on the proliferation of Panc-1 and Capan-1 cells over 6 days. (A) Panc-1 and **(B)** Capan-1 cells were treated with 20 and 100 μM curcumin mono-sulfate for 6 days with repeated daily dosing. Data are expressed relative to the DMSO solvent control, which is set at 100% and represent the average +SEM of three independent experiments, each performed in triplicate, Significant differences compared to the control were determined using a student's T-test and are indicated, where * = $p \leq 0.05$.

5.2.3. Effect of curcumin mono-sulfate on sphere growth in Capan-1 and Panc-1 cells

To determine whether curcumin mono-sulfate can affect sphere growth or number, single cell suspensions of Panc-1 and Capan-1 cells were plated in ultra-low attachment plates under non-adherent conditions and treated with concentrations of 20 or 100 μM every 3 days for a period of 2 weeks. The number of spheres formed by Panc-1 cells decreased significantly in the presence of 100 μM curcumin mono-sulfate, by 60±30%, but there was no effect at the lower

exposure. Additionally, neither treatment had a significant effect on sphere size (Figure 5.3A, B & E). Capan-1 sphere number and size was significantly reduced at both concentrations (Figure 5.3). At 20 µM, sphere number and size was reduced by 45±15% and 37±12%, respectively. No spheres were observed following 100 µM exposure (Figure 5.3C-E).

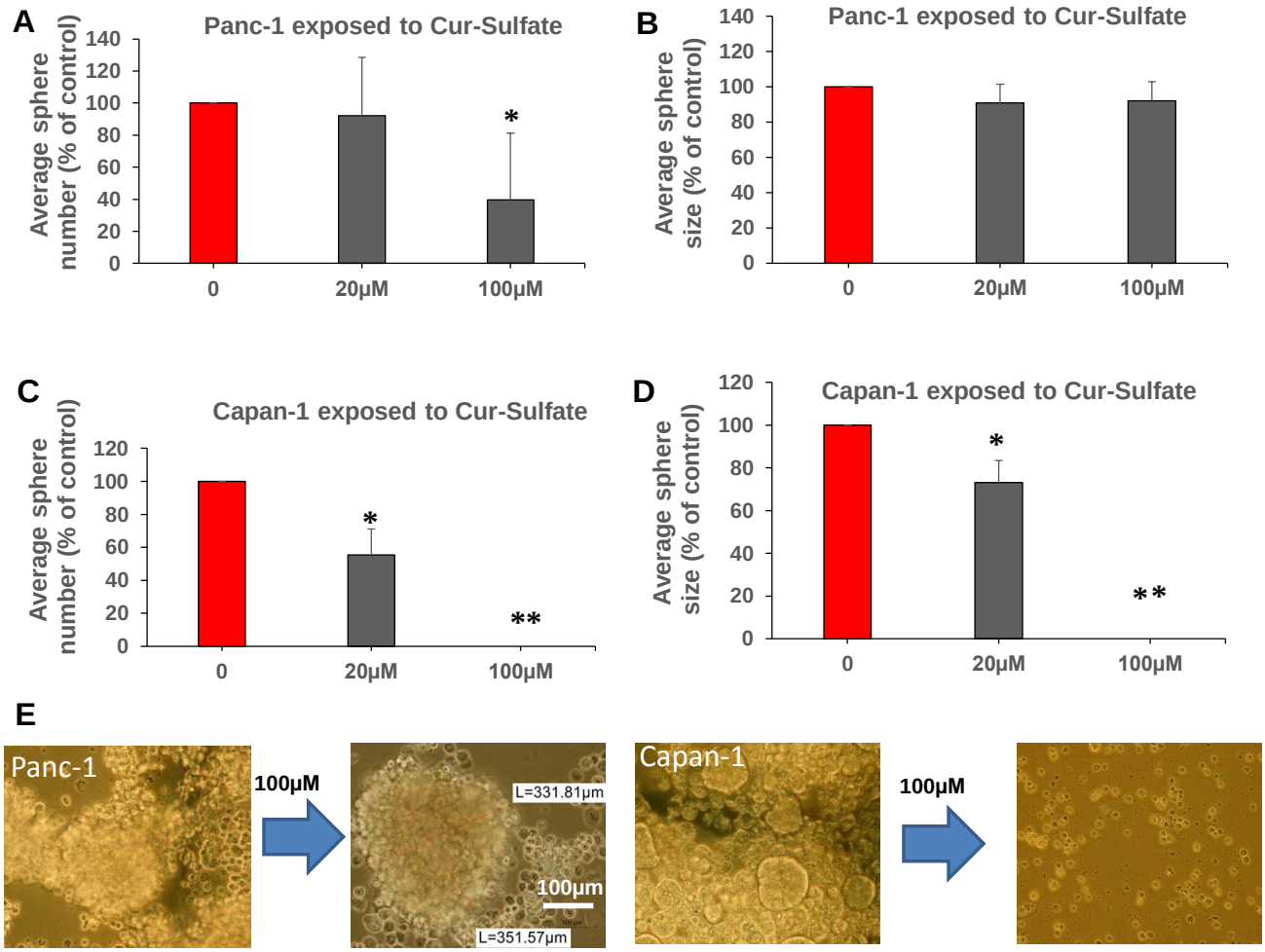


Figure 5.3. Effects of curcumin mono-sulfate on Panc-1 (A&B) and Capan-1 (C&D) sphere growth. Cells were treated with 20 or 100 µM curcumin mono-sulfate for two weeks, with fresh addition of the compound every three days. Data are expressed relative to the DMSO solvent control, which is set at 100% and represent the average +SEM of three independent experiments, each performed in triplicate. Significant differences compared to the control were determined using a student's T-test and are shown, where * = p≤0.05 and **= p≤0.0001 **(E)** Shows representative images obtained by microscopy of Capan-1 and Panc-1 cells exposed to 100 µM curcumin sulfate for 2 weeks (20X objective). Scale bar= 100 µm.

Unfortunately, it was not possible to test the activity of curcumin mono-glucuronide in these cell lines. This is because it is not commercially available, and is difficult to synthesize in high yields to generate the relatively large quantities required for cell treatments. It has been reported by Pal *et al*. (266) that curcumin mono-glucuronide as well as the di-glucuronide do not suppress the proliferation in these cell lines: U266 (multiple myeloma), A549 (lung adenocarcinoma), and Jurkat (human T cell leukemia).

5.3. Cellular uptake and metabolism of curcumin by pancreatic cancer cell lines

After establishing that there was a difference in sensitivity to the curcumin sulfate metabolite, it was important to determine why this differential may exist between the cell lines. To this end, the uptake and metabolism of curcuminoids in both the Panc-1 and Capan-1 cell lines was investigated.

5.3.1. Determination of retention times, limit of detection and limit of quantitation for curcumin and curcumin metabolites

Figure 5.4 shows chromatograms generated by analysis of a mixture of increasing concentrations of curcumin, curcumin mono-sulfate and curcumin mono-glucuronide synthetic standards. Retention times were 15.77 min for curcumin mono-glucuronide, 20.1 min for the mono-sulfate and 23.53 min for curcumin, which is consistent with the greater hydropholicity of the conjugates compared to the parent. Standard curves were produced for each curcumin conjugate and the parent, and used to calculate the Limit of Detection (LOD). The LOD was approximately 5 ng for all three compounds (mono-glucuronide, mono-sulfate and curcuminoids) (Figure 5.4). The Limit of Quantification (LOQ) was approximately 20 ng for all three compounds (Figure 5.4) based on a signal/noise ratio of 3 to 1. Linearity was calculated for the mono-glucuronide, mono-sulfate and curcumin using calibration curves (R^2 = 0.9948, R^2 = 0.9986

and R^2 = 0.9999, respectively) (Appendix 7.10). The percentage recovery for mono-glucuronide, mono-sulfate and curcumin was 59%, 70% and 77%, respectively.

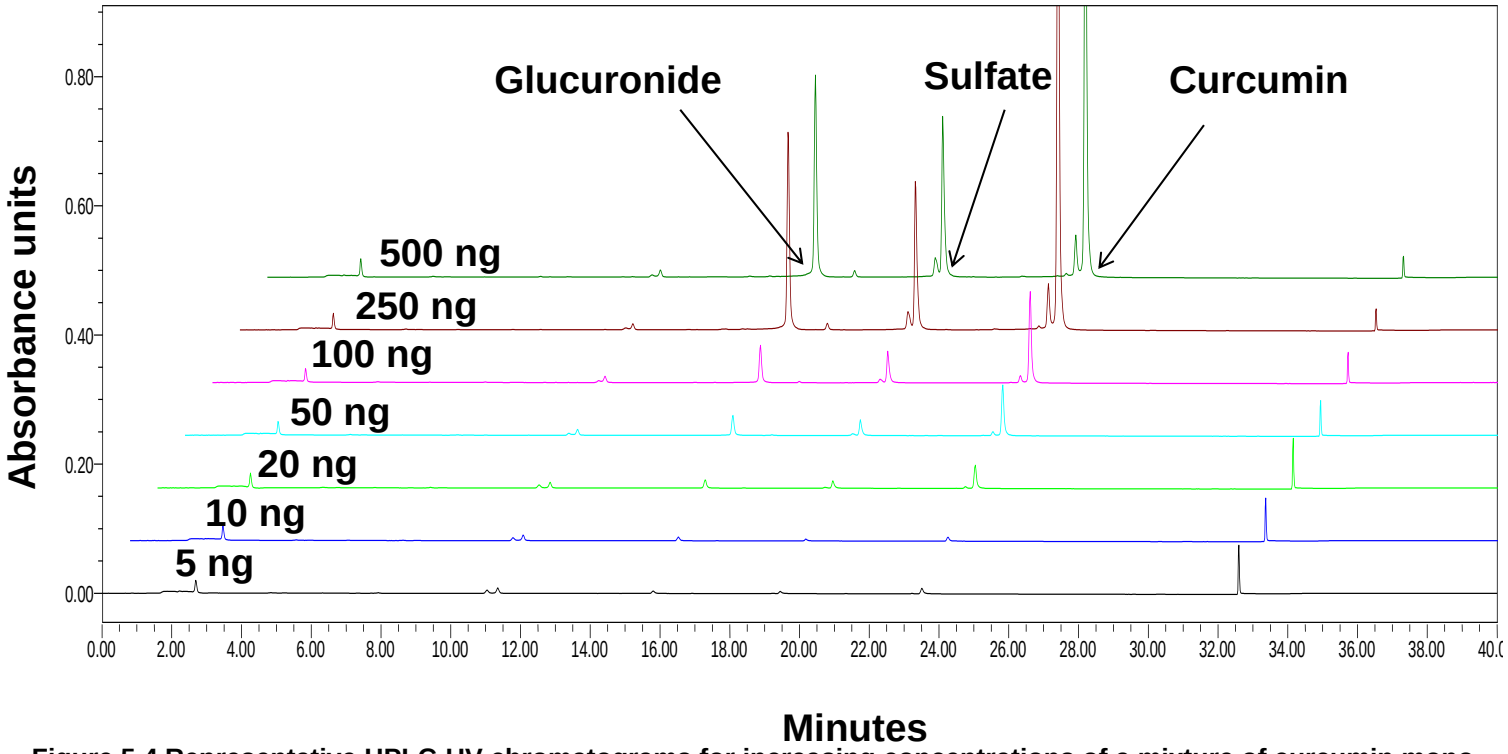


Figure 5.4 Representative HPLC-UV chromatograms for increasing concentrations of a mixture of curcumin mono-glucuronide, mono-sulfate and curcumin. To an aliquot of 100 µL of cell homogenate 1 µL of curcumin mono-glucuronide, mono-sulfate and curcumin stock solution was added to give concentration ranging from 10 ng - 1000 ng/100 µL of cell homogenate. After extraction and drying the samples were re-suspended in 100 µL of mobile phase and 50 µL of this solution was injected onto column giving calibration curve ranging from 5 ng -500 ng on column. Injection of DMSO solvent alone was used as a control. The two peaks at 11 min and 32.5 min were also present in control extracts without any addition of curcuminoids (Appendix 7.11). Data are from three experiments with single injections performed for each (+SD).

5.3.2. Stability of curcumin and its metabolites in Capan-1 and Panc-1 medium

In order to accurately assess the cellular uptake and metabolism of curcumin, its mono-glucuronide and mono-sulfate, it was first necessary to determine whether these three compounds were stable in the different media types required by the cell lines. Curcumin was very stable in the medium used for Capan-1 cells at 0, 15 and 30 min, as quantified by HPLC, with concentrations of 1529, 1578 and 1557 ng/mL, respectively (Figure 5.5A). A gradual decrease was then observed, with levels reaching 799 ng/mL at 48 h, which equates to a 50% reduction. Curcumin mono-glucuronide in Capan-1 medium was less stable than curcumin over a 48 h incubation, with concentrations falling from 5108 ng/mL to 1553 ng/mL at 48 h, representing a decrease of 70% (Figure 5.5B). No change in curcumin mono-sulfate levels was observed for the first 6 h in Capan-1 medium, but it then dropped from 2718 ng/mL to 956 ng/mL at 48 h, which is a decrease of 65% (Figure 5.5C).

Curcumin was very stable in Panc-1 medium for up to 30 min, with levels corresponding to 1717, 1540 and 1513 ng/mL, at 0, 15 and 30 min, respectively. A gradual decrease was then observed, with a final concentration of 934 ng/mL detected after 48 h, which is a reduction of 46% (Figure 5.5D). Overall, curcumin was slightly more stable in Panc-1 medium than Capan-1 medium, based on the proportion remaining after 2 days. Curcumin mono-glucuronide had a similar stability in Panc-1 medium to Capan-1 medium over the course of 48 h, with an initial concentration of 3120 ng/mL falling to 920 ng/mL, which represents a decrease of 71% (Figure 5.5E). Curcumin mono-sulfate was very stable in Panc-1 medium for the first 24 h but decreased by 33% to 8028 ng/mL at 48 h (Figure 5.5F).

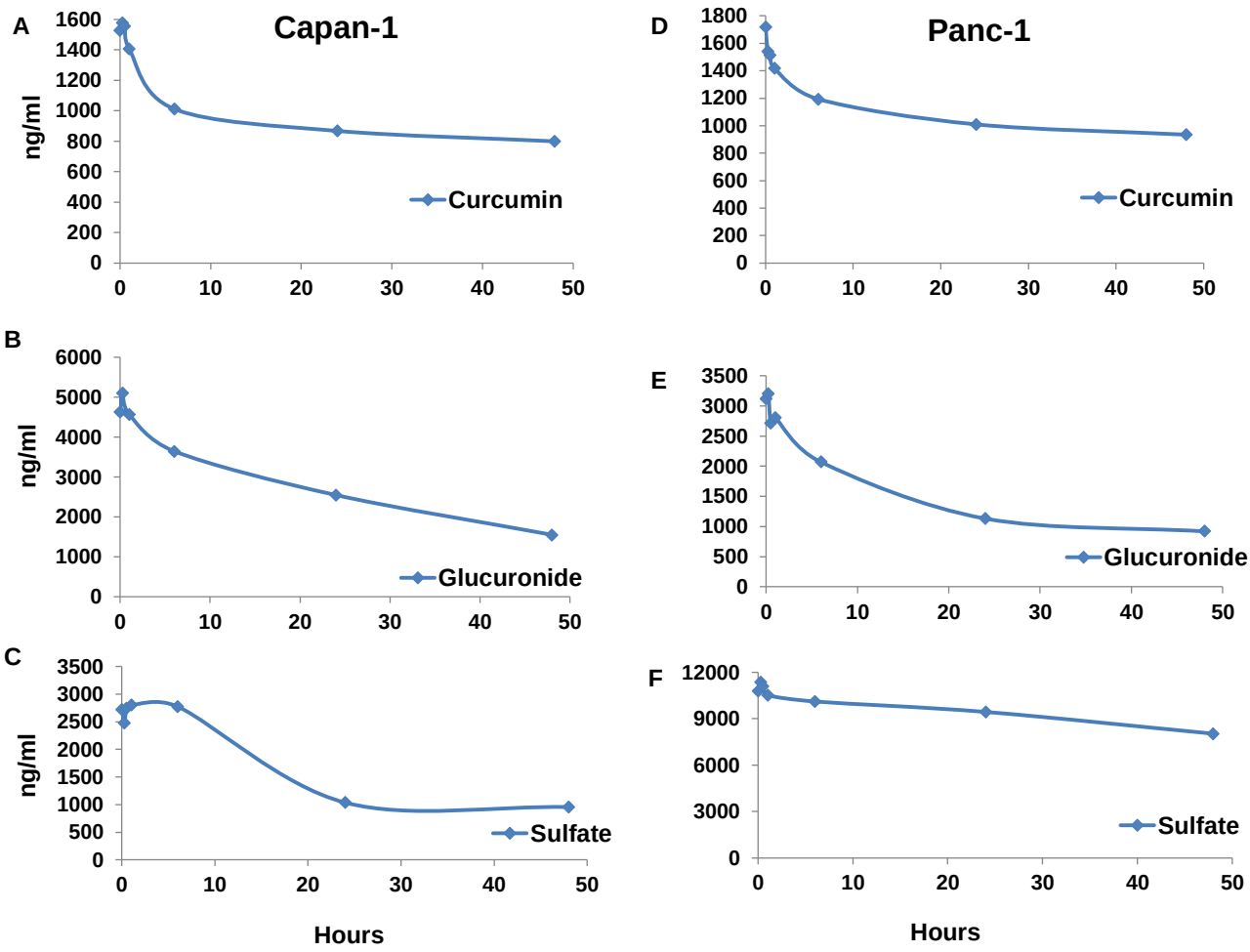


Figure 5.5 Stability of curcumin and its metabolite in Capan-1 and Panc-1 medium over 48 h. Media was spiked with 5 µM curcumin or its mono-glucuronide/sulfate metabolites and concentrations were monitored over 2 days at the following time points: 0, 0.25, 0.5, 1, 6, 24 and 48 h (37°C, 5% CO_2). Stability of curcumin and its conjugates in Capan-1 medium **(A-C)** and Panc-1 medium **(D-F)**. Liquid phase extraction was performed and curcumin/metabolites quantified by HPLC analysis with UV detection at 428nm. Data are from one experiment.

5.3.3. Analysis of media in the presence of cells, following incubation with curcumin

After establishing the stability of curcumin and its metabolites in media alone, cells were incubated with each of the compounds and levels analysed in the media over time.

5.3.3.1. Analysis of media following incubation of Capan-1 cells with curcumin

Following incubation of Capan-1 cells with curcumin, levels of curcumin in the media dropped from 1450±41 ng/mL to 555±66 ng/mL, a decrease of 72 % over 48 h (Figure 5.6). Curcumin glucuronide was detected as a metabolic product from 15 min onwards. The maximum concentration of curcumin detected in the media was 1450±41 ng/mL at 30 min, whilst peak levels of curcumin mono-glucuronide were evident at 6 h (1559±34 ng/mL). No curcumin sulfate was observed at any time point (Figure 5.6B).

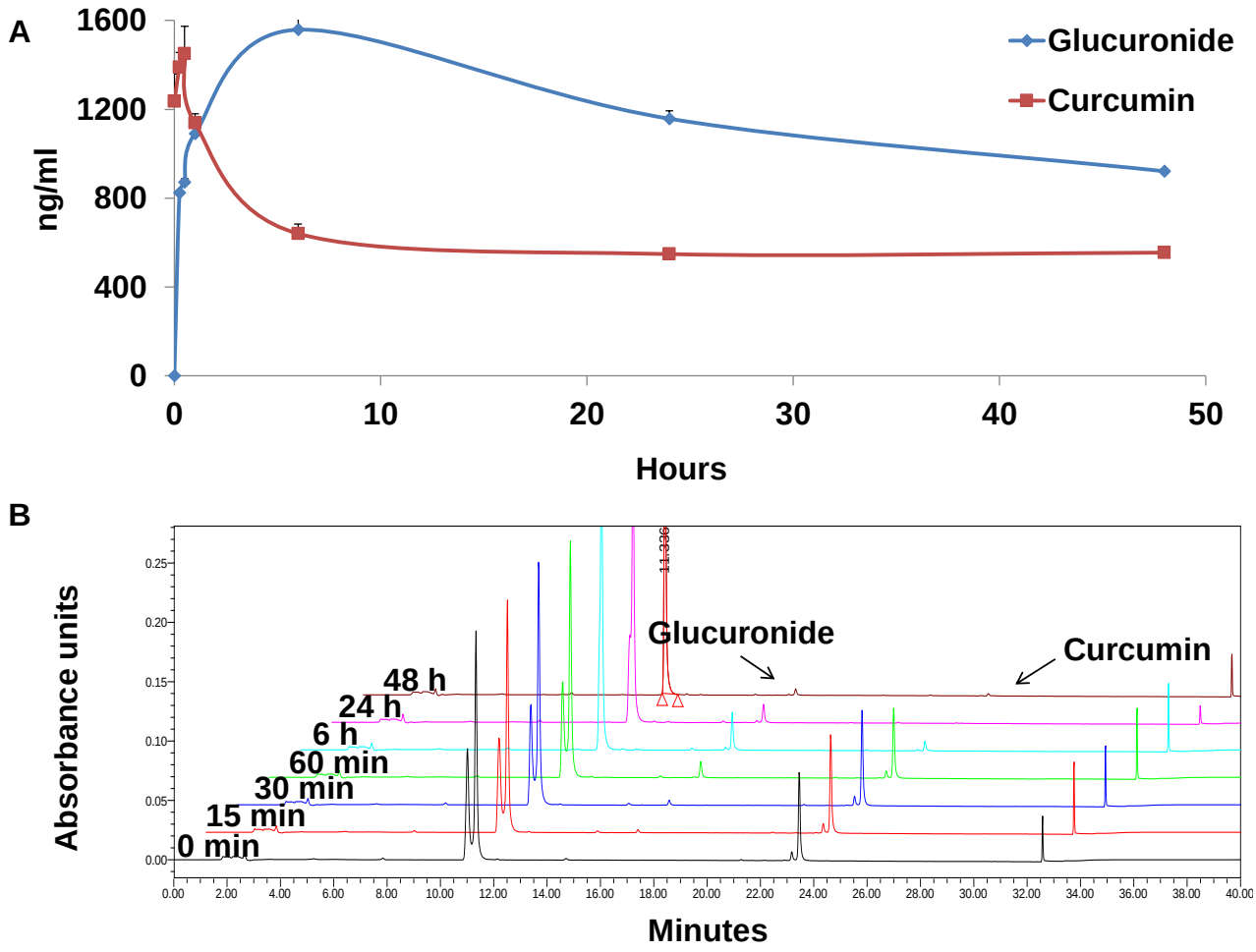


Figure 5.6 Concentration of curcumin and its metabolites in media, following treatment of Capan-1 cells with curcumin. (A) Demonstrates changes in media concentration of curcumin and curcumin glucuronide over time following exposure of cells to 5µM curcumin for 0, 0.25, 0.5, 1, 6, 24 and 48 h. **(B)** Shows representative chromatograms for each time point. Data are the average (+SD) of three experiments with a single injection performed for each.

5.3.3.2. Analysis of media following incubation of Capan-1 cells with curcumin mono-glucuronide

When Capan-1 cells were incubated with curcumin mono-glucuronide it was detected in the media from 0 h (4873±90 ng/mL) and fell gradually over time to 1625±74 ng/mL at 48 h, which is a decrease of 67% (Figure 5.7A). This percentage decrease is only just below that observed for curcumin mono-glucuronide in media without cells (70%), which suggests the metabolite may not be taken up by the cells to an appreciable extent. Curcumin was not detected at

any time point examined, meaning it was either not formed, or was present at or below the LOD.

5.3.3.3. Analysis of media following incubation of Capan-1 cells with curcumin mono-sulfate

When Capan-1 cells were incubated with curcumin mono-sulfate it was detected in the media from 0 h (2853±46 ng/mL), and the levels only decreased slightly over 48 h by ~17% to 2392±36 ng/mL (Figure 5.7B). The magnitude of reduction is very much smaller than that found for curcumin mono-sulfate in media alone, without cells (67%). Curcumin was not detected at any time point, indicating that if formed, the levels are at or below the LOD.

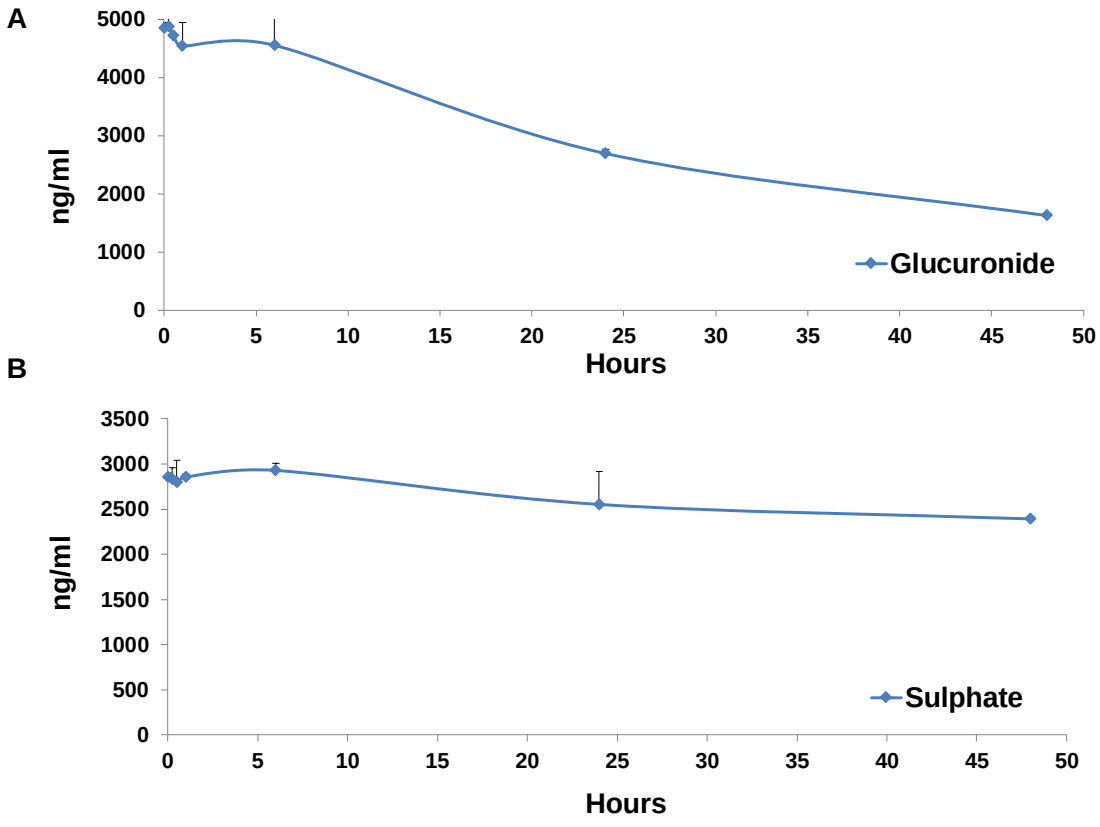


Figure 5.7 Concentration of curcumin mono-glucuronide and mono-sulfate in media, following exposure of Capan-1 cells to the individual metabolites (5 µM). Concentration of curcumin mono-glucuronide **(A)** and mono-sulfate **(B)** in media determined by HPLC-UV analysis. Data are the average of three experiments with single injection performed (+SD).

5.3.3.4. Analysis of media following incubation of Panc-1 cells with curcumin

When Panc-1 cells were incubated with curcumin the maximum concentrations were detected at 0 min (1521±66 ng/mL) then it gradually disappeared from the media over time and was undetectable by 48 h (Figure 5.8A), being below the assay LOD. Curcumin conjugates were not detected at any time point, indicating that if formed they are at or below the LOD.

5.3.3.5. Analysis of media following incubation of Panc-1 cells with curcumin mono-glucuronide

Following incubation of Panc-1 cells with curcumin mono-glucuronide, levels in the media dropped from 3320±163 ng/mL to 1383±94 ng/mL, a decrease of 59% over 48 h (Figure 5.8B). This degree of degradation was slightly lower than that observed in media from Capan-1 cells (67%). Curcumin and other curcumin metabolites were not detected over the course of the incubation, and so were considered at or below the LOD.

5.3.3.6. Analysis of media following incubation of Panc-1 cells with curcumin mono-sulfate

Following incubation of Panc-1 cells with curcumin mono-sulfate, levels in the media dropped from 11199±1167 ng/mL to 7890±355 ng/mL, a decrease of 30% over 48 h (Figure 5.8C). However, the curcumin sulfate degradation in Panc-1 media with cells was nearly same as that degraded in Panc-1 media without cells. Curcumin and other curcumin metabolites were not detected, indicating that if formed they must be at or below the LOD.

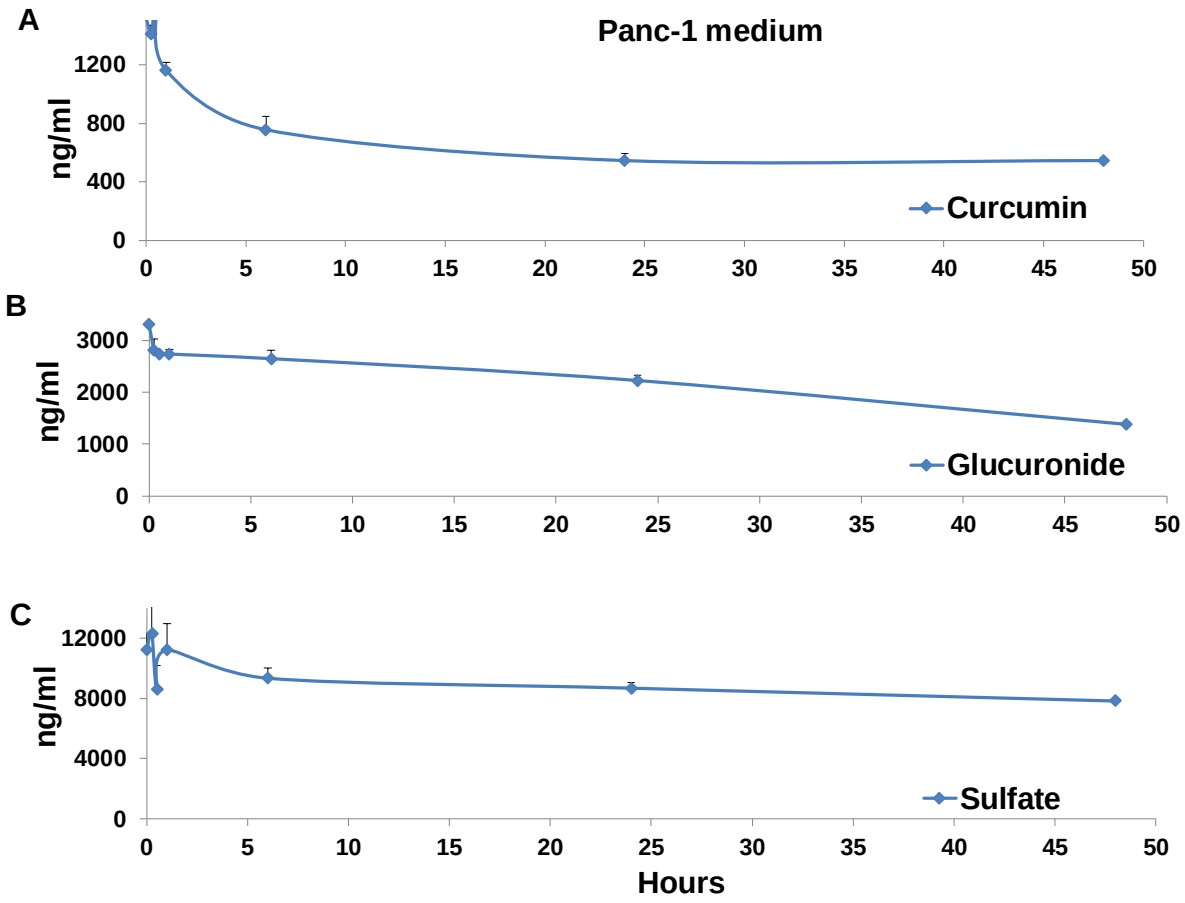


Figure 5.8 Concentration of curcumin and its metabolites in media over 48 h following exposure of Panc-1 cells to (A) curcumin, (B) curcumin mono-glucuronide and (C) curcumin mono-sulfate. Data are the average of three independent experiments, with single injections performed (+SD).

5.4. Cellular uptake and intracellular metabolism of curcumin and its conjugates

After analysis of the media from incubations of Capan-1 and Panc-1 cells with curcumin and it metabolites, the next stage was to determine the intracellular uptake and metabolism of these compounds in the different cell lines.

5.4.1. Analysis of intracellular curcumin/metabolites in Capan-1 cells following incubation with curcumin

Analysis of Capan-1 cellular extracts revealed that curcumin was taken up gradually by Capan-1 cells and rapidly metabolised into curcumin mono-glucuronide within 15 min (Figure 5.9). It was only possible to accurately quantify intracellular levels of curcumin from the cell pellets obtained up to 6 h after exposure, also there were peaks for 24 h and 48 h but were below the LOQ and just above LOD. Curcumin mono-glucuronide was first detectable at 15 min, but curcumin mono-sulfate was not detected at any time point. The maximum intracellular concentrations of curcumin and its mono-glucuronide were both generated after 1 h and reached 0.33±0.07 ng/mg and 1±0.3 ng/mg, respectively. When Capan-1 cells were treated with 5 µM of curcumin mono-sulfate or mono-glucuronide, no metabolites or parent curcumin were detected at any time point, suggesting they are not taken up by the cells (Figure 7.12 and 13 in appendix).

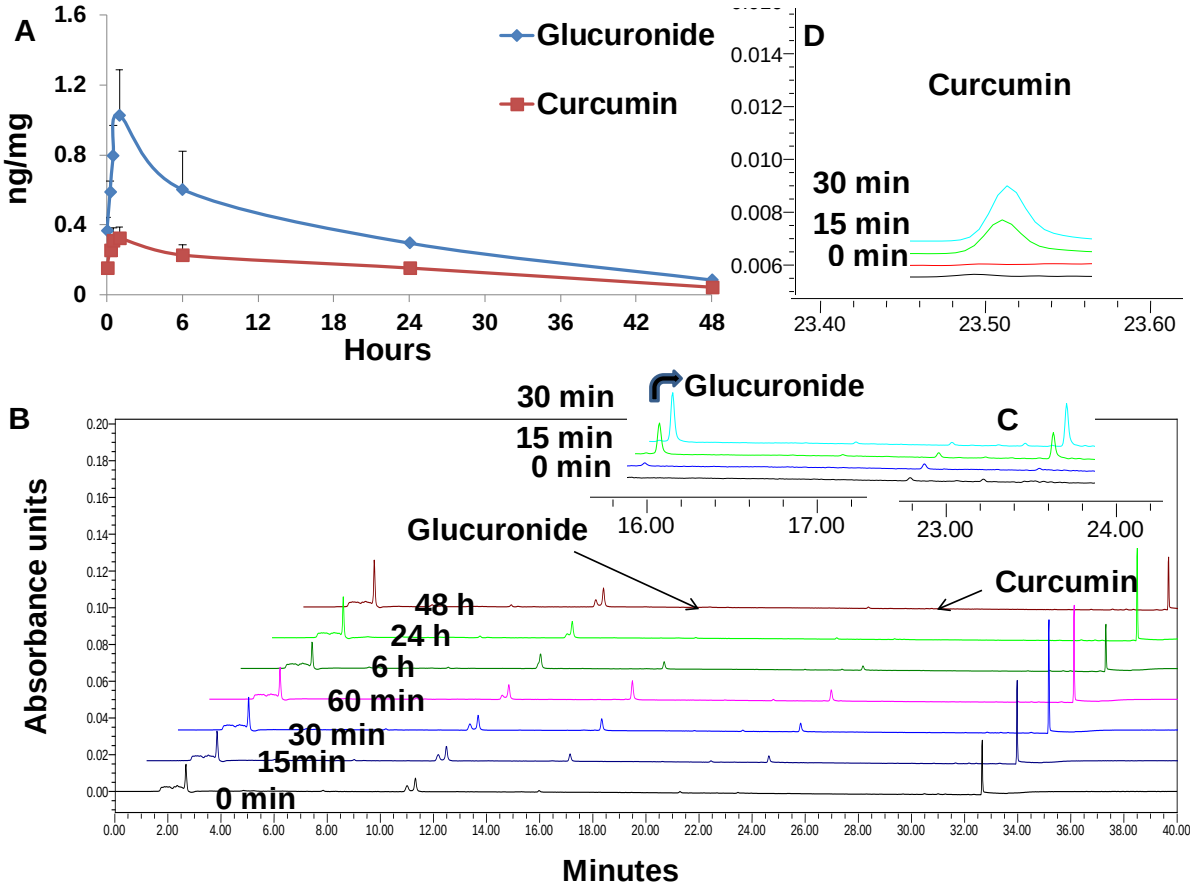


Figure 5.9 Cellular uptake and metabolism of curcumin by Capan-1 cells. (A) Graph demonstrates intracellular concentrations of curcumin and its mono-glucuronide over 48 h when Capan-1 cells were exposed to 5 µM curcumin. **(B)** Shows representative HPLC-UV chromatograms at each time point. **(C)** Magnified peaks for mono-glucuronide and curcumin at 16 and 23.5 min where Capan-1 cells were incubated for 0, 15 and 30 min with 5 µM of curcumin. **(D)** Higher magnification is included to show peaks for curcumin. Data are the average (+SD) of three experiments with a single injection performed for each.

5.4.2. Analysis of intracellular curcumin/metabolites in Panc-1 following treatment with curcumin

Analysis of the intracellular contents after incubation of Panc-1 cells with curcumin showed maximum concentrations were achieved after 30 min and were ~10-fold higher than the levels previously detected in Capan-1 cells (3.22±0.18 ng/mg versus 0.33±0.07 ng/mg) (Figure 5.10). At time points greater than 6 h, curcumin was at or below the LOD. Neither curcumin mono-glucuronide nor

mono-sulfate could be detected at any time point. Consistent with the results obtained for Capan-1 cells, when Panc-1 cells were incubated with the mono-conjugates no intracellular curcuminoids were detected at any time point (Figure 7.13 in appendix). A data summary for the metabolism studies can be found in appendix (Figure 7.14-16).

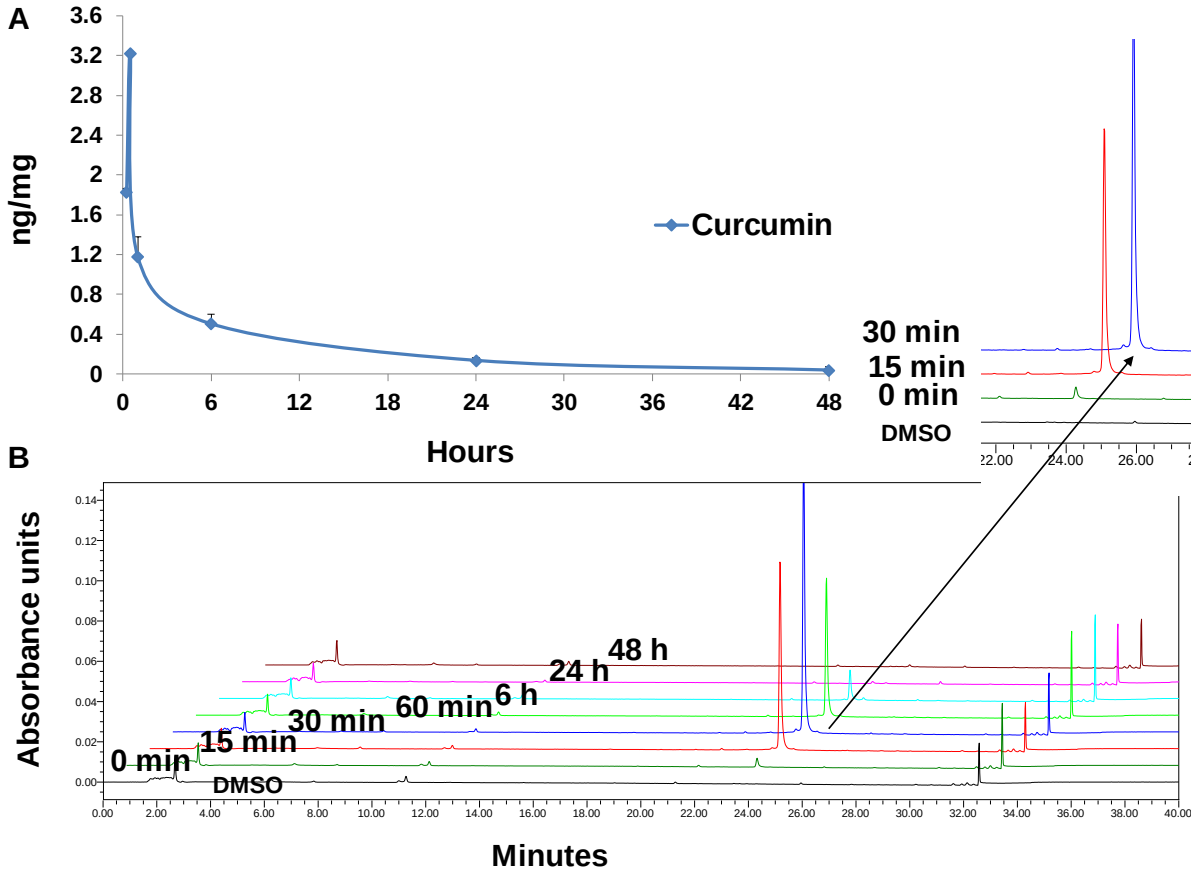


Figure 5.10 Cellular uptake and metabolism of curcumin by Panc-1 cells. (A) Demonstrates levels of intracellular curcumin over 48 h, following treatment of Panc-1 cells with 5 µM curcumin. **(B)** Shows representative HPLC-UV chromatograms of cellular extracts, with detection at 428nm. The retention time of curcumin is ~23.5 min. Data are the average (+SD) of three experiments with a single injection performed for each.

5.5. Discussion:

It has been suggested that curcumin and resveratrol metabolites may have anti-cancer efficacy, and therefore contribute to the overall effects of these compounds *in vivo* (173, 198, 222, 223, 289). The work described in this chapter focused on the potential role of two major curcumin metabolites and their uptake by pancreatic cancer cell lines. The anti-tumour effects of curcumin mono-sulfate and -glucuronide conjugates were explored using a proliferation assay and sphere formation assay to specifically investigate the effects on stem-like cells. To this end, the cytotoxicity and growth inhibitory properties of curcumin mono-sulfate was assessed in the Capan-1 and Panc-1 cell lines by measuring changes in cell number over six days and sphere number/size over two weeks. At the time of this experiment, curcumin glucuronide metabolites were not available in sufficient quantities due to difficulties in the synthesis, meaning it was not possible to assess the activity of curcumin mono-glucuronide. The concentrations chosen for curcumin mono-sulfate were initially dictated by their reported ability to inhibit COX-2 expression in colorectal cancer cell lines (222). Differential sensitivity was observed with the mono-sulfate, with the Capan-1 cells exhibiting greater growth inhibition than the Panc-1 cell line. Enhanced sensitivity to the mono-sulfate was also observed in Capan-1 cells under sphere forming conditions; generally Capan-1 cells were more sensitive. In addition, the results indicated that curcumin mono-sulfate was less potent than parent curcumin in terms of growth inhibition and sphere reduction, in both cell lines. It is clearly demonstrated that curcumin mono-sulfate did not induce any growth inhibition in either of the cell lines at low concentrations but significant activity was apparent at very high concentrations.

The cytotoxic potential of curcumin sulfate and glucuronide metabolites has also been investigated in a variety of other tumour cell lines and they reported that no inhibition of cell proliferation (266). To understand why these two cell lines responded differently to curcumin and its metabolites, the uptake of these compounds by Capan-1 and Panc-1 cells was investigated using HPLC. To my knowledge, the cellular uptake of curcumin and its metabolites in pancreatic cancer cell lines has not previously been carried out. The results showed that

curcumin can be taken up by both cell lines but only Capan-1 cells generate detectable levels of metabolites in the form of curcumin mono-glucuronide. At the concentrations used in the uptake studies the mono-sulfate and mono-glucuronide conjugates were not internalised by either cell line, which would explain why they did not have any effect at low clinically achievable concentrations. The antiproliferative activity of curcumin mono-sulfate at higher concentrations could indicate that a proportion is able to cross the cell membrane when present in higher amounts; alternatively it may hydrolyse in the media and the resulting curcumin may be taken up by the cells. Another possibility is that curcumin mono-sulfate is able to mediate activity via interaction with proteins on the cell surface. Further experiments investigating its interaction with membrane component of these two cell lines could be performed by sub-cellular fractionation to begin to address this possibility.

The mono-sulfate and mono-glucuronide metabolites are less lipophilic than parent curcumin and are unlikely to be capable of crossing cellular membranes by passive diffusion. The more hydrophilic/lipophobic sulfate group is polar or charged, which increases the interaction with water making it more difficult to enter cells by diffusion. Therefore, the conjugated curcumin metabolites are likely to need active transport mechanisms that require energy for transmembrane passage as they are anionic conjugates. This explains the lack of detectable uptake for these metabolites compared to curcumin itself. It has been suggested that (305) ABCG2 preferentially transports sulfate conjugates. The organic anion transporter SLC22A9 and the organic anion–transporting polypeptides (OATPs) SLCO1B1 and SLCO1B3 also play a role in the uptake of sulfated and glucuronidated compounds; the basal expression of these proteins could be examined in the two cell lines to ascertain whether they are present, as the results indicate they may be absent or present at low levels.

Other points for consideration are the enzymes responsible for curcumin glucuronidation and sulfation (uridine diphosphate glucuronosyl transferases (UGTs) and sulfotransferases (SULTs), respectively); differences in the expression of the particular isoforms involved may explain only Capan-1 cells were able to generate detectable metabolites. There does not appear to be any

information available in the literature comparing expression levels for these enzymes in the two cell lines, therefore this would have to be investigated in future experiments. It has been shown that UGT is active in microsomes from human liver and intestine and is responsible for the glucuronidation of various curcuminoids (306). Five major sulfotransferases have been reported in human tissues with varying expression in different tissues (307).

Interestingly, Panc-1 cells had ~10-fold higher peak intracellular concentrations of curcumin compared to Capan-1 cells, however this was due to a rapid sharp uptake within the first 30 min followed by a quick reduction in levels, possibly due to efficient efflux. Consequently, curcumin concentrations were similar in both cell lines after 6 h. Further investigations are required to identify the efflux transporters responsible in both cell lines; this could be done through the basal gene expression profiles for efflux transporters.

Even though cytotoxicity studies in diverse cell lines have shown that the antiproliferative effects of curcumin improve with higher cellular uptake (308) the results obtained here do not support this conclusion. This raises the question of whether curcumin cellular uptake is not an appropriate predictor of effectiveness in the particular cell lines used (308) but that efficacy may correlate with the ability of cells to actually metabolise curcumin, suggesting the metabolites may have intrinsic activity.

Furthermore, cellular growth inhibition (Chapter 3), targeting stem cell markers and sphere inhibition (Chapter 4) by curcumin in the Capan-1 cells were more obvious than in the Panc-1 cells. It would be a worth mentioning that curcumin metabolites, particularly the glucuronide, inhibit the assembly of microtubule proteins under cell-free conditions, indicating intrinsic activity of the glucuronides (304) and it has been suggested any physiological efficacy elicited by curcumin in distant organ rather than intestinal tract are potentially due to curcumin metabolites (309). There is another possibility which needs to be investigated, that metabolites are converted back to the parent intracellularly as has been shown for resveratrol (289).

To sum up the results obtained in this chapter the lower exposures of curcumin mono-sulfate, up to 5 µM, did not have any significant effect on Panc-1 cells but there was a small reduction in the number of Capan-1 cells at 5 µM. When exposed to higher concentrations (20, 100 µM), similar results were seen which means the sensitivity of both cell lines directly depends on curcumin sulfate concentrations in ultra-low attachment plates under non-adherent as well as adherent conditions. The stability of curcumin and it metabolites in media without cells showed that overall curcumin was more stable in Panc-1 medium than Capan-1 medium. Curcumin mono-glucuronide had a similar stability in Panc-1 and Capan-1 medium over the course of the 48 h incubation, whilst the mono-sulfate was more stable in the Panc-1 medium. In addition, when the cells were exposed to curcumin and its metabolites, both cell lines were able to internalize curcumin but only Capan-1 cells were capable of metabolising it to the mono-glucuronide. When the cells were incubated with the metabolites neither curcumin mono-sulfate or curcumin mono-glucuronide could be detected intracellularly, which means both cell lines were unable to internalize them or the levels entering were below the level of detection. As Capan-1 cells are the most sensitive to curcumin, yet are more resistant to gemcitabine than Panc-1 cells (114), it may be that combination treatment of capan-1 with gemcitabine + curcumin would enhance the efficacy of gemcitabine. Many studies have used combination treatment successfully; including combination of curcumin with gemcitabine or other drugs (215, 217, 277, 310).

6.1 Chapter Six: Conclusion

6.1 Combination of dietary agents for pancreatic cancer

Amongst all types of cancers, pancreatic cancer is known as a particularly silent and significant killer, due to the fact that it is amongst the most aggressive of the solid malignancies with an extremely high mortality rate (31-36). This is primarily due to late diagnosis and metastatic spread, and the occurrence of chemo-radio resistance. It is likely that the initiation, maintenance and recurrence of pancreatic cancer is mediated by a specific subset of cells known as Cancer Stem Cells. Therefore, a rational approach towards both cancer prevention and the development of better treatments is to focus on targeting this cellular population. Current chemotherapy regimens for pancreatic cancer do not target CSCs, and, despite significant toxicities, response rates and overall survival have changed little over the past 4 decades. The use of diet-derived agents in both the prevention and therapeutic setting has gained impetus, with increasing numbers of small phase I/II clinical studies suggesting that there may be potential for benefit in some patients (197, 214, 220, 251). Both curcumin and resveratrol have been used individually in cellular/animal models of pancreatic cancer and have been shown to have chemopreventive/therapeutic potential and do not seem to be associated with significant side effect burdens (175, 216, 218, 251). However, these two compounds have yet to be used in combination to assess whether this may prove to be more efficacious in the pancreatic cancer setting, compared to either agent alone. In other models of malignancy, curcumin and resveratrol in combination inhibit colon cancer cell growth by causing a reduction in cell proliferation and the induction of apoptosis (260). A combination of liposomally encapsulated curcumin and resveratrol has been used in the prostate-specific PTEN knockout mouse model (264).

There is increasing evidence for both curcumin and resveratrol as agents that are able to target CSCs, but only a few studies have been published to date in pancreatic cancer. The effect of curcumin on CSCs has been assessed both *in vivo* and *in vitro* by utilizing several different markers indicative of CSC properties including, side populations, tumour-sphere formation, cell-surface marker assays and enzyme activity (187, 231, 233). Furthermore, resveratrol has been shown

to inhibit the self-renewal ability of PCSCs obtained from human primary tumours and KrasG12D mice *in vitro* (254). Nanog and other transcription factors involved in maintenance of pluripotency, including Sox2 and Oct4a, were also downregulated by resveratrol (254, 257).

In addition to the pancreatic epithelial lines, pancreatic stellate cells (PSCs), can contribute up to 90% of the pancreatic tumour mass. These cells play a very important role in pancreatic cancer, such as production of dense desmoplasia to form barriers preventing chemotherapeutic drugs from getting to the cancer cells. In a study by Masamune *et al*. (2006), curcumin blocked pancreatic stellate cell activation (140, 282) but there is no report in the literature on the effects of resveratrol or a combination of resveratrol and curcumin on this cell type.

The overall goal of this project was to investigate the activities, and evaluate the potential efficacy, of two dietary agents, resveratrol and curcumin, both alone and in combination. These could be used with current approved chemotherapeutic drugs (gemcitabine) for pancreatic cancer, as potential new anticancer agents targeting CSCs as well as stromal cells. Anti-proliferative and stem-cell targeting abilities of curcumin and resveratrol have been shown in different cancers, but the combined efficacy of these agents has not been assessed in pancreatic cancer and stellate cell lines, prior to initiation of this project. Furthermore, there is little information regarding the potential for efficacy of curcumin or resveratrol metabolites in pancreatic cancer.

This research sought to identify whether low, clinically-achievable concentrations of these agents may have potential utility for the prevention or treatment of pancreatic cancer, either alone or in combination, and to examine their effects on key drivers of the carcinogenic process such as the PCSC.

6.2 Anti-proliferative activity of curcumin and resveratrol and molecular mechanisms of growth inhibition

Values for the IC_{50} were calculated only for cell lines where 50% cell reduction was observed. Capan-1 and RLT-PSC cell lines were the most sensitive to curcumin, with IC_{50} concentrations of 1.7 and 2.25 µM, respectively. Following combined exposure after 6 days of daily dosing, enhanced anti-proliferative effects were observed for the first time in pancreatic cancer cell lines. The main outcome observed was that across all cell lines, the addition of very low curcumin concentrations (0.1 µM) to 5 µM resveratrol, resulted in an enhancement of the anti-proliferative activity of resveratrol at this concentration. The growth inhibition data also indicated that a combination of both resveratrol and curcumin at clinically achievable concentrations of 0.1 µM induced significant growth inhibition in 2 out of 4 cell lines. The processes underlying the cell growth inhibition were determined; Capan-1 cells underwent apoptosis whilst in the Panc-1 and AsPC-1 cells, cell cycle arrest occurred. Whilst this is in keeping with proposed endpoint mechanisms observed in other studies (284), this is the first study to suggest that curcumin may support induction of apoptosis/cell cycle arrest in pancreatic cell lines at such a low concentration. This exposure is within a similar order of magnitude to the low systemic concentrations observed in clinical trials following oral curcumin administration (186, 218).

6.3 Curcumin and resveratrol for targeting PCSCs in pancreatic cancer cell lines

Various CSC populations have been isolated from both clinical pancreatic cancer tissues and from a variety of pancreatic cancer cell lines. However, as with many other malignancies, there is still no consensus as to which population represents the most important, with regards to CSC hierarchy and contribution to pancreatic

cancer progression. Out of the prospective markers for investigation, there are several which are gaining favour within the literature which may provide representation of cellular subsets linked with prognosis and treatment response (187, 231, 257). Therefore, in this project the decision was made to investigate two different populations, namely $CD24^{+}/CD44^{+}$ using cell surface markers and ALDH-1^{High} as a marker of internal cellular aldehyde dehydrogenase activity (114, 115, 118). The expression level of the embryonic transcription factor Nanog was also considered, since its overexpression can correlate with transformation from pre-malignant to malignant conditions (119, 120, 128). The different CSC populations in Panc-1 cells had very different responses to curcumin and resveratrol, when exposed to each compound individually. The population identified as being double positive for $CD24^{+}/CD44^{+}$ decreased significantly when incubated with 5 µM curcumin, but resveratrol had no significant effect. However, resveratrol significantly reduced the fraction of cells with ALDH-1^{High} activity, whilst curcumin did not have a clear effect at the highest concentration. This observation provides a good indication that using a single compound will not eliminate all sources of tumourigenic populations or all cellular progenies. Additionally, use of the sphere formation assay in Capan-1 cells produced spheres that were the most sensitive to both the single and combination treatments. Here, addition of 0.1 µM curcumin to 5 µM resveratrol brought about a significant reduction in spheroid number that was greater than either compound alone. Other evidence for a combined treatment approach for elimination of PCSCs has indicated that it is possible in *in vivo* models, to enhance stem cell targeting efficacy by a greater extent than for either agent alone (109, 138). With the combination of resveratrol and curcumin, there is the added advantage that both of these agents have a favourable toxicity profile, and that more studies are now utilising the single agents in clinical combination with chemotherapy agents.

Next, I investigated how curcumin might be targeting PCSCs. Here, the ALDH-1^{High} population was isolated from Capan-1 cells which had been exposed to curcumin. Following Western blot analysis, it could be determined that curcumin caused a significant reduction of Nanog expression in this stem cell population. Unpublished work conducted in this laboratory by Dr A. Karmokar has found

similar results in colorectal cancer stem cell models, whereby a clinically achievable concentration of curcumin (~0.1 μM) selectively targets the ALDH-1^{High} population, with this decreased activity correlating with significant down-regulation of Nanog expression. Here it has also been established that in these cells, specific binding sites for curcumin exist within the Nanog protein. I did not explore the effect of resveratrol as Dr A. Karmokar did not show any correlation between resveratrol treatment and Nanog protein levels. The results of this chapter have demonstrated that different cell lines have different stem cell populations and varying sensitivities to both compounds individually. The PCSC heterogeneity in patient samples with pancreatic cancer was also observed when analysis was undertaken for 12 primary tissue samples (Chapter four 4.7).

The use of combinations was shown in some cases to be capable of targeting PCSCs at the lower, more clinically achievable concentrations. Therefore, targeting multiple CSC populations within pancreatic cancer using compounds such as curcumin and resveratrol is a sensible and plausible strategy for cancer prevention or as a combined adjuvant in the treatment setting.

6.4 Potential for efficacy of curcumin metabolites, and their cellular uptake and metabolism

It is clearly demonstrated that curcumin sulfate did not exhibit anti-proliferative activity at clinically achievable concentrations (0.1-5 μM). Previous data has similarly suggested that it is the parent compound, rather than curcumin metabolites which may be responsible for the majority of its anti-cancer efficacy (222, 266, 311). However, neither the sulfate nor glucuronide could be internalised by either cell line and only the Capan-1 cells were capable of metabolising the parent compound. Overall the results indicate that curcumin metabolites are less active than the parent compound but could contribute additional effects to the parent when present intracellularly.

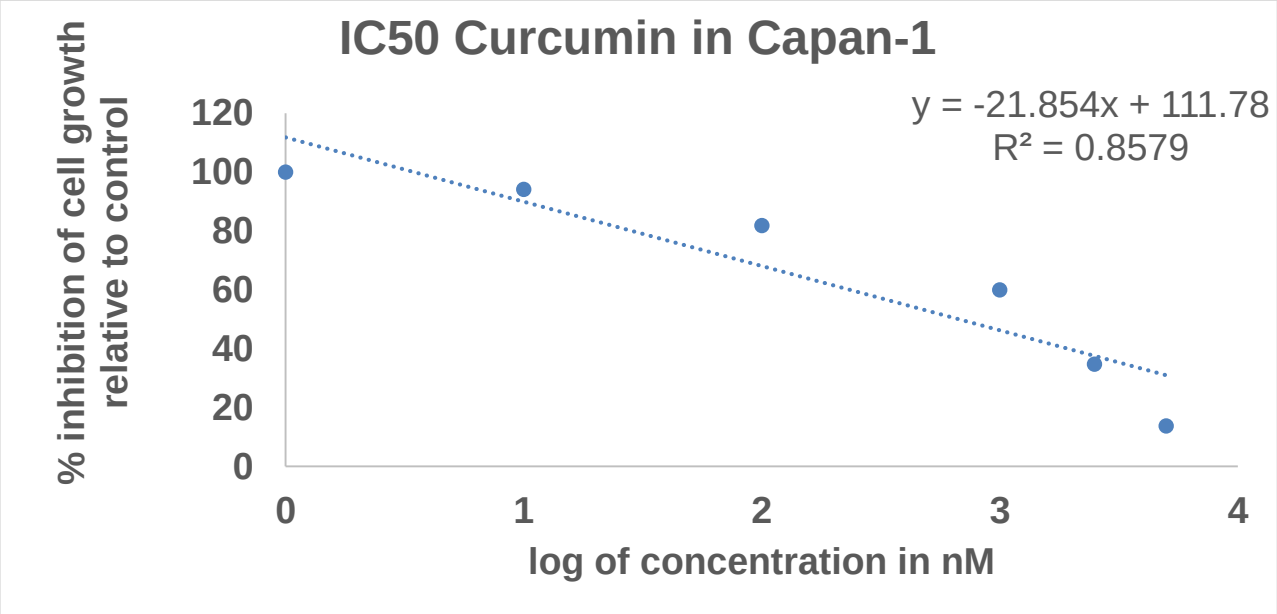
6.6 Future Objectives

It is important to determine which PCSC populations represent the best therapeutic target. However, most CSC populations are extremely labile and may differ between patients and also following intervention strategies. Furthermore, trying to recapitulate PCSC expression in *in vitro* models will prove difficult due to the occurrence of phenotypic switching, which is very dependent on environmental stimuli (94, 112, 118, 293). In order to overcome this issue which leads to an extremely heterogenous PCSC population, it may be that single targeted agents do not provide the most efficacious way forward. Rather, it is the combination of agents that are capable of targeting different sub-populations which may provide the better PCSC targeting strategy.

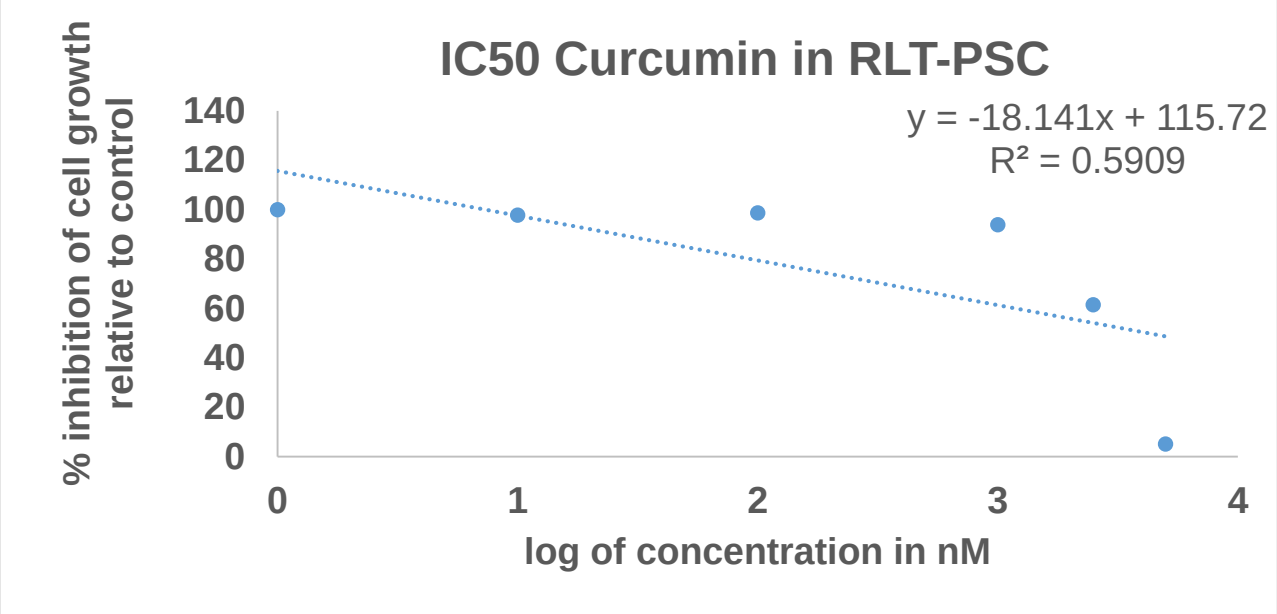
Interestingly, it is the lower concentrations of curcumin that engender significant interest for preventive/therapeutic potential, with a hormesis-like effect observed, particularly within the PCSC population (Unpublished data by Dr A. Karmokar). Further studies are required in order to translate this from cell lines into *in vivo* pre-clinical models and ultimately into the clinic. Due to the nature of late presentation and diagnosis of pancreatic cancer, administration of combinations involving curcumin and resveratrol is most likely to be undertaken in conjunction with first line chemotherapy. Clinical studies administering curcumin in combination with gemcitabine have been undertaken (215), proving this combination to be safe and tolerable. However, for the first time in decades, standard of care may be changing. Other intervention options for those with good performance status include FOLFIRINOX and nab-paclitaxel, neither of which have been investigated in combination with diet-derived agents. Presumably it would therefore be worth investigating curcumin and resveratrol in combination with these new regimens in future studies.

Appendices

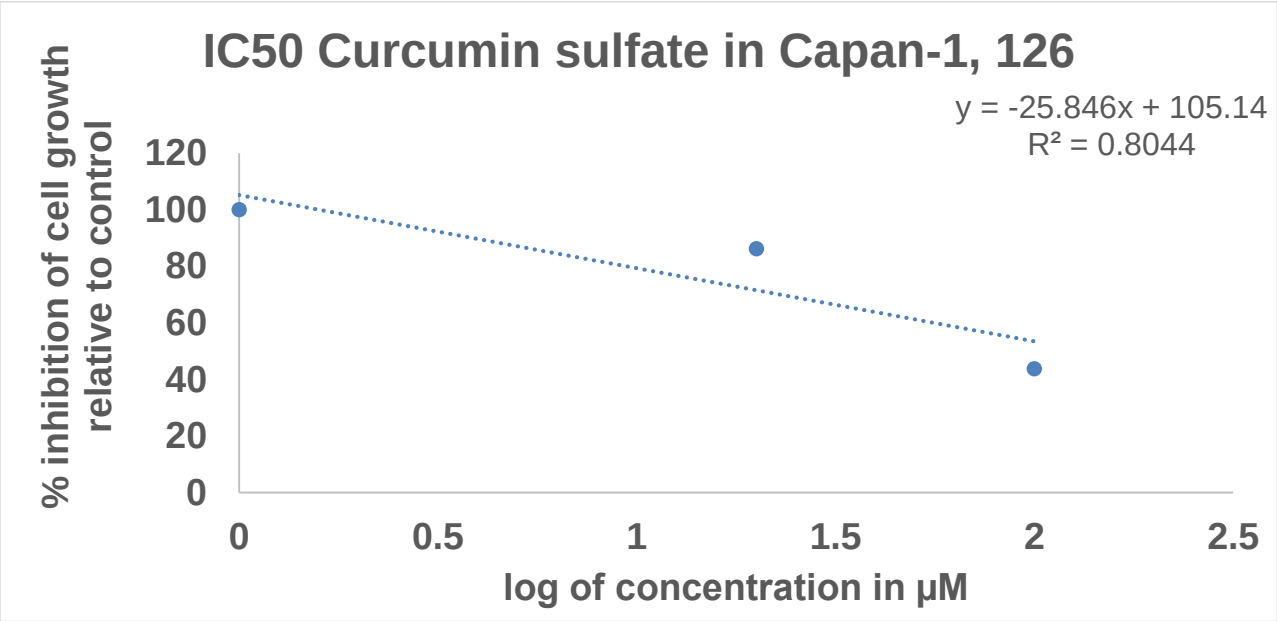
7.1 IC_{50} Calculation via linear regression method for Curcumin in Capan-1



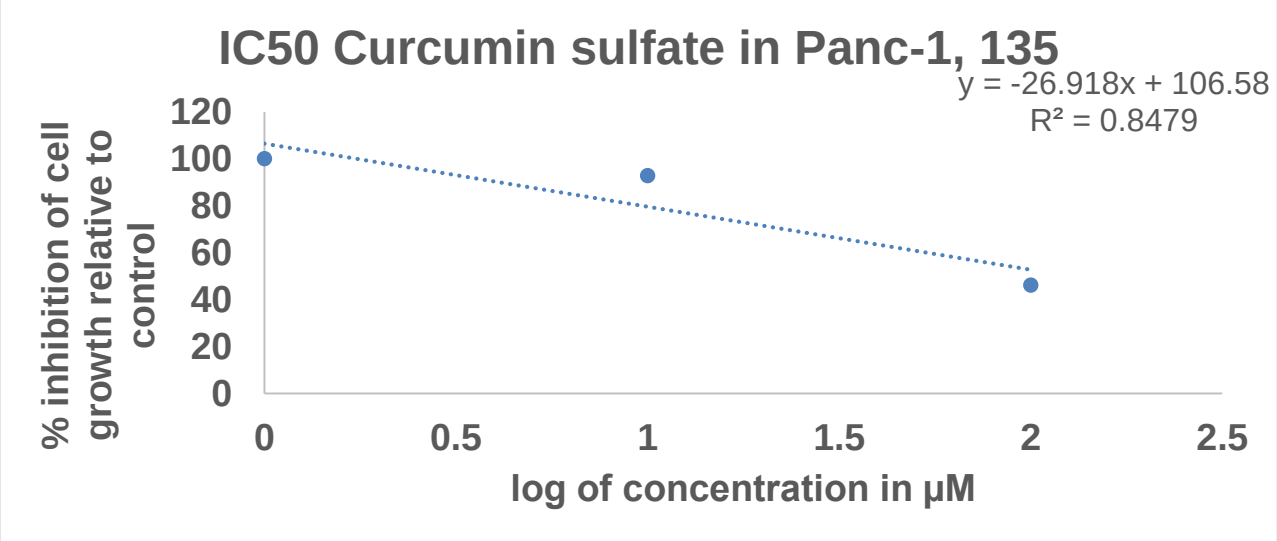
7.2 IC_{50} Calculation via linear regression method for Curcumin in Panc-1



7.3 IC_{50} calculation via linear regression for curcumin sulfate in Capan-1, IC_{50}=126 µM.



7.4 IC_{50} calculation via linear regression method for curcumin sulfate in Panc-1, IC_{50}=135 µM.



7.5 IC_{50} for curcumin and resveratrol in pancreatic cancer cell lines

Pancreatic Cell line	IC_{50} Curcumin	Reference	IC_{50} resveratrol	Reference
Capan-1 (Pancreas)	5.4 µM	(Li et al., 2004)		
Panc-1(Pancreas)	25 µM, 16.50 µg/ml, 19.6 µM, 26 µM, 25 µM	(Parasramka and Gupta, 2012), (Ramachandran et al., 2010), (Sutaria et al., 2012), (LEV-ARI et al., 2006)	70 ± 10 µmol/L, 78.3 ± 9.6 µmol/L	Kotha et al., 2006, Cui et al., 2010
AsPC-1 (Pancreas)	11 µM	(Li et al., 2004)	123.1 ± 6.5 µmol/L	Cui et al., 2010
BxPC-3 (Pancreas)	5.4 µM, 10 µM, 14.08 µg/ml	(Li et al., 2004), (Parasramka and Gupta, 2012), (Ramachandran et al., 2010)	71.85 ± 1.55 µM, 76.1 ± 7.8 µmol/L	(Azmi et al., 2013), Cui et al., 2010
Capan-2 (Pancreas)	46 µM	(Li et al., 2004)		
HS766-T (Pancreas)	7 µM	(Li et al., 2004)		
MIA PaCa-2 (Pancreas)	19.6 µM, 18 µM	(Sutaria et al., 2012)		

7.6 IC_{50} for curcumin and resveratrol in different cancer cell lines

Cell line	IC_{50} Curcumin	Reference	IC_{50} resveratrol	Reference
PC-14 (Liver)	10 µM	(LEV-ARI et al., 2006)		
H1299 (Liver)	20 µM	(LEV-ARI et al., 2006)		
HCT116 (Colon)	10.91 µM	(Cen et al., 2009)		
HT-29 (Colon)	13.31 µM	(Cen et al., 2009)	72.9 ± 2.4 µM	(Azmi et al., 2013)
SW480 (Colon)	10.26 µM	(Cen et al., 2009)		
WI-38 (Colon)	48.82 µM	(Cen et al., 2009)		
DU-145 (prostate)			107.92 ± 1.57 µM, 25 ± 11 µmol/L	(Azmi et al., 2013), Kotha et al., 2006

7.7 Cell cycle arrest by curcumin.

Table 3.1 Appendix.			
Cell lines	Cell cycle arrest	Curcumin concentration	Reference
MDA-MB-231 (Breast)	G0/G1	50 µM	Kotha et al., 2006
Panc-1,(PANC-1, BxPC-3 and AsPC-1)	G0/G1 X	50 µM, (78.3 µM, 76.1 µM and 123.1 µM)	Kotha et al., 2006 (Cui J 2010)
,(PANC-1 and AsPC-1) (Pancreas)	X x G0/G1	(100 µM)	(Ding XZ, Adrian TE 2002)
DU145 (Prostate)	G0/G1	50 µM	Kotha et al., 2006
MDA-MB-468 (Breast)	S	50 µM	Kotha et al., 2006
COLO 357 (Pancreas)	S	50 µM	Kotha et al., 2006
CEM-C7H (Blood)	S	20 µM	Bernhard et al., 2000
A431 (Skin)	G1	50 µM	Ahmad et al., 2001

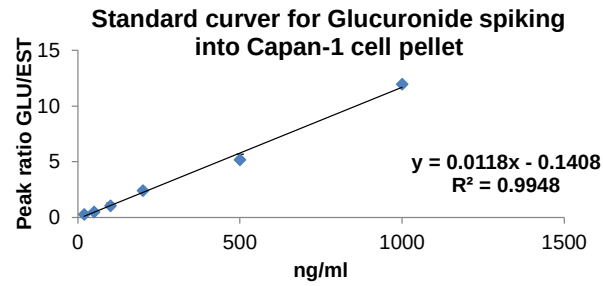
7.8 Shows maximum concentration for Curcumin, Cur-Glucuronide and Cur-Sulfate exposure to Capan-1 and Panc-1 in media.

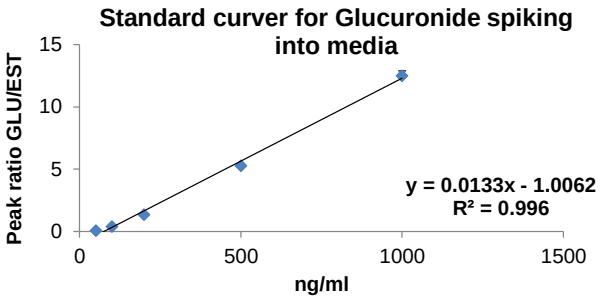
Cell lines		curcumin Media	Cur-Glu Media	Cur-Sul Media
Capan-1	C	1450±41 ng/ml	4873±90 ng/ml	2853±46 ng/ml
	T	30 min	0	0
Panc-1	C	1521±66 ng/ml	3320±163 ng/ml	11199±167 ng/ml
	T	0	0	0
Cell lines		**curcumin Cell pellets**	**Cur-Glu Cell Pellets**	**Cur-Sul Cell Pellets**
Capan-1	C	0.33±0.07 ng/mg	At or below LOD	At or below LOD
	T	60 min	0	0
Panc-1	C	3.22±0.18 ng/mg	At or below LOD	At or below LOD
	T	30 min	0	0

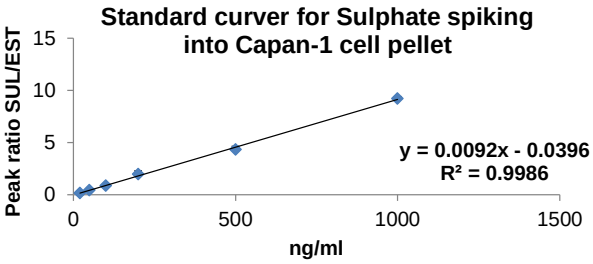
7.9 Shows concentration of metabolites for Curcumin exposure to Capan-1 in medium and cell pellets intracellularly.

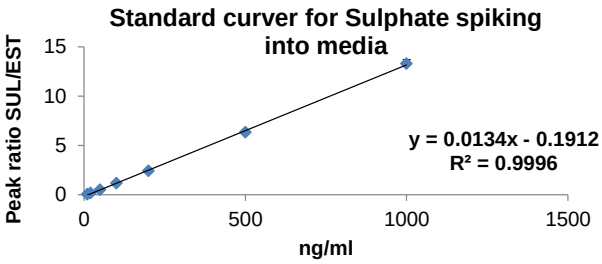
Cell lines		Curcumin Media	Cur-Glu Media	Cur-Sul Media
Capan-1	C		155841 ng/ml	At or below LOD
	T		6	0
Cell lines		**Curcumin Cell pellets**	**Cur-GlCell Pellets**	**Cur-Sul Cell Pellets**
Capan-1	C		1±0.3 ng/mg	At or below LOD
	T		60	0

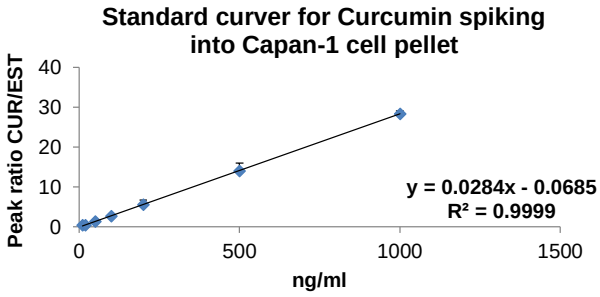
7.10 Standard curve for Mono-Glucuronide, Mono-Sulfate spiking into Capan-1 cell pellet and their media.

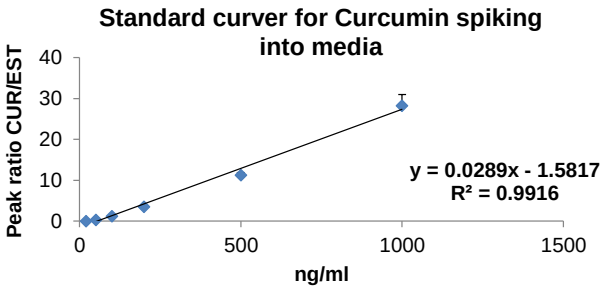




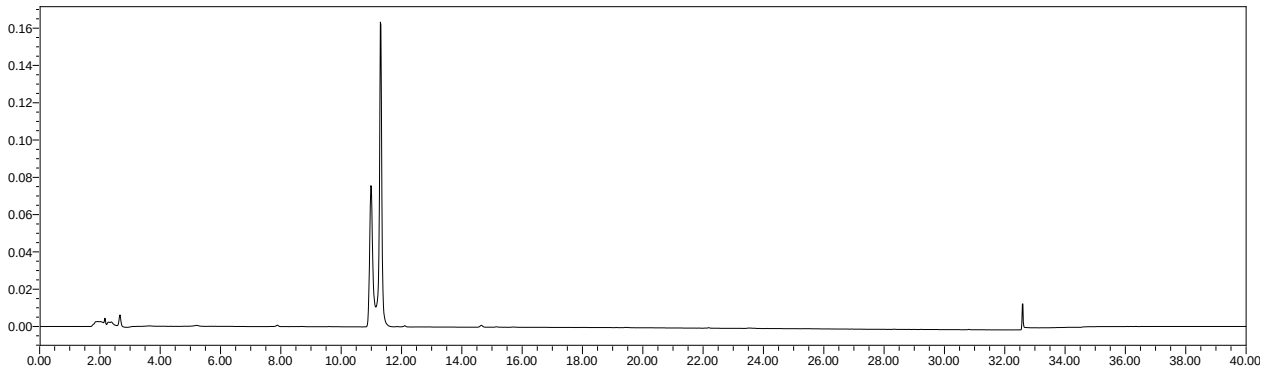




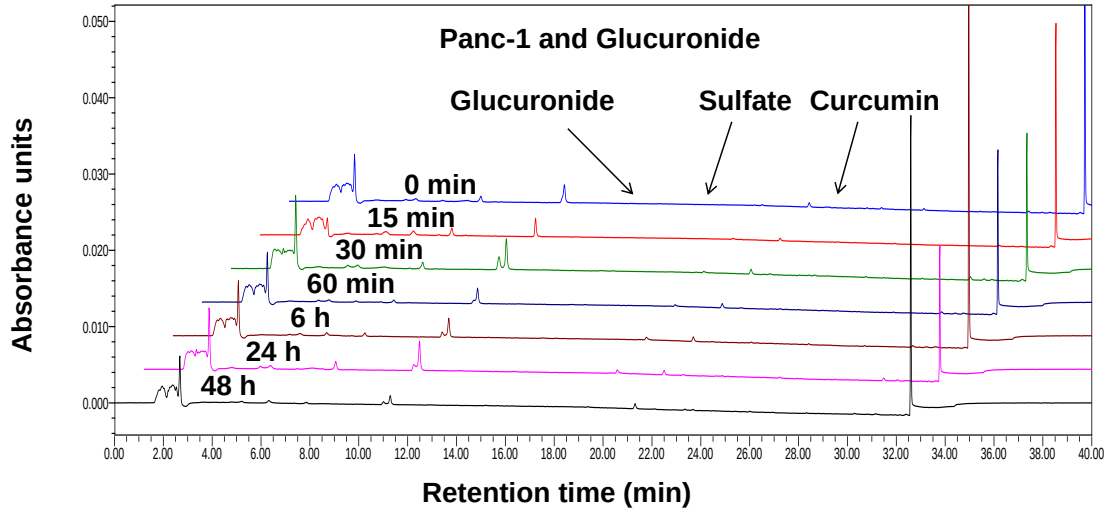




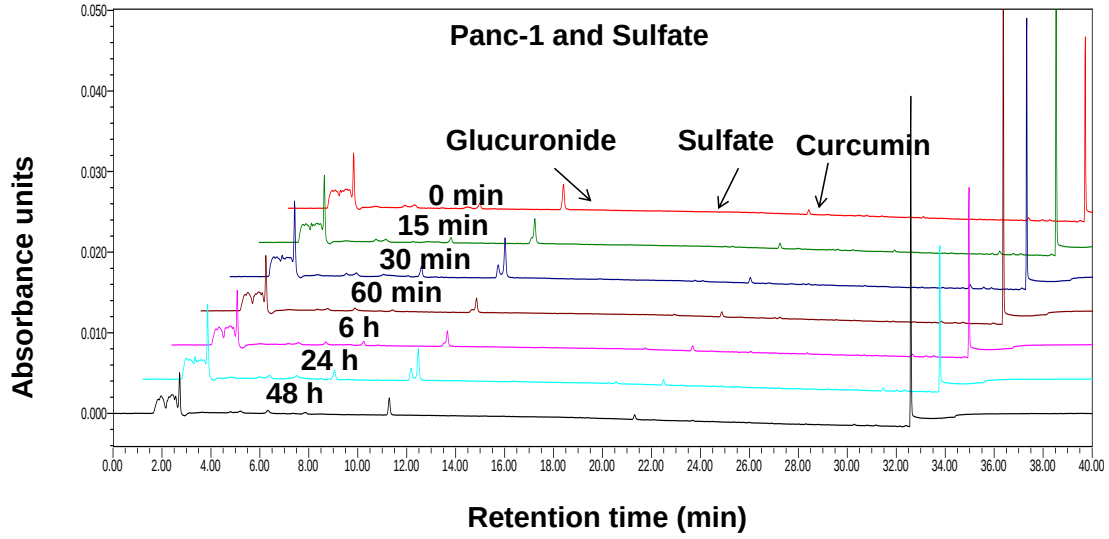
7.11 Chromatography for injecting of DMSO only two peaks at 11 and 32.5 min.



7.12 Representative HPLC-UV chromatograms of Panc-1 cell pellets following exposure with Glucuronide. It was not possible to quantify any intracellular amounts of Glucuronide or its parent compound as it was at or below the LOD.



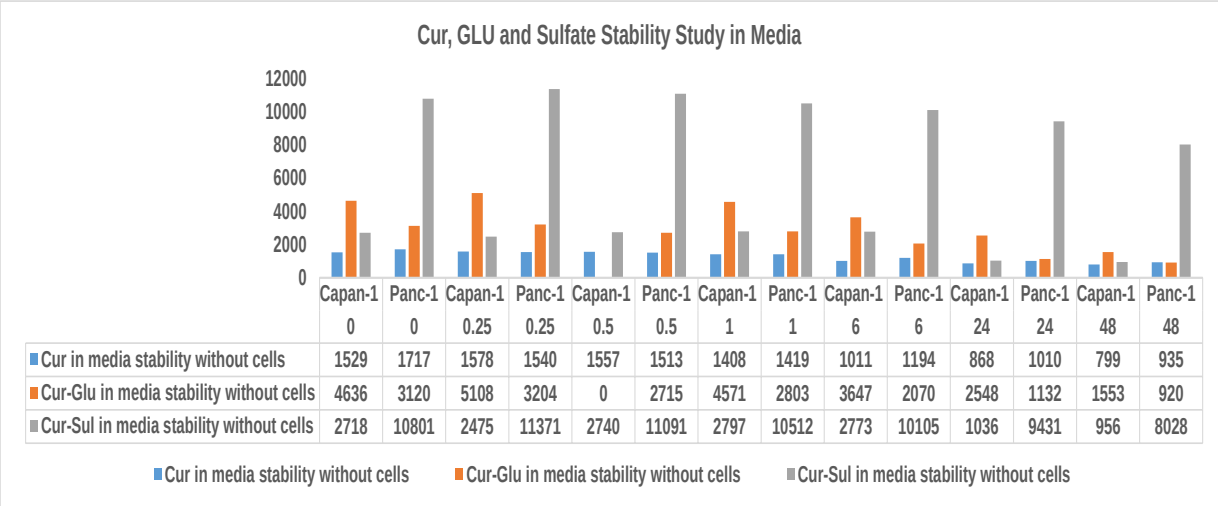
7.13 Representative HPLC-UV chromatograms of Panc-1 cell pellets following exposure with sulfate. It was not possible to quantify any intracellular amounts of sulfate or its parent compound as it was at or below the LOD.



7.14 Levels of curcumin, curcumin; curcumin mono-glucuronide and curcumin mono-sulfate in cells and media. Following treatment of Capan-1 and Panc-1 cell lines with 5uM curcumin, curcumin glucuronide and curcumin sulfate over 48 h. N=3, ±S.D.

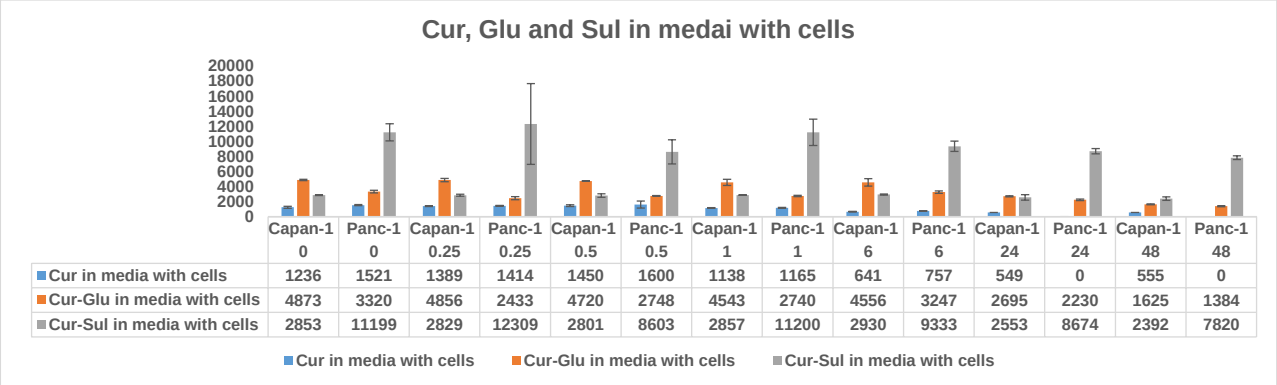
		Capan-1		Panc-1	
		Maximum Concentration	**Time (h)**	**Maximum concentration**	**Time (h)**
[Media] (ng/mL)	**Curcumin**	1450±41 ng/ml	0.5	1521±66 ng/ml	0
	Glucuronide	4873±90 ng/ml	0	3320±163 ng/ml	0
	Sulfate	2853±46 ng/ml	0	11199±167 ng/ml	0
[Intracellular] (ng/mg)	**Curcumin**	0.33±0.07 ng/mg	1	3.22±0.18 ng/mg	0.5
	Glucuronide		0		
	Sulfate	At or below LOD	0	At or below LOD	0

7.15 Only curcumin and it metabolites in media to study their stabilities.



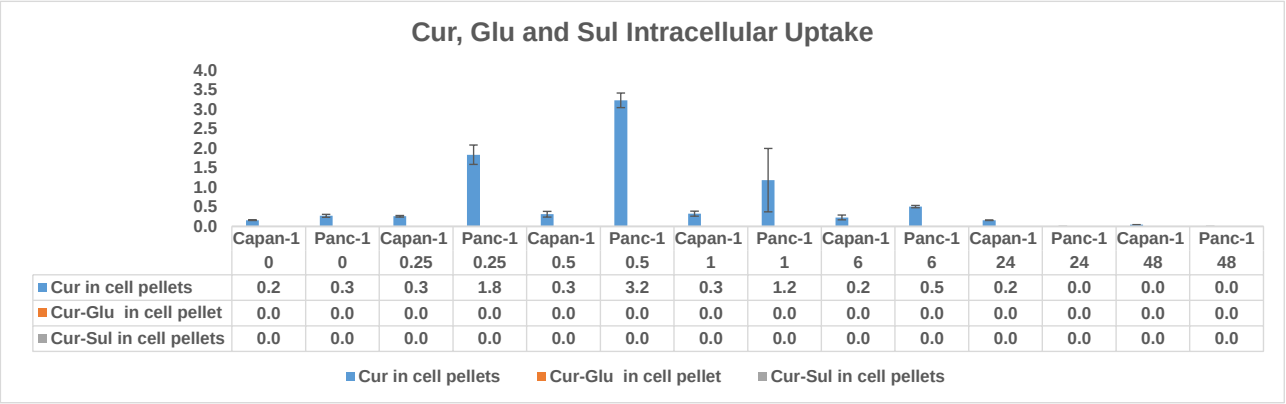
	Capan-1 0	Panc-1 0	Capan-1 0.25	Panc-1 0.25	Capan-1 0.5	Panc-1 0.5	Capan-1 1	Panc-1 1	Capan-1 6	Panc-1 6	Capan-1 24	Panc-1 24	Capan-1 48	Panc-1 48
Cur in media stability without cells	1529	1717	1578	1540	1557	1513	1408	1419	1011	1194	868	1010	799	935
Cur-Glu in media stability without cells	4636	3120	5108	3204	0	2715	4571	2803	3647	2070	2548	1132	1553	920
Cur-Sul in media stability without cells	2718	10801	2475	11371	2740	11091	2797	10512	2773	10105	1036	9431	956	8028

7.16 Curcumin and its metabolites after exposed to media with cells for 48 h.



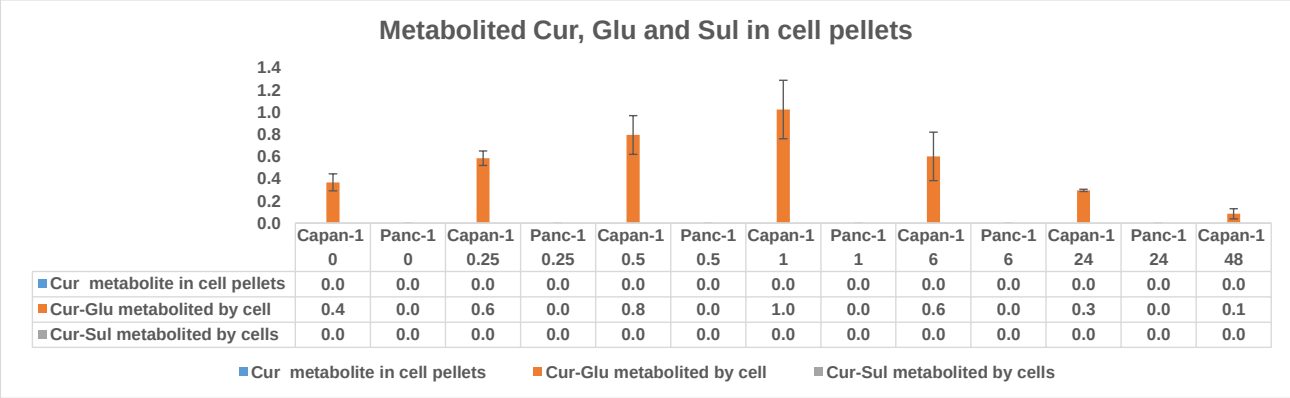
	Capan-1 0	Panc-1 0	Capan-1 0.25	Panc-1 0.25	Capan-1 0.5	Panc-1 0.5	Capan-1 1	Panc-1 1	Capan-1 6	Panc-1 6	Capan-1 24	Panc-1 24	Capan-1 48	Panc-1 48
Cur in media with cells	1236	1521	1389	1414	1450	1600	1138	1165	641	757	549	0	555	0
Cur-Glu in media with cells	4873	3320	4856	2433	4720	2748	4543	2740	4556	3247	2695	2230	1625	1384
Cur-Sul in media with cells	2853	11199	2829	12309	2801	8603	2857	11200	2930	9333	2553	8674	2392	7820

7.17 Curcumin and its metabolites after exposed to cells for 48 h and extraction was carried out from cell pellets.



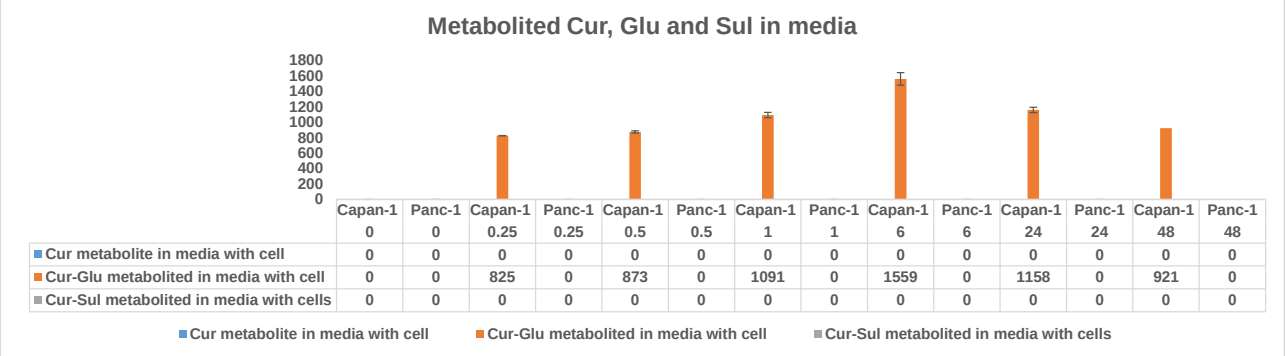
	Capan-1 0	Panc-1 0	Capan-1 0.25	Panc-1 0.25	Capan-1 0.5	Panc-1 0.5	Capan-1 1	Panc-1 1	Capan-1 6	Panc-1 6	Capan-1 24	Panc-1 24	Capan-1 48	Panc-1 48
Cur in cell pellets	0.2	0.3	0.3	1.8	0.3	3.2	0.3	1.2	0.2	0.5	0.2	0.0	0.0	0.0
Cur-Glu in cell pellet	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cur-Sul in cell pellets	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

7.18 Curcumin only exposed to cells for 48 h and its metabolites were extracted from cell pellet.



	Capan-1 0	Panc-1 0	Capan-1 0.25	Panc-1 0.25	Capan-1 0.5	Panc-1 0.5	Capan-1 1	Panc-1 1	Capan-1 6	Panc-1 6	Capan-1 24	Panc-1 24	Capan-1 48
Cur metabolite in cell pellets	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cur-Glu metabolited by cell	0.4	0.0	0.6	0.0	0.8	0.0	1.0	0.0	0.6	0.0	0.3	0.0	0.1
Cur-Sul metabolited by cells	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

7.19 curcumin only exposed to cells for 48 h and its metabolites were extracted from cell media.



	Capan-1 0	Panc-1 0	Capan-1 0.25	Panc-1 0.25	Capan-1 0.5	Panc-1 0.5	Capan-1 1	Panc-1 1	Capan-1 6	Panc-1 6	Capan-1 24	Panc-1 24	Capan-1 48	Panc-1 48
Cur metabolite in media with cell	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cur-Glu metabolited in media with cell	0	0	825	0	873	0	1091	0	1559	0	1158	0	921	0
Cur-Sul metabolited in media with cells	0	0	0	0	0	0	0	0	0	0	0	0	0	0

8. References

1. Foulds L. The experimental study of tumor progression: a review. Cancer research. 1954;14(5):327-39.
2. Renan MJ. How many mutations are required for tumorigenesis? Implications from human cancer data. Molecular carcinogenesis. 1993;7(3):139-46.
3. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194(4260):23-8.
4. Schedin P, Elias A. Multistep tumorigenesis and the microenvironment. Breast Cancer Research. 2004;6(2):93-101.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
6. Bishop JM. The molecular genetics of cancer. Science. 1987;235(4786):305-11.
7. Ponder BA. Molecular genetics of cancer. BMJ : British Medical Journal. 1992;304(6836):1234-6.
8. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer. 2001;1(2):157-62.
9. Loechler EL. Environmental Carcinogens and Mutagens. eLS: John Wiley & Sons, Ltd; 2001.
10. Pitot HC. Multistage carcinogenesis--genetic and epigenetic mechanisms in relation to cancer prevention. Cancer detection and prevention. 1993;17(6):567-73.
11. Pitot HC. The molecular biology of carcinogenesis. Cancer. 1993;72(3 Suppl):962-70.
12. Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. Environmental health perspectives. 1993;100:9-20.
13. Frank SA. Dynamics of Cancer: Incidence, Inheritance, and Evolution. Princeton (NJ): Princeton University Press
Steven A Frank; 2007.
14. UK CR. Cancer Research UK 2015 [updated 2015]. Available from: http://www.cancerresearchuk.org/.
15. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367(6464):645-8.
16. Chen J-H, Hales CN, Ozanne SE. DNA damage, cellular senescence and organismal ageing: causal or correlative? Nucleic Acids Research. 2007;35(22):7417-28.
17. Ralston A. Environmental mutagens, cell signalling and DNA repair. Nature Education 2008; 1(1):114: 1():114.
18. Pray L. DNA replication and causes of mutation. Nature Education. Nature Education. 2008;1(1):214.
19. Surh Y-J. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer. 2003;3(10):768-80.
20. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
21. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicologic pathology. 2007;35(4):495-516.
22. Olsson M, Zhivotovsky B. Caspases and cancer. Cell death and differentiation. 2011;18(9):1441-9.

23. Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. Trends in cell biology. 1995;5(1):21-6.
24. Cohen GM. Caspases: the executioners of apoptosis. The Biochemical journal. 1997;326 (Pt 1):1-16.
25. McDonald ER, III, El-Deiry W. Mammalian Cell Death Pathways. In: El-Deiry W, editor. Death Receptors in Cancer Therapy. Cancer Drug Discovery and Development: Humana Press; 2005. p. 1-41.
26. Weinberg RA. The Biology Of Cancer: Sri Harsha; 2007.
27. Choudhuri T, Pal S, Das T, Sa G. Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. The Journal of biological chemistry. 2005;280(20):20059-68.
28. Sa G, Das T. Anti cancer effects of curcumin: cycle of life and death. Cell division. 2008;3:14.
29. Diehl JA. Cycling to cancer with cyclin D1. Cancer biology & therapy. 2002;1(3):226-31.
30. Pandol SJ SRCMCLS. The Exocrine Pancreas 2010.
31. Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. Gut. 2007;56(8):1134-52.
32. Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer. 2002;2(12):897-909.
33. De La OJ, Murtaugh LC. Notch and Kras in pancreatic cancer: at the crossroads of mutation, differentiation and signaling. Cell cycle (Georgetown, Tex). 2009;8(12):1860-4.
34. Chen A, Xu J. Activation of PPAR{gamma} by curcumin inhibits Moser cell growth and mediates suppression of gene expression of cyclin D1 and EGFR. American journal of physiology Gastrointestinal and liver physiology. 2005;288(3):G447-56.
35. Larsson SC, Hakanson N, Permert J, Wolk A. Meat, fish, poultry and egg consumption in relation to risk of pancreatic cancer: a prospective study. International journal of cancer Journal international du cancer. 2006;118(11):2866-70.
36. Wong HH, Lemoine NR. Pancreatic cancer: molecular pathogenesis and new therapeutic targets. Nature reviews Gastroenterology & hepatology. 2009;6(7):412-22.
37. Hidalgo M. Pancreatic cancer. The New England journal of medicine. 2010;362(17):1605-17.
38. Cascinu S, Falconi M, Valentini V, Jelic S. Pancreatic cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2010;21 Suppl 5:v55-8.
39. Yachida S, White CM, Naito Y, Zhong Y, Brosnan JA, Macgregor-Das AM, et al. Clinical significance of the genetic landscape of pancreatic cancer and implications for identification of potential long-term survivors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(22):6339-47.
40. Macgregor-Das AM, Iacobuzio-Donahue CA. Molecular pathways in pancreatic carcinogenesis. Journal of surgical oncology. 2013;107(1):8-14.
41. Saiki Y, Horii A. Molecular pathology of pancreatic cancer. Pathology International. 2014;64(1):10-9.
42. Gungor C, Hofmann BT, Wolters-Eisfeld G, Bockhorn M. Pancreatic cancer. British journal of pharmacology. 2014;171(4):849-58.
43. Fukushige S, Horii A. Road to early detection of pancreatic cancer: Attempts to utilize epigenetic biomarkers. Cancer letters. 2014;342(2):231-7.

44. Wang P, Chen L, Zhang J, Chen H, Fan J, Wang K, et al. Methylation-mediated silencing of the miR-124 genes facilitates pancreatic cancer progression and metastasis by targeting Rac1. Oncogene. 2014;33(4):514-24.
45. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell. 1988;53(4):549-54.
46. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes & development. 2006;20(10):1218-49.
47. Javle M, Li Y, Tan D, Dong X, Chang P, Kar S, et al. Biomarkers of TGF-beta signaling pathway and prognosis of pancreatic cancer. PLoS One. 2014;9(1):e85942.
48. Boschman CR, Stryker S, Reddy JK, Rao MS. Expression of p53 protein in precursor lesions and adenocarcinoma of human pancreas. The American journal of pathology. 1994;145(6):1291-5.
49. Tempero MA, Arnoletti JP, Behrman S, Ben-Josef E, Benson AB, Berlin JD, et al. Pancreatic Adenocarcinoma: Clinical Practice Guidelines in Oncology. Journal of the National Comprehensive Cancer Network : JNCCN. 2010;8(9):972-1017.
50. Distler M, Aust D, Weitz J, Pilarsky C, Grützmann R. Precursor lesions for sporadic pancreatic cancer: panIN, IPMN, and MCN. BioMed research international. 2014;2014.
51. Hruban RH, Maitra A, Kern SE, Goggins M. Precursors to Pancreatic Cancer. Gastroenterology clinics of North America. 2007;36(4):831-vi.
52. Neesse A, Michl P, Frese KK, Feig C, Cook N, Jacobetz MA, et al. Stromal biology and therapy in pancreatic cancer. Gut. 2011;60(6):861-8.
53. Evans A, Costello E. The role of inflammatory cells in fostering pancreatic cancer cell growth and invasion. Frontiers in physiology. 2012;3:270.
54. Steele CW, Jamieson NB, Evans TRJ, McKay CJ, Sansom OJ, Morton JP, et al. Exploiting inflammation for therapeutic gain in pancreatic cancer. Br J Cancer. 2013;108(5):997-1003.
55. . Journal of Korean medical science.
56. Ling S, Feng T, Jia K, Tian YU, Li YAN. Inflammation to cancer: The molecular biology in the pancreas (Review). Oncology Letters. 2014;7(6):1747-54.
57. Nitecki SS, Sarr MG, Colby TV, van Heerden JA. Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? Ann Surg. 1995;221(1):59-66.
58. Yamamoto T, Yagi S, Kinoshita H, Sakamoto Y, Okada K, Uryuhara K, et al. Long-term survival after resection of pancreatic cancer: a single-center retrospective analysis. World J Gastroenterol. 2015;21(1):262-8.
59. Vikas Bhardwaj SMT, James C.K. Lai and Alok Bhushan. , Prof. Sanjay Srivastava (Ed.). Failure of Pancreatic Cancer Chemotherapy: Consequences of Drug Resistance Mechanisms, Pancreatic Cancer - Molecular Mechanism and Targets. InTech Journals. 2012.
60. Kaiser MH, Ellenberg SS. Pancreatic cancer: Adjuvant combined radiation and chemotherapy following curative resection. Archives of Surgery. 1985;120(8):899-903.
61. Moertel CG, Frytak S, Hahn RG, O'Connell MJ, Reitemeier RJ, Rubin J, et al. Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil: The Gastrointestinal Tumor Study Group. Cancer. 1981;48(8):1705-10.

62. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. The New England journal of medicine. 2011;364(19):1817-25.
63. Snady H, Bruckner H, Cooperman A, Paradiso J, Kiefer L. Survival advantage of combined chemoradiotherapy compared with resection as the initial treatment of patients with regional pancreatic carcinoma. An outcomes trial. Cancer. 2000;89(2):314-27.
64. Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1997;15(6):2403-13.
65. Shore S, Raraty MG, Ghaneh P, Neoptolemos JP. Review article: chemotherapy for pancreatic cancer. Alimentary pharmacology & therapeutics. 2003;18(11-12):1049-69.
66. Spears CP, Shani J, Shahinian AH, Wolf W, Heidelberger C, Danenberg PV. Assay and time course of 5-fluorouracil incorporation into RNA of L1210/0 ascites cells in vivo. Molecular pharmacology. 1985;27(2):302-7.
67. Ghoshal K, Jacob ST. An alternative molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. Biochem Pharmacol. 1997;53(11):1569-75.
68. Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003;3(5):330-8.
69. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. Cancer research. 1991;51(22):6110-7.
70. de Sousa Cavalcante L, Monteiro G. Gemcitabine: Metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. European Journal of Pharmacology. 2014;741(0):8-16.
71. Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer research. 1998;58(19):4349-57.
72. Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, et al. The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(20):6956-61.
73. Guidance on the use of gemcitabine for the treatment of pancreatic cancer [Internet]. 2001. Available from: https://www.nice.org.uk/guidance/ta25/resources/guidance-guidance-on-the-use-of-gemcitabine-for-the-treatment-of-pancreatic-cancer-pdf.
74. Seufferlein T, Bachet JB, Van Cutsem E, Rougier P. Pancreatic adenocarcinoma: ESMO-ESDO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2012;23 Suppl 7:vii33-40.
75. Vaccaro V, Sperduti I, Vari S, Bria E, Melisi D, Garufi C, et al. Metastatic pancreatic cancer: Is there a light at the end of the tunnel? World Journal of Gastroenterology : WJG. 2015;21(16):4788-801.
76. Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development (Cambridge, England). 1990;110(4):1001-20.
77. Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. Physiological reviews. 2005;85(2):635-78.

78. Burdon T, Chambers I, Stracey C, Niwa H, Smith A. Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. Cells, tissues, organs. 1999;165(3-4):131-43.
79. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105-11.
80. Dick JE. Stem cell concepts renew cancer research. Blood. 2008;112(13):4793-807.
81. Clevers H. The cancer stem cell: premises, promises and challenges. Nature medicine. 2011;17(3):313-9.
82. Lander AD. The 'stem cell' concept: is it holding us back? Journal of biology. 2009;8(8):70.
83. Visvader JE. Cells of origin in cancer. Nature. 2011;469(7330):314-22.
84. Rosen JM, Jordan CT. The increasing complexity of the cancer stem cell paradigm. Science. 2009;324(5935):1670-3.
85. J. F. The Transmission of Leukemia of Mice with a Single Cell. American Association for Cancer Research. 1937;31; 276.
86. Medema JP. Cancer stem cells: The challenges ahead. Nat Cell Biol. 2013;15(4):338-44.
87. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100(7):3983-8.
88. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature. 2007;445(7123):111-5.
89. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer research. 2005;65(23):10946-51.
90. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. Cell death and differentiation. 2008;15(3):504-14.
91. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer research. 2007;67(3):1030-7.
92. M. K. Culture and Assay Systems Utilized for Cancer Stem Cell Research 2012. Available from: bdbiosciences.com.
93. Borovski T, De Sousa EMF, Vermeulen L, Medema JP. Cancer stem cell niche: the place to be. Cancer research. 2011;71(3):634-9.
94. Li Y, Laterra J. Cancer stem cells: distinct entities or dynamically regulated phenotypes? Cancer research. 2012;72(3):576-80.
95. Chen K, Huang Y-h, Chen J-l. Understanding and targeting cancer stem cells: therapeutic implications and challenges. Acta pharmacologica Sinica. 2013;34(6):732-40.
96. Ahmed Hama PC, and Maryam Mehrpour.
CANCER STEM CELLS AND AUTOPHAGY: FACTS AND PERSPECTIVES. ournal of Cancer Stem Cell Research. 2014;2:e1005 (July 07, 2014)

97. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 1992;255(5052):1707-10.
98. Wang YJ, Bailey JM, Rovira M, Leach SD. Sphere-forming assays for assessment of benign and malignant pancreatic stem cells. Methods in molecular biology (Clifton, NJ). 2013;980:281-90.

99. Gou S, Liu T, Wang C, Yin T, Li K, Yang M, et al. Establishment of clonal colony-forming assay for propagation of pancreatic cancer cells with stem cell properties. Pancreas. 2007;34(4):429-35.
100. Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. Cell stem cell. 2011;8(5):486-98.
101. Gottesman MM. Mechanisms of cancer drug resistance. Annual review of medicine. 2002;53:615-27.
102. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5(4):275-84.
103. Moitra K, Lou H, Dean M. Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development. Clinical pharmacology and therapeutics. 2011;89(4):491-502.
104. Vinogradov S, Wei X. Cancer stem cells and drug resistance: the potential of nanomedicine. Nanomedicine (London, England). 2012;7(4):597-615.
105. Simeone DM. Pancreatic cancer stem cells: implications for the treatment of pancreatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(18):5646-8.
106. Vaz A. A concise review on the current understanding of pancreatic cancer stem cells. Cancer Stem Cell Research 2014. 2014;2:e1004.
107. Rasheed ZA, Yang J, Wang Q, Kowalski J, Freed I, Murter C, et al. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. Journal of the National Cancer Institute. 2010;102(5):340-51.
108. Li C, Wu JJ, Hynes M, Dosch J, Sarkar B, Welling TH, et al. c-Met is a marker of pancreatic cancer stem cells and therapeutic target. Gastroenterology. 2011;141(6):2218-27.e5.
109. Fitzgerald TL, McCubrey JA. Pancreatic cancer stem cells: association with cell surface markers, prognosis, resistance, metastasis and treatment. Advances in biological regulation. 2014;56:45-50.
110. Significance of CD44 and CD24 as Cancer Stem Cell Markers: An Enduring Ambiguity. Clinical and Developmental Immunology. 2012;2012:11.
111. Hong WK, Spitz MR, Lippman SM. Cancer chemoprevention in the 21st century: genetics, risk modeling, and molecular targets. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2000;18(21 Suppl):9s-18s.
112. Wei HJ, Yin T, Zhu Z, Shi PF, Tian Y, Wang CY. Expression of CD44, CD24 and ESA in pancreatic adenocarcinoma cell lines varies with local microenvironment. Hepatobiliary & pancreatic diseases international : HBPD INT. 2011;10(4):428-34.
113. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell stem cell. 2007;1(3):313-23.
114. Lee HJ, You DD, Choi DW, Choi YS, Kim SJ, Won YS, et al. Significance of CD133 as a cancer stem cell markers focusing on the tumorigenicity of pancreatic cancer cell lines. Journal of the Korean Surgical Society. 2011;81(4):263-70.
115. Kim MP, Fleming JB, Wang H, Abbruzzese JL, Choi W, Kopetz S, et al. ALDH activity selectively defines an enhanced tumor-initiating cell population relative to CD133 expression in human pancreatic adenocarcinoma. PLoS One. 2011;6(6):e20636.
116. Duffy MJ, Shering S, Sherry F, McDermott E, O'Higgins N. CA 15-3: a prognostic marker in breast cancer. The International journal of biological markers. 2000;15(4):330-3.
117. Duong HQ, Yi YW, Kang HJ, Bae I, Jang YJ, Kwak SJ, et al. Combination of dasatinib and gemcitabine reduces the ALDH1A1 expression and the proliferation of

gemcitabine-resistant pancreatic cancer MIA PaCa-2 cells. International journal of oncology. 2014;44(6):2132-8.
118. Abel EV, Simeone DM. Biology and clinical applications of pancreatic cancer stem cells. Gastroenterology. 2013;144(6):1241-8.
119. Wen J, Park JY, Park KH, Chung HW, Bang S, Park SW, et al. Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. Pancreas. 2010;39(5):622-6.
120. Herreros-Villanueva M, Bujanda L, Billadeau DD, Zhang JS. Embryonic stem cell factors and pancreatic cancer. World J Gastroenterol. 2014;20(9):2247-54.
121. Lu Y, Zhu H, Shan H, Lu J, Chang X, Li X, et al. Knockdown of Oct4 and Nanog expression inhibits the stemness of pancreatic cancer cells. Cancer letters. 2013;340(1):113-23.
122. Wang X, Liu Q, Hou B, Zhang W, Yan M, Jia H, et al. Concomitant targeting of multiple key transcription factors effectively disrupts cancer stem cells enriched in side population of human pancreatic cancer cells. PLoS One. 2013;8(9):e73942.
123. Liu A, Yu X, Liu S. Pluripotency transcription factors and cancer stem cells: small genes make a big difference. Chinese journal of cancer. 2013;32(9):483-7.
124. Polvani S, Tarocchi M, Tempesti S, Mello T, Ceni E, Buccoliero F, et al. COUP-TFII in pancreatic adenocarcinoma: clinical implication for patient survival and tumor progression. International journal of cancer Journal international du cancer. 2014;134(7):1648-58.
125. Lonardo E, Hermann PC, Mueller MT, Huber S, Balic A, Miranda-Lorenzo I, et al. Nodal/Activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. Cell stem cell. 2011;9(5):433-46.
126. Luo W, Li S, Peng B, Ye Y, Deng X, Yao K. Embryonic stem cells markers SOX2, OCT4 and Nanog expression and their correlations with epithelial-mesenchymal transition in nasopharyngeal carcinoma. PLoS One. 2013;8(2):e56324.
127. Herreros-Villanueva M, Zhang JS, Koenig A, Abel EV, Smyrk TC, Bamlet WR, et al. SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells. Oncogenesis. 2013;2:e61.
128. Wang Z, Oron E, Nelson B, Razis S, Ivanova N. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell stem cell. 2012;10(4):440-54.
129. Hage C, Rausch V, Giese N, Giese T, Schonsiegel F, Labsch S, et al. The novel c-Met inhibitor cabozantinib overcomes gemcitabine resistance and stem cell signaling in pancreatic cancer. Cell death & disease. 2013;4:e627.
130. Rajeshkumar NV, Rasheed ZA, Garcia-Garcia E, Lopez-Rios F, Fujiwara K, Matsui WH, et al. A combination of DR5 agonistic monoclonal antibody with gemcitabine targets pancreatic cancer stem cells and results in long-term disease control in human pancreatic cancer model. Molecular cancer therapeutics. 2010;9(9):2582-92.
131. Jimeno A, Feldmann G, Suarez-Gauthier A, Rasheed Z, Solomon A, Zou GM, et al. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Molecular cancer therapeutics. 2009;8(2):310-4.
132. Feldmann G, Dhara S, Fendrich V, Bedja D, Beaty R, Mullendore M, et al. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. Cancer research. 2007;67(5):2187-96.
133. Feldmann G, Fendrich V, McGovern K, Bedja D, Bisht S, Alvarez H, et al. An orally bioavailable small-molecule inhibitor of Hedgehog signaling inhibits tumor

initiation and metastasis in pancreatic cancer. Molecular cancer therapeutics. 2008;7(9):2725-35.
134. Mullendore ME, Koorstra JB, Li YM, Offerhaus GJ, Fan X, Henderson CM, et al. Ligand-dependent Notch signaling is involved in tumor initiation and tumor maintenance in pancreatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(7):2291-301.
135. Zhang GN, Liang Y, Zhou LJ, Chen SP, Chen G, Zhang TP, et al. Combination of salinomycin and gemcitabine eliminates pancreatic cancer cells. Cancer letters. 2011;313(2):137-44.
136. Li L, Hao X, Qin J, Tang W, He F, Smith A, et al. Antibody against CD44s inhibits pancreatic tumor initiation and postradiation recurrence in mice. Gastroenterology. 2014;146(4):1108-18.
137. Padhye SS, Guin S, Yao HP, Zhou YQ, Zhang R, Wang MH. Sustained expression of the RON receptor tyrosine kinase by pancreatic cancer stem cells as a potential targeting moiety for antibody-directed chemotherapeutics. Molecular pharmaceutics. 2011;8(6):2310-9.
138. Rausch V, Liu L, Kallifatidis G, Baumann B, Mattern J, Gladkich J, et al. Synergistic activity of sorafenib and sulforaphane abolishes pancreatic cancer stem cell characteristics. Cancer research. 2010;70(12):5004-13.
139. Yen WC, Fischer MM, Hynes M, Wu J, Kim E, Beviglia L, et al. Anti-DLL4 has broad spectrum activity in pancreatic cancer dependent on targeting DLL4-Notch signaling in both tumor and vasculature cells. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(19):5374-86.
140. Erkan M, Adler G, Apte MV, Bachem MG, Buchholz M, Detlefsen S, et al. StellaTUM: current consensus and discussion on pancreatic stellate cell research. Gut. 2012;61(2):172-8.
141. Wang Z, Li J, Chen X, Duan W, Ma Q, Li X. Disrupting the Balance between Tumor Epithelia and Stroma is a Possible Therapeutic Approach for Pancreatic Cancer. Medical science monitor : international medical journal of experimental and clinical research. 2014;20:2002-6.
142. Al-Assar O, Demiciorglu F, Lunardi S, Gaspar-Carvalho MM, McKenna WG, Muschel RM, et al. Contextual regulation of pancreatic cancer stem cell phenotype and radioresistance by pancreatic stellate cells. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2014;111(2):243-51.
143. Rodova M, Fu J, Watkins DN, Srivastava RK, Shankar S. Sonic hedgehog signaling inhibition provides opportunities for targeted therapy by sulforaphane in regulating pancreatic cancer stem cell self-renewal. PLoS One. 2012;7(9):e46083.
144. Gao Z, Wang X, Wu K, Zhao Y, Hu G. Pancreatic stellate cells increase the invasion of human pancreatic cancer cells through the stromal cell-derived factor-1/CXCR4 axis. Pancreatology : official journal of the International Association of Pancreatology (IAP) [et al]. 2010;10(2-3):186-93.
145. Spector I, Honig H, Kawada N, Nagler A, Genin O, Pines M. Inhibition of pancreatic stellate cell activation by halofuginone prevents pancreatic xenograft tumor development. Pancreas. 2010;39(7):1008-15.
146. Froeling FE, Feig C, Chelala C, Dobson R, Mein CE, Tuveson DA, et al. Retinoic acid-induced pancreatic stellate cell quiescence reduces paracrine Wnt-beta-catenin signaling to slow tumor progression. Gastroenterology. 2011;141(4):1486-97, 97.e1-14.
147. Ansari D, Chen BC, Dong L, Zhou MT, Andersson R. Pancreatic cancer: translational research aspects and clinical implications. World J Gastroenterol. 2012;18(13):1417-24.

148. Sporn MB. Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer research. 1976;36(7 pt 2):2699-702.
149. Wu X, Patterson S, Hawk E. Chemoprevention--history and general principles. Best practice & research Clinical gastroenterology. 2011;25(4-5):445-59.
150. Davis JS, Wu X. Current state and future challenges of chemoprevention. Discovery medicine. 2012;13(72):385-90.
151. Steward WP, Brown K. Cancer chemoprevention: a rapidly evolving field. Br J Cancer. 2013;109(1):1-7.
152. Levin B. An overview of preventive strategies for pancreatic cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 1999;10 Suppl 4:193-6.
153. Tsao AS, Kim ES, Hong WK. Chemoprevention of cancer. CA: a cancer journal for clinicians. 2004;54(3):150-80.
154. Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2010;154(2):103-16.
155. Sheweita SA. Drug-metabolizing enzymes: mechanisms and functions. Curr Drug Metab. 2000;1(2):107-32.
156. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. Journal of the National Cancer Institute. 1998;90(18):1371-88.
157. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. Jama. 2006;295(23):2727-41.
158. Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG, et al. The influence of finasteride on the development of prostate cancer. The New England journal of medicine. 2003;349(3):215-24.
159. Redman MW, Tangen CM, Goodman PJ, Lucia MS, Coltman CA, Jr., Thompson IM. Finasteride does not increase the risk of high-grade prostate cancer: a bias-adjusted modeling approach. Cancer prevention research (Philadelphia, Pa). 2008;1(3):174-81.
160. Andriole GL, Kirby R. Safety and Tolerability of the Dual 5α-Reductase Inhibitor Dutasteride in the Treatment of Benign Prostatic Hyperplasia. European Urology. 2003;44(1):82-8.
161. Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet. 2007;369(9573):1603-13.
162. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. The Lancet Oncology. 2012;13(5):518-27.
163. Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S, et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. Lancet. 2011;378(9809):2081-7.
164. Zhang X, Smith-Warner SA, Chan AT, Wu K, Spiegelman D, Fuchs CS, et al. Aspirin use, body mass index, physical activity, plasma C-peptide, and colon cancer risk in US health professionals. American journal of epidemiology. 2011;174(4):459-67.
165. Barnes CJ, Lee M. Chemoprevention of spontaneous intestinal adenomas in the adenomatous polyposis coli Min mouse model with aspirin. Gastroenterology. 1998;114(5):873-7.

166. Rothwell PM, Price JF, Fowkes FG, Zanchetti A, Roncaglioni MC, Tognoni G, et al. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. Lancet. 2012;379(9826):1602-12.
167. Parkin DM, Boyd L, Walker LC. 16. The fraction of cancer attributable to lifestyle and environmental factors in the UK in 2010. Br J Cancer. 2011;105(S2):S77-S81.
168. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. Journal of the National Cancer Institute. 1981;66(6):1191-308.
169. Dorai T, Aggarwal BB. Role of chemopreventive agents in cancer therapy. Cancer letters. 2004;215(2):129-40.
170. Li Y, Ellis KL, Ali S, El-Rayes BF, Nedeljkovic-Kurepa A, Kucuk O, et al. Apoptosis-inducing effect of chemotherapeutic agents is potentiated by soy isoflavone genistein, a natural inhibitor of NF-kappaB in BxPC-3 pancreatic cancer cell line. Pancreas. 2004;28(4):e90-5.
171. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science. 1997;275(5297):218-20.
172. Cui J, Sun R, Yu Y, Gou S, Zhao G, Wang C. Antiproliferative effect of resveratrol in pancreatic cancer cells. Phytotherapy research : PTR. 2010;24(11):1637-44.
173. Patel KR, Brown VA, Jones DJ, Britton RG, Hemingway D, Miller AS, et al. Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. Cancer research. 2010;70(19):7392-9.
174. Howells LM, Moiseeva EP, Neal CP, Foreman BE, Andreadi CK, Sun YY, et al. Predicting the physiological relevance of in vitro cancer preventive activities of phytochemicals. Acta pharmacologica Sinica. 2007;28(9):1274-304.
175. Brown VA, Patel KR, Viskaduraki M, Crowell JA, Perloff M, Booth TD, et al. Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis. Cancer research. 2010;70(22):9003-11.
176. Oommen S, Anto RJ, Srinivas G, Karunagaran D. Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. Eur J Pharmacol. 2004;485(1-3):97-103.
177. Shakibaei M, Mobasheri A, Lueders C, Busch F, Shayan P, Goel A. Curcumin enhances the effect of chemotherapy against colorectal cancer cells by inhibition of NF-kappaB and Src protein kinase signaling pathways. PLoS One. 2013;8(2):e57218.
178. Goel A, Kunnumakkara AB, Aggarwal BB. Curcumin as "Curecumin": From kitchen to clinic. Biochemical Pharmacology. 2008;75(4):787-809.
179. Prasad S, Tyagi AK, Aggarwal BB. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res Treat. 2014;46(1):2-18.
180. Shishodia S, Chaturvedi MM, Aggarwal BB. Role of curcumin in cancer therapy. Current problems in cancer. 2007;31(4):243-305.
181. Prasad S, Gupta SC, Tyagi AK, Aggarwal BB. Curcumin, a component of golden spice: from bedside to bench and back. Biotechnology advances. 2014;32(6):1053-64.
182. Kunnumakkara AB, Guha S, Krishnan S, Diagaradjane P, Gelovani J, Aggarwal BB. Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and inhibition of nuclear factor-kappaB-regulated gene products. Cancer research. 2007;67(8):3853-61.

183. Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer research. 2003;23(1a):363-98.
184. Aggarwal BB, Harikumar KB. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. The international journal of biochemistry & cell biology. 2009;41(1):40-59.
185. Irving GRB, Howells LM, Sale S, Kralj-Hans I, Atkin WS, Clark SK, et al. Prolonged biologically active colonic tissue levels of curcumin achieved after oral administration - a clinical pilot study including assessment of patient acceptability. Cancer prevention research (Philadelphia, Pa). 2013;6(2):119-28.
186. Howells LM, Mahale J, Sale S, McVeigh L, Steward WP, Thomas A, et al. Translating curcumin to the clinic for lung cancer prevention: evaluation of the preclinical evidence for its utility in primary, secondary, and tertiary prevention strategies. The Journal of pharmacology and experimental therapeutics. 2014;350(3):483-94.
187. Li Y, Zhang T. Targeting cancer stem cells by curcumin and clinical applications. Cancer letters. 2014;346(2):197-205.
188. Stan SD, Singh SV, Brand RE. Chemoprevention strategies for pancreatic cancer. Nature reviews Gastroenterology & hepatology. 2010;7(6):347-56.
189. Lev-Ari S, Vexler A, Starr A, Ashkenazy-Voghera M, Greif J, Aderka D, et al. Curcumin augments gemcitabine cytotoxic effect on pancreatic adenocarcinoma cell lines. Cancer investigation. 2007;25(6):411-8.
190. Lev-Ari S, Zinger H, Kazanov D, Yona D, Ben-Yosef R, Starr A, et al. Curcumin synergistically potentiates the growth inhibitory and pro-apoptotic effects of celecoxib in pancreatic adenocarcinoma cells. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2005;59 Suppl 2:S276-80.
191. Mukherjee P, Basu GD, Tinder TL, Subramani DB, Bradley JM, Arefayene M, et al. Progression of pancreatic adenocarcinoma is significantly impeded with a combination of vaccine and COX-2 inhibition. Journal of immunology (Baltimore, Md : 1950). 2009;182(1):216-24.
192. Kallifatidis G, Rausch V, Baumann B, Apel A, Beckermann BM, Groth A, et al. Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. Gut. 2009;58(7):949-63.
193. Harikumar KB, Kunnumakkara AB, Sethi G, Diagaradjane P, Anand P, Pandey MK, et al. Resveratrol, a multitargeted agent, can enhance antitumor activity of gemcitabine in vitro and in orthotopic mouse model of human pancreatic cancer. International journal of cancer Journal international du cancer. 2010;127(2):257-68.
194. Azmi AS, Ahmad A, Banerjee S, Rangnekar VM, Mohammad RM, Sarkar FH. Chemoprevention of pancreatic cancer: characterization of Par-4 and its modulation by 3,3' diindolylmethane (DIM). Pharmaceutical research. 2008;25(9):2117-24.
195. Health tUSNIo. clinicaltrials.gov https://clinicaltrials.gov/: https://clinicaltrials.gov/.
196. Irving GR, Karmokar A, Berry DP, Brown K, Steward WP. Curcumin: the potential for efficacy in gastrointestinal diseases. Best practice & research Clinical gastroenterology. 2011;25(4-5):519-34.
197. Dhillon N, Aggarwal BB, Newman RA, Wolff RA, Kunnumakkara AB, Abbruzzese JL, et al. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(14):4491-9.

198. Heger M, van Golen RF, Broekgaarden M, Michel MC. The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. Pharmacological reviews. 2014;66(1):222-307.
199. Wongcharoen W, Phrommintikul A. The protective role of curcumin in cardiovascular diseases. International journal of cardiology. 2009;133(2):145-51.
200. Fu Y, Zheng S, Lin J, Ryerse J, Chen A. Curcumin protects the rat liver from CCl4-caused injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation. Molecular pharmacology. 2008;73(2):399-409.
201. Baum L, Lam CW, Cheung SK, Kwok T, Lui V, Tsoh J, et al. Six-month randomized, placebo-controlled, double-blind, pilot clinical trial of curcumin in patients with Alzheimer disease. Journal of clinical psychopharmacology. 2008;28(1):110-3.
202. Reddy S, Rishi AK, Xu H, Levi E, Sarkar FH, Majumdar AP. Mechanisms of curcumin- and EGF-receptor related protein (ERRP)-dependent growth inhibition of colon cancer cells. Nutrition and cancer. 2006;55(2):185-94.
203. Xia Y, Jin L, Zhang B, Xue H, Li Q, Xu Y. The potentiation of curcumin on insulin-like growth factor-1 action in MCF-7 human breast carcinoma cells. Life sciences. 2007;80(23):2161-9.
204. Chadalapaka G, Jutooru I, Chintharlapalli S, Papineni S, Smith R, 3rd, Li X, et al. Curcumin decreases specificity protein expression in bladder cancer cells. Cancer research. 2008;68(13):5345-54.
205. Singh S, Aggarwal BB. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. The Journal of biological chemistry. 1995;270(42):24995-5000.
206. Andreadi CK, Howells LM, Atherfold PA, Manson MM. Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols. Molecular pharmacology. 2006;69(3):1033-40.
207. Beevers CS, Li F, Liu L, Huang S. Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. International journal of cancer Journal international du cancer. 2006;119(4):757-64.
208. Verma SP, Goldin BR, Lin PS. The inhibition of the estrogenic effects of pesticides and environmental chemicals by curcumin and isoflavonoids. Environmental health perspectives. 1998;106(12):807-12.
209. Lev-Ari S, Starr A, Vexler A, Karaush V, Loew V, Greif J, et al. Inhibition of pancreatic and lung adenocarcinoma cell survival by curcumin is associated with increased apoptosis, down-regulation of COX-2 and EGFR and inhibition of Erk1/2 activity. Anticancer research. 2006;26(6b):4423-30.
210. Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH. Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. Cancer. 2006;106(11):2503-13.
211. Sun M, Estrov Z, Ji Y, Coombes KR, Harris DH, Kurzrock R. Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells. Molecular cancer therapeutics. 2008;7(3):464-73.
212. Abdollahi A, Aguda BD, Ali S, Alian OM, Amundadottir LT, Azmi AS, et al. Contributors. In: Azmi AS, editor. Molecular Diagnostics and Treatment of Pancreatic Cancer. Oxford: Academic Press; 2014. p. xvii-xx.
213. Kanai M. Therapeutic applications of curcumin for patients with pancreatic cancer. World Journal of Gastroenterology : WJG. 2014;20(28):9384-91.
214. Kanai M, Yoshimura K, Asada M, Imaizumi A, Suzuki C, Matsumoto S, et al. A phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with

gemcitabine-resistant pancreatic cancer. Cancer chemotherapy and pharmacology. 2011;68(1):157-64.
215. Epelbaum R, Schaffer M, Vizel B, Badmaev V, Bar-Sela G. Curcumin and gemcitabine in patients with advanced pancreatic cancer. Nutrition and cancer. 2010;62(8):1137-41.
216. Kanai M, Otsuka Y, Otsuka K, Sato M, Nishimura T, Mori Y, et al. A phase I study investigating the safety and pharmacokinetics of highly bioavailable curcumin (Theracurmin) in cancer patients. Cancer chemotherapy and pharmacology. 2013;71(6):1521-30.
217. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Molecular pharmaceutics. 2007;4(6):807-18.
218. Irving GR, Howells LM, Sale S, Kralj-Hans I, Atkin WS, Clark SK, et al. Prolonged biologically active colonic tissue levels of curcumin achieved after oral administration--a clinical pilot study including assessment of patient acceptability. Cancer prevention research (Philadelphia, Pa). 2013;6(2):119-28.
219. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer research. 2001;21(4b):2895-900.
220. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(20):6847-54.
221. Cuomo J, Appendino G, Dern AS, Schneider E, McKinnon TP, Brown MJ, et al. Comparative absorption of a standardized curcuminoid mixture and its lecithin formulation. Journal of natural products. 2011;74(4):664-9.
222. Ireson C, Orr S, Jones DJ, Verschoyle R, Lim CK, Luo JL, et al. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer research. 2001;61(3):1058-64.
223. Ireson CR, Jones DJ, Orr S, Coughtrie MW, Boocock DJ, Williams ML, et al. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2002;11(1):105-11.
224. Dempe JS, Pfeiffer E, Grimm AS, Metzler M. Metabolism of curcumin and induction of mitotic catastrophe in human cancer cells. Molecular Nutrition & Food Research. 2008;52(9):1074-81.
225. Shoji M, Nakagawa K, Watanabe A, Tsuduki T, Yamada T, Kuwahara S, et al. Comparison of the effects of curcumin and curcumin glucuronide in human hepatocellular carcinoma HepG2 cells. Food Chemistry. 2014;151(0):126-32.
226. Pari L, Murugan P. Tetrahydrocurcumin: effect on chloroquine-mediated oxidative damage in rat kidney. Basic & clinical pharmacology & toxicology. 2006;99(5):329-34.
227. Suresh D, Srinivasan K. Studies on the in vitro absorption of spice principles--curcumin, capsaicin and piperine in rat intestines. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2007;45(8):1437-42.
228. Lin YL, Liu YK, Tsai NM, Hsieh JH, Chen CH, Lin CM, et al. A Lipo-PEG-PEI complex for encapsulating curcumin that enhances its antitumor effects on curcumin-

sensitive and curcumin-resistance cells. Nanomedicine : nanotechnology, biology, and medicine. 2012;8(3):318-27.
229. Fong D, Yeh A, Naftalovich R, Choi TH, Chan MM. Curcumin inhibits the side population (SP) phenotype of the rat C6 glioma cell line: towards targeting of cancer stem cells with phytochemicals. Cancer letters. 2010;293(1):65-72.
230. Zhuang W, Long L, Zheng B, Ji W, Yang N, Zhang Q, et al. Curcumin promotes differentiation of glioma-initiating cells by inducing autophagy. Cancer science. 2012;103(4):684-90.
231. Kakarala M, Brenner DE, Korkaya H, Cheng C, Tazi K, Ginestier C, et al. Targeting breast stem cells with the cancer preventive compounds curcumin and piperine. Breast cancer research and treatment. 2010;122(3):777-85.
232. Almanaa TN, Geusz ME, Jamasbi RJ. Effects of curcumin on stem-like cells in human esophageal squamous carcinoma cell lines. BMC complementary and alternative medicine. 2012;12:195.
233. Yu Y, Kanwar SS, Patel BB, Nautiyal J, Sarkar FH, Majumdar AP. Elimination of Colon Cancer Stem-Like Cells by the Combination of Curcumin and FOLFOX. Translational oncology. 2009;2(4):321-8.
234. Chachay VS, Kirkpatrick CMJ, Hickman IJ, Ferguson M, Prins JB, Martin JH. Resveratrol – pills to replace a healthy diet? British Journal of Clinical Pharmacology. 2011;72(1):27-38.
235. Pervaiz S. Resveratrol--from the bottle to the bedside? Leukemia & lymphoma. 2001;40(5-6):491-8.
236. Matsuoka A, Furuta A, Ozaki M, Fukuhara K, Miyata N. Resveratrol, a naturally occurring polyphenol, induces sister chromatid exchanges in a Chinese hamster lung (CHL) cell line. Mutation research. 2001;494(1-2):107-13.
237. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, et al. Resveratrol: a review of preclinical studies for human cancer prevention. Toxicology and applied pharmacology. 2007;224(3):274-83.
238. Boocock DJ, Faust GE, Patel KR, Schinas AM, Brown VA, Ducharme MP, et al. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2007;16(6):1246-52.
239. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006;444(7117):337-42.
240. Barger JL, Kayo T, Vann JM, Arias EB, Wang J, Hacker TA, et al. A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS One. 2008;3(6):e2264.
241. Niles RM, Cook CP, Meadows GG, Fu YM, McLaughlin JL, Rankin GO. Resveratrol is rapidly metabolized in athymic (nu/nu) mice and does not inhibit human melanoma xenograft tumor growth. The Journal of nutrition. 2006;136(10):2542-6.
242. Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res. 2005;49(5):482-94.
243. Shakibaei M, Csaki C, Nebrich S, Mobasheri A. Resveratrol suppresses interleukin-1beta-induced inflammatory signaling and apoptosis in human articular chondrocytes: potential for use as a novel nutraceutical for the treatment of osteoarthritis. Biochem Pharmacol. 2008;76(11):1426-39.

244. Lee YJ, Hwang IS, Lee YJ, Lee CH, Kim SH, Nam HS, et al. Knockdown of Bcl-xL enhances growth-inhibiting and apoptosis-inducing effects of resveratrol and clofarabine in malignant mesothelioma H-2452 cells. Journal of Korean medical science. 2014;29(11):1464-72.
245. Benitez DA, Hermoso MA, Pozo-Guisado E, Fernandez-Salguero PM, Castellon EA. Regulation of cell survival by resveratrol involves inhibition of NF kappa B-regulated gene expression in prostate cancer cells. The Prostate. 2009;69(10):1045-54.
246. St Leger AS, Cochrane AL, Moore F. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. Lancet. 1979;1(8124):1017-20.
247. Vidavalur R, Otani H, Singal PK, Maulik N. Significance of wine and resveratrol in cardiovascular disease: French paradox revisited. Experimental & Clinical Cardiology. 2006;11(3):217-25.
248. Timmers S, Hesselink MK, Schrauwen P. Therapeutic potential of resveratrol in obesity and type 2 diabetes: new avenues for health benefits? Annals of the New York Academy of Sciences. 2013;1290:83-9.
249. Ma T, Tan M-S, Yu J-T, Tan L. Resveratrol as a Therapeutic Agent for Alzheimer's Disease. BioMed research international. 2014;2014:350516.
250. Nguyen AV, Martinez M, Stamos MJ, Moyer MP, Planutis K, Hope C, et al. Results of a phase I pilot clinical trial examining the effect of plant-derived resveratrol and grape powder on Wnt pathway target gene expression in colonic mucosa and colon cancer. Cancer management and research. 2009;1:25-37.
251. Howells LM, Berry DP, Elliott PJ, Jacobson EW, Hoffmann E, Hegarty B, et al. Phase I randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases--safety, pharmacokinetics, and pharmacodynamics. Cancer prevention research (Philadelphia, Pa). 2011;4(9):1419-25.
252. Popat R, Plesner T, Davies F, Cook G, Cook M, Elliott P, et al. A phase 2 study of SRT501 (resveratrol) with bortezomib for patients with relapsed and or refractory multiple myeloma. British journal of haematology. 2013;160(5):714-7.
253. Carter LG, D'Orazio JA, Pearson KJ. Resveratrol and cancer: focus on in vivo evidence. Endocrine-Related Cancer. 2014;21(3):R209-R25.
254. Shankar S, Nall D, Tang SN, Meeker D, Passarini J, Sharma J, et al. Resveratrol inhibits pancreatic cancer stem cell characteristics in human and KrasG12D transgenic mice by inhibiting pluripotency maintaining factors and epithelial-mesenchymal transition. PLoS One. 2011;6(1):e16530.
255. Shankar S, Srivastava RK. Involvement of Bcl-2 family members, phosphatidylinositol 3'-kinase/AKT and mitochondrial p53 in curcumin (diferulolylmethane)-induced apoptosis in prostate cancer. International journal of oncology. 2007;30(4):905-18.
256. Pandey PR, Okuda H, Watabe M, Pai SK, Liu W, Kobayashi A, et al. Resveratrol suppresses growth of cancer stem-like cells by inhibiting fatty acid synthase. Breast cancer research and treatment. 2011;130(2):387-98.
257. Shen YA, Lin CH, Chi WH, Wang CY, Hsieh YT, Wei YH, et al. Resveratrol Impedes the Stemness, Epithelial-Mesenchymal Transition, and Metabolic Reprogramming of Cancer Stem Cells in Nasopharyngeal Carcinoma through p53 Activation. Evidence-based complementary and alternative medicine : eCAM. 2013;2013:590393.
258. Cassileth BR, Deng G. Complementary and alternative therapies for cancer. The oncologist. 2004;9(1):80-9.

259. Demain AL, Vaishnav P. Natural products for cancer chemotherapy. Microbial Biotechnology. 2011;4(6):687-99.
260. Majumdar AP, Banerjee S, Nautiyal J, Patel BB, Patel V, Du J, et al. Curcumin synergizes with resveratrol to inhibit colon cancer. Nutrition and cancer. 2009;61(4):544-53.
261. Swamy MV, Citineni B, Patlolla JM, Mohammed A, Zhang Y, Rao CV. Prevention and treatment of pancreatic cancer by curcumin in combination with omega-3 fatty acids. Nutrition and cancer. 2008;60 Suppl 1:81-9.
262. Parasramka MA, Gupta SV. Synergistic effect of garcinol and curcumin on antiproliferative and apoptotic activity in pancreatic cancer cells. Journal of oncology. 2012;2012:709739.
263. Wang Z, Desmoulin S, Banerjee S, Kong D, Li Y, Deraniyagala RL, et al. Synergistic Effects of Multiple Natural Products in Pancreatic Cancer Cells. Life sciences. 2008;83(7-8):293-300.
264. Narayanan NK, Nargi D, Randolph C, Narayanan BA. Liposome encapsulation of curcumin and resveratrol in combination reduces prostate cancer incidence in PTEN knockout mice. International journal of cancer Journal international du cancer. 2009;125(1):1-8.
265. Du Q, Hu B, An HM, Shen KP, Xu L, Deng S, et al. Synergistic anticancer effects of curcumin and resveratrol in Hepa1-6 hepatocellular carcinoma cells. Oncology reports. 2013;29(5):1851-8.
266. Pal A, Sung B, Prasad BAB, Schuber PT, Prasad S, Aggarwal BB, et al. Curcumin Glucuronides: Assessing the Proliferative Activity against Human Cell Lines. Bioorganic & medicinal chemistry. 2014;22(1):435-9.
267. Sun H, Ma H, Hong G, Sun H, Wang J. Survival improvement in patients with pancreatic cancer by decade: A period analysis of the SEER database, 1981-2010. Sci Rep. 2014;4.
268. Haqq J, Howells LM, Garcea G, Metcalfe MS, Steward WP, Dennison AR. Pancreatic stellate cells and pancreas cancer: Current perspectives and future strategies. European Journal of Cancer. 2014;50(15):2570-82.
269. Britton RG, Horner-Glister E, Pomenya OA, Smith EE, Denton R, Jenkins PR, et al. Synthesis and biological evaluation of novel flavonols as potential anti-prostate cancer agents. European journal of medicinal chemistry. 2012;54:952-8.
270. Griffiths M, Sundaram H. Drug design and testing: profiling of antiproliferative agents for cancer therapy using a cell-based methyl-[3H]-thymidine incorporation assay. Methods in molecular biology (Clifton, NJ). 2011;731:451-65.
271. Smith PJ, Soues S, Gottlieb T, Falk SJ, Watson JV, Osborne RJ, et al. Etoposide-induced cell cycle delay and arrest-dependent modulation of DNA topoisomerase II in small-cell lung cancer cells. Br J Cancer. 1994;70(5):914-21.
272. Attardi LD, de Vries A, Jacks T. Activation of the p53-dependent G1 checkpoint response in mouse embryo fibroblasts depends on the specific DNA damage inducer. Oncogene. 0000;23(4):973-80.
273. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. Lancet. 2004;363(9414):1049-57.
274. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer cell. 2006;10(6):515-27.
275. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature. 2012;483(7391):603-307.

276. Kumar R, Chaudhary K, Singla D, Gautam A, Raghava GPS. Designing of promiscuous inhibitors against pancreatic cancer cell lines. Sci Rep. 2014;4.
277. Hagmann W, Jesnowski R, Löhr JM. Interdependence of Gemcitabine Treatment, Transporter Expression, and Resistance in Human Pancreatic Carcinoma Cells. Neoplasia (New York, NY). 2010;12(9):740-7.
278. SHI Xin GN-r, HUO Ming-dong. The mechanism by which pancreatic cancer cells acquire drug resistance against 5-FU and gemcitabine. Chinese Journal of General Surgery. 2002.
279. Nath S, Daneshvar K, Roy LD, Grover P, Kidiyoor A, Mosley L, et al. MUC1 induces drug resistance in pancreatic cancer cells via upregulation of multidrug resistance genes. Oncogenesis. 2013;2(6):e51.
280. Hasima N, Aggarwal BB. Cancer-linked targets modulated by curcumin. International journal of biochemistry and molecular biology. 2012;3(4):328-51.
281. Chan EWC, Lim YY, Wong SK, Lim KK, Tan SP, Lianto FS, et al. Effects of different drying methods on the antioxidant properties of leaves and tea of ginger species. Food Chemistry. 2009;113(1):166-72.
282. Masamune A, Suzuki N, Kikuta K, Satoh M, Satoh K, Shimosegawa T. Curcumin blocks activation of pancreatic stellate cells. Journal of cellular biochemistry. 2006;97(5):1080-93.
283. Schwer CI, Guerrero AM, Humar M, Roesslein M, Goebel U, Stoll P, et al. Heme oxygenase-1 inhibits the proliferation of pancreatic stellate cells by repression of the extracellular signal-regulated kinase1/2 pathway. The Journal of pharmacology and experimental therapeutics. 2008;327(3):863-71.
284. Sahu RP, Batra S, Srivastava SK. Activation of ATM/Chk1 by curcumin causes cell cycle arrest and apoptosis in human pancreatic cancer cells. Br J Cancer. 2009;100(9):1425-33.
285. Jaiswal AS, Marlow BP, Gupta N, Narayan S. Beta-catenin-mediated transactivation and cell-cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. Oncogene. 2002;21(55):8414-27.
286. Schaaf C, Shan B, Buchfelder M, Losa M, Kreutzer J, Rachinger W, et al. Curcumin acts as anti-tumorigenic and hormone-suppressive agent in murine and human pituitary tumour cells in vitro and in vivo. Endocr Relat Cancer. 2009;16(4):1339-50.
287. Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. Major cell death pathways at a glance. Microbes and infection / Institut Pasteur. 2009;11(13):1050-62.
288. Grabowska W, Kucharewicz K, Wnuk M, Lewinska A, Suszek M, Przybylska D, et al. Curcumin induces senescence of primary human cells building the vasculature in a DNA damage and ATM-independent manner. Age. 2015;37(1):7.
289. Patel KR, Andreadi C, Britton RG, Horner-Glister E, Karmokar A, Sale S, et al. Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence. Sci Transl Med. 2013;5(205):205ra133.
290. Waldman T, Zhang Y, Dillehay L, Yu J, Kinzler K, Vogelstein B, et al. Cell-cycle arrest versus cell death in cancer therapy. Nature medicine. 1997;3(9):1034-6.
291. Deer EL, Gonzalez-Hernandez J, Coursen JD, Shea JE, Ngatia J, Scaife CL, et al. Phenotype and genotype of pancreatic cancer cell lines. Pancreas. 2010;39(4):425-35.
292. Hasima N, Aggarwal BB. Targeting proteasomal pathways by dietary curcumin for cancer prevention and treatment. Current medicinal chemistry. 2014;21(14):1583-94.
293. Bao GF, Philip PA, Azmi AS. Chapter 13 - Systems Biology of Pancreatic Cancer Stem Cells. In: Azmi AS, editor. Molecular Diagnostics and Treatment of Pancreatic Cancer. Oxford: Academic Press; 2014. p. 297-322.

294. Jaiswal KR, Xin HW, Anderson A, Wiegand G, Kim B, Miller T, et al. Comparative testing of various pancreatic cancer stem cells results in a novel class of pancreatic-cancer-initiating cells. Stem cell research. 2012;9(3):249-60.
295. Penchev VR, Rasheed ZA, Maitra A, Matsui W. Heterogeneity and targeting of pancreatic cancer stem cells. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(16):4277-84.
296. Chen L, Fan J, Chen H, Meng Z, Chen Z, Wang P, et al. The IL-8/CXCR1 axis is associated with cancer stem cell-like properties and correlates with clinical prognosis in human pancreatic cancer cases. Sci Rep. 2014;4.
297. Cabarcas SM, Sun L, Mathews L, Thomas S, Zhang X, Farrar WL. The differentiation of pancreatic tumor-initiating cells by vitronectin can be blocked by Cilengitide. Pancreas. 2013;42(5):861-70.
298. Ishizawa K, Rasheed ZA, Karisch R, Wang Q, Kowalski J, Susky E, et al. Tumor-initiating cells are rare in many human tumors. Cell stem cell. 2010;7(3):279-82.
299. Nalls D, Tang SN, Rodova M, Srivastava RK, Shankar S. Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells. PLoS One. 2011;6(8):e24099.
300. Shankar S, Ganapathy S, Hingorani SR, Srivastava RK. EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer. Frontiers in bioscience : a journal and virtual library. 2008;13:440-52.
301. Anand P, Thomas SG, Kunnumakkara AB, Sundaram C, Harikumar KB, Sung B, et al. Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. Biochemical Pharmacology. 2008;76(11):1590-611.
302. Sandur SK, Pandey MK, Sung B, Ahn KS, Murakami A, Sethi G, et al. Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. Carcinogenesis. 2007;28(8):1765-73.
303. Pari L, Amali DR. Protective role of tetrahydrocurcumin (THC) an active principle of turmeric on chloroquine induced hepatotoxicity in rats. J Pharm Pharm Sci. 2005;8(1):115-23.
304. Pfeiffer E, Hoehle SI, Walch SG, Riess A, Solyom AM, Metzler M. Curcuminoids form reactive glucuronides in vitro. Journal of agricultural and food chemistry. 2007;55(2):538-44.
305. Suzuki M, Suzuki H, Sugimoto Y, Sugiyama Y. ABCG2 transports sulfated conjugates of steroids and xenobiotics. The Journal of biological chemistry. 2003;278(25):22644-9.
306. Hoehle SI, Pfeiffer E, Metzler M. Glucuronidation of curcuminoids by human microsomal and recombinant UDP-glucuronosyltransferases. Mol Nutr Food Res. 2007;51(8):932-8.
307. Riches Z, Stanley EL, Bloomer JC, Coughtrie MW. Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT "pie". Drug Metab Dispos. 2009;37(11):2255-61.
308. Kunwar A, Barik A, Mishra B, Rathinasamy K, Pandey R, Priyadarsini KI. Quantitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. Biochimica et Biophysica Acta (BBA) - General Subjects. 2008;1780(4):673-9.
309. Dempe JS, Scheerle RK, Pfeiffer E, Metzler M. Metabolism and permeability of curcumin in cultured Caco-2 cells. Mol Nutr Food Res. 2013;57(9):1543-9.
310. Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, et al. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of

miR-200 and miR-21 expression by curcumin or its analogue CDF. Cancer research. 2010;70(9):3606-17.
311. Pan M-H, Lin-Shiau S-Y, Lin J-K. Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IκB kinase and NFκB activation in macrophages. Biochemical pharmacology. 2000;60(11):1665-76.