KINGSTON UNIVERSITY

ASSESSMENT OF PROTEASE ACTIVITY IN ENDOTHELIAL CELLS AND ITS ROLE IN TUMOUR ANGIOGENESIS AND SPREAD.

A thesis submitted in partial fulfilment of the requirements of Kingston University for degree of Doctor of Philosophy

In collaboration with

Department of Periodontology, St. Bartholomew's and the Royal London School of Medicine and Dentistry.

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Declaration

I hereby declare that whilst registered for a research degree at Kingston University, I have not been a registered candidate or enrolled student at any other academic or professional institution.

I declare that the materials contained in this thesis have not been used in any other submission for an academic award. All the sources of investigation have been duly acknowledged.

This thesis has been composed by myself and is a result of my own investigation.

Thakur

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Sapna Thakur BSc (Hons)

Dedication

To my loving and supporting parents, Ambika Singh Thakur and Kusum Thakur

without them I wouldn't be where I am today.

I LOVE YOU BOTH

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Angiogenesis is essential for the growth of a tumour, as it provides tumour cells with nutrients and oxygen for their survival. As the tumour expands, neovascularisation is facilitated by the release of enzymes called proteases, which degrade extracellular matrix and facilitate the metastatic spread of cancer.

TNFα and IL-1β are potent cytokines that share the ability to stimulate angiogenesis, hence their possible significance in metastasis has been a focus of intense research. TNFα and IL-1β have been shown to regulate the activity of proteases such as MMP's and serine protease.

The aim of this study was to investigate the effect of TNFα and IL-1β on cathepsin B and DPP IV activity and their protein levels in HRT 18, HT 29 cells and HUVEC's. Further experiments were conducted to assess the viability of the cells upon treatment with the cytokines. In addition the potency of inhibitors Mu-Phe-Hph-FMK, for cathepsin B enzyme, and Gly-Pro-Gly-Gly, for DPP IV enzyme were assayed in the three cell lines studied.

Addition of TNF α and IL-1β resulted in the reduction of intracellular cathepsin B and DPP IV activity and an increase in its extracellular activity in HRT 18 and HT 29 cells, suggesting that the cytokines induced the release of the enzymes or may have inactivated intracellular enzyme while activating the latent extracellular enzyme. However, in HUVECs, both the cytokines led to an increase in intracellular as well as extracellular cathepsin B activity, possibly by activating the latent form of enzyme present within and outside the cell. With respect to DPP IV, there was an increased intracellular and extracellular activity with TNFα, but with IL-1β, an increase in intracellular activity and a decrease in extracellular activity were observed, suggesting involvement of a different mechanism for the exopeptidase enzyme in HUVECs.

The two Cytokines had a cytotoxic/cytostatic effect on all three cell lines, with prominent reduction in cell viability of HUVECs.

In conclusion the varied response of intracellular and extracellular activity and protein levels of cathepsin B and DPP IV, in different cell lines suggests that TNFα and IL-1β may act as important modulators of proteases in the process of angiogenesis in cancer and normal endothelial cells. Thus understanding the pleiotropic nature of these cytokines will further broaden the knowledge of involvement of these cytokines in cancer progression/cancer regression.

ABC	Avidin biotin complex
aFGF	Acidic fibroblast growth factor
Ab	Antibody
Ag	Antigen
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAM	Cellular adhesion molecules
CA-074	N-[L-3-*trans*-Propylcarbamoyloxirane-2-carbonyl]-Ile-Pro-OH
$CuSO_4.5H_2O$	Copper sulphate solution
DAB	3,3'-diaminobenzidine
ddw	Double distilled water
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.C	Endothelial cells
ECM	Extracellular matrix
ECGS	Endothelial cell growth supplement
EGF	Epidermal growth factor
FasL	Fas Ligand
FCS	Fetal calf serum
FGF	Fibroblast growth factor
g	Relative centrifugal force
Gly-Pro-AMC	Gly-Pro 7amido-4Methyl Coumarin hydrobromide
HAF	Human angiogenic factor
HBSS	Hanks' balanced salts with calcium and magnesium
HRP	Horseradish Peroxidase
HRT 18	Human rectum adenocarcinoma
HT 29	Human colon carcinoma
HUVEC	Human umbilical vein endothelial cells
IFNγ	Interferon gamma
IFNβ	Interferon beta
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL-1β	Interleukin-1beta
kDa	Kilo Dalton
$KNaC_4H_4O_6.4H_2O$	Potassium sodium tartrate solution
m	Milli
M	Molar
MEM	Minimum essential medium with Earle's salts and L-Glutamine
MES	Monohydrate sigmaultra
MPA	Medroxyprogesterone acetate
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide

Mu-Phe-Hph-FMK	MeOSuc-Phe-HomoPhe Fluoromethyl Ketone
Na_2CO_3	Sodium carbonate
NaCL	Sodium chloride
NaOH	Sodium hydroxide
NEAA	Non essential amino acid
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PD-ECGF	Platelet derived endothelial growth factor
PF	Platelet factor
RNA	Ribonucleic acid
RPM	Revolution per minute
RPMI	RPMI 1640 medium with L-Glutamine
SDS	Sodium dodecyl sulphate
TBS	Tris buffer saline
TEMED	N,N,N'N'-tetramethylethylenediamine
TF	Tissue factor
TGF β	Transforming growth factor beta
TMB	3,3',5,5'-tetramethylbenzidine
TNF α	Tumour necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
Tris-HCL	Tris (hydroxymethyl)methylamine hydrochloride
Triton X-100	Polyoxyethylene-p-isooctylphenol
Trypsin-EDTA	Trypsin/ethylenediaminetetraacetic acid
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WST-1	Water-soluble tetrazolium (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)
XTT	Sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate
Z-ARG-ARG-AMC	Nα-CBZ-Arg-Arg 7-amido 4-Methyl Coumarin hydrochloride

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1.0 Angiogenesis

Angiogenesis is the formation of new capillaries from pre-existing vasculature (O'Byrne & Steward 2001) by migration and proliferation of endothelial cells (Griffioen & Molema 2000). Under physiological conditions such as during the female reproductive cycle, during pregnancy, during wound healing and in response to tissue hypoxia, angiogenesis is highly regulated by various angiogenic stimulators and inhibitors (Folkman 1992). However, under pathological conditions the regulatory process of angiogenesis is disturbed due to imbalance between angiogenic stimulators and inhibitors, resulting in arthritis, tumour growth, psoriasis, hemangiomas, diabetic retinopathy (due to excessive angiogenesis), coronary artery disease, stroke, and delayed wound healing (due to insufficient angiogenesis) (Bicknell 1994). The process of angiogenesis begins with the localised breakdown of the basement membrane of a postcapillary venule, resulting in the formation of a capillary sprout into the surrounding tissue. The sprout elongates by further migration and proliferation of endothelial cells, and a lumen is gradually formed proximal to the region of proliferation. Connected tubular sprouts anastomise to form a functional capillary loop, and finally vessel maturation is accomplished by reformation of the basement membrane. Angiogenesis is thus characterised by –

1) Modulation of interactions with the extracellular matrix, which requires alterations of cell-matrix contacts.

2) Production of proteolytic enzymes that have the capability of degrading the basement membrane.

3) Initial increase and then decrease in the migration of the cells so as to allow the cells to translocate towards the angiogenic stimulus and to stop once they reach their destination.

4) An increase in proliferation, in order to provide the growing vessel with new cells, and a subsequent return to a quiescent state once the vessel has been formed.

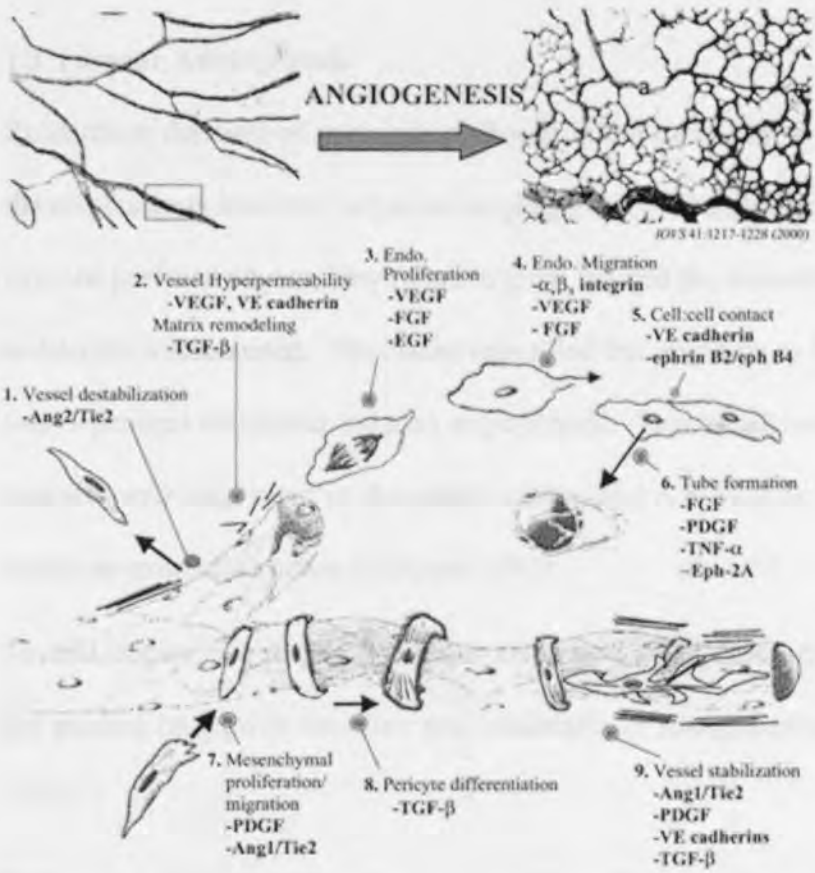


Figure 1.0: *A diagrammatic view of normal angiogenesis, which is dependent on the coordination of several independent processes. (1) Removal of pericytes from the endothelium and destabilization of the vessel by angiopoietin-2 (Ang2) shift endothelial cells from a stable, growth-arrested state to a plastic, proliferative phenotype. (2) Vascular endothelial growth factor (VEGF)-induced hyperpermeability allows for local extravasation of proteases and matrix components from the bloodstream. (3) Endothelial cells proliferate (4) and migrate through the remodelled matrix, (5) they then form tubes through which blood can flow. (7) Mesenchymal cells proliferate and migrate along the new vessel (8) and differentiate into mature pericytes. (9) Establishment of endothelial cell quiescence, strengthening of cell-cell contacts, and elaboration of new matrix stabilise the new vessel.*

(Papetti & Herman 2002)

1.1 Tumour Angiogenesis

Research in the field of angiogenesis began in the early 1960s with the view to investigate the mechanisms involved in tumour angiogenesis. When tumour cells were implanted into isolated perfused organs, they failed to grow beyond the diameter of 1-2mm and also failed to become vascularised. This observation led the scientists to believe that tumour growth was dependent on tumour induced angiogenesis. This idea however was not accepted until tumours were implanted in the rabbit cornea, and new vessels could be observed to grow across an avascular cornea (Folkman 1992).

Several studies now implicate tumour-associated blood vessel formation as a central part in the process of growth, invasion and metastasis of malignancies (Jahroudi & Greenberger 1995).

Subversion of normal physiological functions of certain host cells and tissues is currently regarded as a hallmark of cancer (Hanahan & Weinberg 2000). Indeed, host endothelial cells are believed to play a central role in tumour growth, progression and metastasis, acting as main building blocks of the tumour microvasculature. Experimental evidence uniformly points to the fact that tumour growth cannot proceed without access to, and recruitment of host blood vessels, a process broadly referred to as tumour angiogenesis (Folkman 1995). Nearly 30 years ago, Folkman predicted that because of this "angiogenesis dependence" of solid tumours (now extended also to leukemias and lymphomas), selective inhibition or destruction of the tumour vasculature (using antiangiogenic or antivascular treatment modalities, respectively) could trigger tumour growth inhibition, regression and/or, a state of dormancy and thereby offer a fundamentally new (host cell directed) approach to cancer treatment (Folkman 1971). To date, preclinical studies with endostatin, angiostatin, VEGF antagonists, and many other new generation

angiogenesis inhibitors have convincingly validated the guiding principles of this concept (Folkman 1995).

The dependency of tumour growth on angiogenesis has resulted in optimism and high expectations for therapeutic efficacy of the prospective tumour angiogenesis antagonists, many of which have entered clinical trials (Rak *et al.*, 2002).

While considering the question of variability in tumour responses to antiangiogenic treatments, it may be helpful to revisit the relationship between angiogenesis and genetic tumour progression. Many properties of cancer cells arise as a result of their genetic instability, diversity, and clonal evolution (Nowell 1976; Folkman, Hahnfeldt, Hlatky 2000). The sequential activation of mutant oncogenes and loss of tumour suppressor genes associated with this process (Fearon & Vogelstein 1990) is thought to contribute to, and promote the acquisition of, the angiogenic phenotype by tumour cell clones, mainly through the influence of these transforming genes on expression of endogenous angiogenesis inhibitors e.g., thrombospondin-1 and stimulators e.g., VEGF; (Bouck, Stellmach, Hsu 1996). A threshold change in the net "balance" between these stimulatory and inhibitory influences, in favour of the former, is believed to trigger the initial onset of tumour angiogenesis the so called "angiogenic switch" (Folkman 1995). However, expression profiles of various angiogenesis regulators undoubtedly continue to evolve in parallel with genetic tumour progression, as do properties of tumour-associated microvascular networks (Relf *et al.*, 1997). Thus, tumour angiogenesis, although influenced by microenvironmental conditions *e.g.*, hypoxia, can be viewed as being, to a large extent, genetically driven. As such, angiogenesis is a means by which genetic alterations in cancer cells secure a host dependent support mechanism for tumour cell survival. This is consistent with the observation that when neovasculature is compromised,

tumour cells are prone to undergo apoptosis, mainly as a result of hypoxia, and metabolic stress (Folkman 1995).

Research into angiogenesis has opened many prospects in the field of medicine such as: -

- *Prognosis* - Quantification of stained blood vessels by microscopy has made it possible to predict the risk of metastasis. For instance, in patients with invasive breast cancer increase in blood vessel number is directly proportional to increasing risk of metastasis (Heimann *et al.*, 1998). In addition, studies by Engel & co-workers (1996) have suggested that the number of microvessels in sections of invasive colorectal adenocarcinoma immunohistochemically stained with CD31 may be an important independent predictor of tumour recurrence and time to recurrence.
- *Stimulation of angiogenesis* - in the case of chronic wounds, where the process of angiogenesis can be accelerated by cytokines (Nath & Gulati 1998).
- *Inhibition of angiogenesis* - in the treatment of life-threatening hemangiomas in children (Folkman & Ingber 1992).

1.2 Proteolytic Enzymes

Proteases are enzymes which catalyse the hydrolysis of peptide bonds, they occur naturally in all organisms and constitute 1-5% of the gene content. Proteases are widely distributed in cells, and they are either soluble, or associated with plasma membranes and subcellular organelles, or secreted (Barrett, Rawlings, Woessner 2003). The presence of intracellular proteolytic activity has been reported in numerous tissues, the most intensively studied of which have been the brush border membranes of the intestine and kidney, the anterior pituitary, leucocytes and erythrocytes. This extensive distribution of proteases in both organs and subcellular compartments suggests that they play a key role in both

physiological and pathophysiological situations. They play a key role in the metabolism of proteins, zymogen activation, activation of complimentary system, and in blood coagulation and fibrinolysis. They are also involved in regulation of blood pressure, in immune response reproduction, maturation of peptides and proteins, growth and development. Their role in pathological condition has been widely demonstrated in certain inflammatory processes, bacterial, viral and parasitic infections, chronic degenerative and immunological diseases, and allergic reactions and finally cancer (Nduwimana *et al.*, 1995).

Proteases have been the centre of interest because of their prominent involvement in cancer progression and metastasis (Decock, Paridaens, Tanja 2005). These enzymes facilitate the dissemination of tumours by degrading the ECM proteins that form the structural barriers that cells must cross to reach the vasculature (Szpaderska & Frankfater 2001), they are among the first group of molecules causally involved in metastasis which act as prognostic markers in cancer (Duffy 1996).

1.2.1 Types of Proteases

Proteases can be classified into two groups, depending on their site of action on the polypeptide chain, **endopeptidases** (endo-acting peptide bond hydrolase) and **exopeptidases** (exo-acting peptide bond hydrolase) (Rzychon, Chmiel, Stec-Niemczyk 2004). Endopeptidases are subdivided according to their catalytic mechanism, into four groups namely serine, cysteine, aspartic and metallo endopeptidases (Kurschat & Mauch 2000), each group comprising of several other enzymes as summarised in table 1.0. Proteases from the four classes of endopeptidases have been linked to tumour progression (Berquin & Sloane 1996) by causing degradation of the basement membrane. Proteases are mostly interdependent , for instance several enzymes are secreted as inactive form,

which require activation by other endopeptidases (Ludwig 2005), for example cathepsin B can activate prourokinase to urokinase (a serine protease), which activates plasminogen to plasmin, and in turn plasmin activates stromelysin (a metalloprotease). Reciprocally, procathepsin B can be activated by urokinase. Thus these proteases might facilitate tumour progression both directly and indirectly. Tumour cells frequently produce proteases but may also induce the secretion of proteases by the neighbouring stromal cells. Though this has not yet been demonstrated for cathepsin B (Berquin & Sloane 1996).

Exopeptidases can be further subdivided (section 1.2.1.5), depending on their site of action on the polypeptide chain (Otto & Schirmeister 1997). They are group of enzymes that play a role in the regulation of biologically active peptides. Aminopeptidases and carboxypeptidases are involved in the production of angiotensin, bradykinin, and vasopressin (Mitsui *et al.*, 2004), in the regulation of fibrinolysis (Nesheim *et al.*, 1997.).

Exopeptidases have been implicated in cancer (Matrisian, Sledge, Mohla 2003). For instance, aminopeptidase is highly expressed in bladder, gastric, thyroid, and hepatic carcinomas, and the concentration of its soluble form is also increased in cancer patients (van Hensbergen *et al.*, 2002). In addition, increased activity and expression of aminopeptidase have been functionally correlated with metastasis of cancer cells by promotion of angiogenesis (Pasqualini *et al.*, 2000). Similarly, increased concentration of a lysosomal dipeptidyl-aminopeptidase (DAP II) has been observed in sera of tumour-bearing animals and cancer patients (Kojima *et al.*, 1987). However, carboxypeptidases are more highly expressed in hematopoietic tumour cells (O'Malley et al., 2005).

Class of protease	Types of proteases	Cellular compartment
Cysteine	CTSB	Nucleus/cytosol/endosome-lysosome/plasma membrane
	CTSL	Nucleus/endosome-lysosome/plasma membrane
	CTSS	Endosome-lysosome
	Calpains	Nucleus/cytosol
	Procathepsins B, C, L	Secreted zymogens
Serine	CTSA	Endosome-lysosome
	uPA	Endosome-lysosome
	tPA	Endosome-lysosome
	Plasmin	Endosome-lysosome
	Tripsinogins I & II	Secreted zymogens
Aspartyl	CTSD	Endosome-lysosome
	CTSE	Cytosol/RER and golgi
	Procathepsin D	Secreted Zymogens
Metallo	Meprins A & B	Plasma membrane
	Gelatinases A & B	Plasma membrane
	Pro-MMPs	Secreted zymogens

(Nduwimana *et al.*, 1995; Keppler *et al.*, 1996)

Table 1.0: *Classification of endopeptidases and their cellular compartment.*

1.2.1.1 Serine Proteases

Proteases belonging to the Serine endopeptidases are characterised by the presence of aspartic acid, serine, and histidine. Serine forms a covalent bond with the substrate. They act at a pH ranging from 7-9. Serine endopeptidases consist of plasminogen activators (PA), urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). Their key role is the activation of plasminogen to an active enzyme, plasmin, which is capable of degrading major components of the extracellular matrix.

uPA is the first protease shown to be a prognostic marker in human malignancy, it is a significant marker for breast, ovary, stomach, lung, colon, cervix, uterus and kidney cancer (Goel & Chauhan 1997). The other enzymes that belong to this group are coagulation factors, proteins of the complement system, fibrinolytic enzymes and digestive enzymes. Organophosphates, coumarins, heterocyclic derivatives, boronic acid derivatives and

chloromethyl ketones (small organic compounds) serpins or microbial inhibitors can inhibit these proteases.

1.2.1.2 Cysteine Proteases

Cysteine proteases are characterised by the presence of histidine, cysteine, and aspartic acid. Cysteine (same as serine) forms a covalent intermediate complex with their substrate, because the amino acids at the catalytic sites are very nucleophilic. The enzymes of the papain family (Cathepsin B, H, L, S, papain and bromelain isolated from plants) and calpain family belong to this group (Cathepsin B will be described in more detail in this chapter, section 1.2.2). These proteases work in the pH range 3-8. Cysteine endopeptidases can be inhibited by peptide epoxides and microbial inhibitors. Small proteins of the cystatin family derived from animal tissues can inhibit cathepsins (Nduwimana *et al.*, 1995).

Lysosomal cysteine proteases cathepsin B and cathepsin L have been implicated in tumour spread and metastasis and may serve as prognostic factors for tumour recurrence in human breast cancer (Thomssen *et al.*, 1995). Significant increase in cathepsin H expression has been found in carcinoma of the prostate (Waghray *et al.*, 2002).

1.2.1.3 Aspartate proteases

Aspartate (acid) endopeptidase consists of 2 residues of aspartic acid at the catalytic site. Examples of enzymes belonging to this group are cathepsin D, E, renin and pepsin. They are specifically inhibited by pepstatins. The pH range best suited for this group of enzymes is 2-7 (Nduwimana *et al.*, 1995).

As a lysosomal protease, cathepsin D is a major contributor to protein degradation. As a secreted protease from prostate carcinoma cells, cathepsin D is responsible for the generation of angiostatin, a potent endogeneous inhibitor of angiogenesis (Tsukuba *et al.*,

2000). Cathepsin E is not a lysosomal protease and has a limited cell and tissue distribution. Both cathepsins play important roles in the generation of bioactive proteins and in antigen processing (Tsukuba *et al.*, 2000).

1.2.1.4 Matrix metalloproteases (MMPs)

MMPs, naturally occurring proteases, are a group of zinc enzymes that are involved in extracellular matrix remodelling by degrading one or several connective tissue elements and are also implicated in the progression of cancer (Goel & Chauhan, 1997). All these enzymes are secreted by normal and tumour cells in proenzyme form and converted to an active form by the cleavage of the amino terminal domain. There are three types of MMPs known, which are Type 1 collagenases, type IV collagenases and stromelysins. Type 1 collagenases include interstitial collagenase (MMP1) and neutrophil collagenase (MMP8). Fibroblasts, smooth muscle cells and mononuclear phagocytes all produce the interstitial collagenase. Type IV collagenase (gelatinases) include two forms 72 (MMP2) and 92 (MMP9) KDa. The expression of the gelatinases has been shown to correlate with glioma progression (Levi ćar, Nutall, Lah 2003). Stromelysin broadly comprises of stromelysins 1,2,3 and matrilysin. Large quantities of stromelysin 2 mRNA has been observed in the basement membrane of epidermoid carcinoma of the head and neck, suggesting it has a role in the degradation of the basement membrane. Stromelysin 3 expression has been shown to be associated with the progression of human breast cancer (Goel & Chauhan 1997).

A positive correlation between metalloproteases expression and metastatic behaviour has been demonstrated in various tumours such as breast, prostate, colon, lung, ovarian, and thyroid cancers (Goel & Chauhan 1997).

It is very important that the protease activity is regulated, to prevent inappropriate and uncontrolled protein degradation.

Protease	Target Structure
Metallo	Collagens type I, II, III, and X
Type 1 collagenases	Triple helical portion of type IV collagen, gelatin, collagen V, VII, IX, X, fibronectin and elastin.
Type IV collagenases	Type IV and X collagens, proteoglycan core protein, laminin, globular domains of fibronectin, procollagen I and III, gelatin and non-helical portions of basement membrane collagens.
Stromelysins	
Serine	Fibronectin and type IV collagen.
uPA	
Aspartyl	Proteoglycans and endorphins.
CTSD	
Cysteine	Non-helical regions of collagens of types I-IV, proteoglycans, laminin, and fibronectin.
CTSB	
CTSL	Collagens, laminin, proteoglycans, elastin, fibronectin.

(Goel & Chauhan 1997; Khan *et al.*, 1998)

Table 1.1: *Endopeptidases, and their targets.*

1.2.1.5 Exopeptidase

The exopeptidases are a group of intracellular peptidases whose attack upon a peptide substrate is limited to one or other terminus of the peptide chain (McDonald & Schwabe 1977). Their activities are thought to be essential for the complete breakdown of protein substrates and the release of free amino acids required for the biosynthetic activities and protein turnover of cells (Coffey & De Duve 1968). However, the degradation of cytoplasmic proteins is believed to be initiated by endopeptidases and terminated by exopetidases (McDonald & Schwabe 1977).

Exopetidases have been described according to specificity determinants that include size or identity of the liberated fragment, terminus of origin, and size restrictions on susceptible peptide substrates. The aminopeptidase class has three distinct subdivisions with

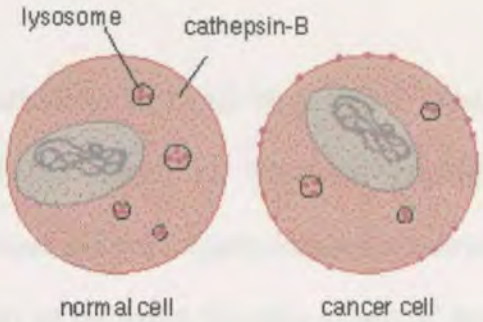
characteristic specificities directed toward the unsubstituted N termini of polypeptide substrates (Nduwimana *et al.*, 1995). These subdivisions are termed aminopeptidases, dipeptidylpeptidases (DPPs) and tripeptidyl peptidases, on the basis of the number of amino acid residues removed at a time. The term praline aminopeptidase is sometimes used to refer to enzymes that specifically act on substrates bearing praline in the penultimate position (McDonald 1985).

The carboxypeptidase group is characteristically active toward the unsubstituted C termini of polypeptide substrates (Nduwimana *et al.*, 1995) and has two subdivisions, carboxypeptidases and peptidyl dipeptidases. The carboxypeptidases remove one amino acid at a time and the peptidyl dipeptidases remove two at a time. The dipeptidases are restricted to the cleavage of unsubstituted dipeptides and the tripeptidases remove a terminal amino acid from one end or the other of unsubstituted tripeptides. The omega peptidases are those enzymes that do not conform to the classical definitions for aminopeptidases and carboxypeptidases, but also include exopeptidases that hyrdrolyse isopeptide bonds which do not link α amino and α carboxyl groups (McDonald 1985).

1.2.2 Cathepsin B

Cathepsin B is a ubiquitous lysosomal cysteine protease. The main function of this enzyme is the intracellular degradation of proteins.

There is also evidence that cathepsin B is implicated in several forms of cancer, and therefore may play an important role in angiogenesis (Keppler *et al.*, 1996). It has been suggested that cathepsin B may be linked with tumour progression through observation that its activity and secretion are increased in most malignant tumours (Podgorski & Sloane 2003).



http://americanscientist.org/articles/98articles/vfig8.gif

Figure 1.1: *Redistribution of cathepsin B on cell surface of tumour cells.*

Proteases such as cathepsin B are normally housed in membrane-bound vesicles, called lysosomes, inside the cell. There, they help degrade cellular proteins to their constituent parts to be excreted or recycled to form new proteins (Stachowiak *et al.*, 2004). Cancer cells seem to express cathepsin B on their membrane surfaces as well (figure 1.1). Cathepsin B is one of several proteases, some of which may be expressed and secreted by normal cell types, that help the cancer cell escape its parent tissue and enter the bloodstream.

Cathepsin B is a two lobed protein, with the active site and the substrate binding cleft located at the interface between the two lobes. Peptide bond cleavage is catalysed by cysteine residue on the left lobe that interacts with histidine residue on the right lobe of the enzyme. More specifically cleavage of the substrate peptide bond is mediated by nucleophilic attack by S^- from the cysteine residue on the carbonyl carbon atom followed by proton donation from histidine (Mort & Buttle 1997).

This cysteine protease also acts as a peptidylpeptidase, an exopeptidase that removes dipeptides from the c-terminus of proteins and peptides. The occluding loop (a structural element unique to cathepsin B) is responsible for this activity. The protease acts as an endopeptidase due to the flexibility of the occluding loop that can adopt a conformation that no longer fits the binding cleft (Mort & Buttle 1997). It is this change in conformation that makes cathepsin B a less effective endopeptidase than other members of its family,

such as cathepsins H, L, M, N, S, and T. Cathepsin B is able to participate in both the early and late stages of lysosomal protein degradation, due to its action as an endopeptidase and peptidylpeptidase (Stachowiak *et al.*, 2004).

The activity of cathepsin B is due to several levels of regulation:- The gene is transcribed as heterogenous nuclear RNA, which is processed to messenger RNA (mRNA) and transported to the cytosol. Preprocathepsin B synthesis takes place on ribosomes associated with the endoplasmic reticulum. Maturation of cathepsin B is coupled with intracellular trafficking (localization and secretion) and includes co-and-post-transcriptional glycosylation and proteolytic processing events. Fully processed cathepsin B is present in the lysosomes. The last step in the regulation of cathepsin B, the inhibition step, has drawn quite a lot of attention since all strategies to reduce cathepsin B activity, in disease states, is aimed at inhibiting the enzyme (Berquin & Sloane 1996).

Many more studies will be required before the mechanism of regulation in each step of the regulatory process of cathepsin B in normal cells, and the alteration of this regulation resulting in increase proteolytic activity in tumour cells is fully understood. Increases in the mRNA and protein levels, increased activity and altered trafficking of cathepsin B have been found to correlate with malignancy of murine and human tumours, suggesting its involvement in the progression of tumour (Keppler *et al.*, 1996). Cathepsin B in tumours has been found to be associated with plasma membrane/endosomal fractions and on the surface of the tumour cells (Moin *et al.*, 1998).

Localization of cathepsin B protein and activity in serial sections has been shown to reveal a distinct pattern in relation to colorectal carcinogenesis. In normal colorectal mucosa, cathepsin B protein was present in the larger part of the epithelium but was active only in the older cells at the colorectal surface (Bleeker *et al.*, 2000). Therefore it has been

postulated that it plays a role in apoptosis in these cells, as well as during regression of cancer of the prostate and mammary glands (Guenette *et al.*, 1994).

Strong immunostaining of cathepsin B in endothelial cells (rat brain microvessels) has been observed during angiogenesis, suggesting its role in tumour growth and progression (Keppler *et al.*, 1996).

Cathepsin B activity has been found to be elevated in a variety of animal and human tumours with the highest activity being at the invasive edge of the malignant tumour. For example, cathepsin B mRNA levels are increased in human colorectal carcinoma compared to normal colorectal tissues. Immunohistochemical studies have shown that cathepsin B staining increases with colorectal carcinoma, which is confirmed by the mRNA studies (Goel & Chauhan 1997).

Another example of increase in cathepsin B expression with tumour progression is found in human gliomas. The abundance of cathepsin B transcripts and the intensity of staining for cathepsin B protein increases progressively in astrocytoma, anaplastic astrocytoma and malignant glioblastoma, and correlate with evidence of clinical invasion assessed by Magnetic Resonance Imaging (Berquin & Sloane 1996). In situ hybridisation and immunohistochemistry techniques have shown increased cathepsin B mRNA and protein levels in cells at the invasive edge of prostate tumours. Hence, staining of cathepsin B may be of diagnostic and prognostic value in human colorectal, glioma as well as colon carcinoma. In lung cancer patients elevated cathepsin B staining is associated with shorter overall survival (Yan, Sameni, Sloane 1998). Cathepsin B activity and protein levels has been shown to be elevated in various other human tumours including breast (Krepela 1987) (Foekens *et al.*, 1998) (Maguire *et al.*, 1998), cervix and ovary, and thyroid (Aisa *et al.*, 1996). In addition higher activity of the enzyme has also been observed in gastric carcinoma (Khan *et al.*, 1998) (Dohchin *et al.*, 2000). Cathepsin B has also been

successfully used as a marker to monitor the differentiation process in engineered cartilage (Grigolo *et al.*, 2003).

Cathepsin B activity has also been detected intracellularly and extracellularly in both human osteoblast-like cells and human osteosarcoma cells, IL-1β has been shown to significantly increase cathepsin B activity in both the cell types intracellularly and extracellularly (Aisa *et al.*, 1996).

1.2.3 Dipeptidyl Peptidase IV

DPP IV is a membrane bound exopeptidase protease which catalyses the cleavage of dipeptides from the N-terminus of polypeptides under definite structural conditions (Fleischer 1994) (Gorrell, Gysbers, McCaughan 2001). When the possible substrate is presented as P_2-P_1-P_1-P_2,---, DPP IV cleaves off P_2-P_1 if the following conditions are fulfilled: the substrate P_1 position must be occupied by proline, hydroxyproline, dehydroproline, or alanine: among these the highest rates are observed for proline in penultimate position (McDonald & Barrett 1986).

DPP IV was discovered by Hopsu-Havu & Glenner (1966) as glycyl-prolyl-β-naphthylamidase and first isolated and partially characterised from rat liver (Hopsu-Havu & Sarimo 1967). This enzyme is exceptional in combining a serine-type protease mechanism with an exopeptidase specificity, while most exopeptidases are metalloproteases (Kenny, Stephenson, Turner 1987). DPP IV is a 110-kDa glycoprotein (Darmoul *et al.*, 1992), and plays an important role in immune regulation, signal transduction, and apoptosis. Furthermore, DPP IV appears to play an important role in tumour progression (Pro & Dang 2004). The substrates of DPPIV are proline-containing peptides and include growth factors, chemokines, neuropeptides, and vasoactive peptides. DPP IV has been implicated in the control of cell growth and differentiation (Tsugiki *et al.*, 1998) and is broadly distributed in various tissues (Goel & Chauhan 1997) and cell types

(Ruiz *et al.*, 1998). It is thought to be a major physiological regulator for some regulatory peptides (Augustyns *et al.*, 1997), neuropeptides, circulating hormones and chemokines (Mentlein 1999). DPP IV is known to have a role in the normal functioning of the liver and digestive tract, but is also implicated in various disease processes (Augustyns *et al.*, 1999). DPP IV expression has been found to be altered in cirrhotic human liver, human liver transplant rejection, rat liver regeneration and rat liver tumour cells. It is also involved in tumour cell invasion and the formation of metastasis (Stange, Kettmann, Holzhausen 2000) (Kikkawa *et al.*, 2005). Loss of DPP IV expression and enzymatic activity has been found to occur during malignant transformation of melanocytes to melanoma. A marked contrast to this has been noted where upregulation of DPP IV on the surface of tumour cells has been reported, in cases of T-cell acute lymphoblastic leukaemia. DPP IV activity is also shown to be higher in cases of basal cell carcinoma than in normal skin or benign lesions (Iwata & Morimoto 1999). It has been found that DPP IV immunostaining or activity staining is a very useful tool for supporting the pathological diagnosis of well-differentiated thyroid carcinoma and it is being utilised in the cytological and histological diagnosis of thyroid tumour (Hildebrandt *et al.*, 1999). Studies by Kholova & co-workers (2003) demonstrated aberrant expression of DPP IV in thyroid carcinomas and showed DPP IV to be a diagnostic tumour marker for malignant thyroid tumours of follicular cell origin.

The upregulation or downregulation of DPP IV appears to be tissue specific and even cell type specific in a variety of malignancies, due to the multifunctional nature of DPP IV.

Several recent studies have shown that DPP IV affects tumour progression in several human malignancies. It has been demonstrated that ovarian carcinoma cell lines (in contrast to thyroid carcinoma) with higher DPP IV expression showed less invasive potential. These findings imply that DPP IV may functionally suppress peritoneal

dissemination and progression of ovarian carcinoma by regulating the expression levels of several molecules associated with carcinoma cell invasion and progression (Kikkawa *et al.*, 2005).

1.3 Proteolytic Inhibitors

Protease activity must be regulated in order to avoid inappropriate and uncontrolled protein degradation. Proteolytic activation must be stopped when the activated molecule has fulfilled its biological role. This prevents for example, the expansion of a clot outside a wound site, which could be cause of thrombosis. Table 1.2 gives a listing of various inhibitors of endopeptidases.

Serine protease inhibitors
➢ AEBSF (230.7 MW) inhibits catalytic activity of the protease's active site.
➢ Antithrombin (60,000 MW) plasma protein that inhibits thrombin and other serine proteases in the blood clotting cascade.
➢ APMSF (352.7 MW) irreversible inhibitor of trypsin-like serine proteases.
➢ Aprotinin (6500 MW) polypeptide that inhibits serine proteases by tightly binding to the active site of the enzyme.
➢ DFP (184.2 MW) irreversible inhibitor of serine proteases and acetylcholine esterase. Very toxic, take care in its preparation and use.
➢ PMSF (174.2 MW) irreversible inhibitor that acts by chemically modifying the active site of the enzyme. Because it is toxic, care must be taken in its preparation and use.
Serine and cysteine protease inhibitors
➢ Antipain (678.2 MW) reversible inhibitor of proteases and of RNA synthesis.
➢ Chymostatin (600 MW) reversible inhibitor of some serine and cysteine proteases.
➢ Leupeptin (475.6 MW) reversible competitive inhibitor of trypsin-like proteases.
➢ TLCK (369.3 MW) inhibits irreversibly by chemically altering the enzyme active site.
➢ TPCK (351.8 MW) irreversibly inhibits by chemically altering the enzyme active site.
Cysteine protease inhibitors
➢ E-64 (357.4 MW) non-competitive irreversible inhibitor of cysteine proteases.
Metallo-protease inhibitors
➢ Amastatin (511 MW) reversible inhibitor. Non-toxic.
➢ Bestatin (244.8 MW) multi-function metallo-protease inhibitor that has anticarcinogenic and immunomodulating properties.
➢ Diprotin (341.5 MW) reversible inhibitor.
➢ EDTA (372.3 MW) reversible inhibitor that acts by chelating enzyme cofactors. May interfere with other metal dependent biological processes.
Aspartic protease inhibitors
➢ Pepstatin (685.9 MW) peptide that provides reversible inhibition.

http://www.sigmaaldrich.com/Area_of_Interest/Biochemicals/Enzyme_Explorer/Key_Resources/Protease_Inhibitors/Broad_Spectrum_Inhib_.html

Table 1.2: *An overview of endopeptidase inhibitors.*

1.3.1 Serine Inhibitors

The activity of the serine proteases is commonly maximal at alkaline pH and is characteristically inhibited by diisopropyl fluorophosphates (DFP) (Bharadwaj, Bharadwaj, Hati 1996) and a variety of naturally occurring protein inhibitors such as soya bean trypsin inhibitor (SBTI), Ovoinhibitor and aprotinin. Phenylmethane sulphonyl fluoride (PMSF) has a similar action to DFP but is not as selective since it can also inactivate cysteine proteases under certain conditions (Barrett 1986). Both serine and cysteine proteases are likewise inhibited by the microbial derived product leupeptin. Another class of inhibitors with reactivity towards serine and cysteine proteases are chloromethyl ketone derivatives of amino acids and peptides. Among the serine proteases, however, different chloromethyl ketones show a degree of specificity for classes of enzyme (Kettner & Shaw 1981). Thus chloromethyl ketone derivatives of lysine (Tos-Lys-CH_2Cl; TLCK), phenylalanine (Tos-Phe- CH_2Cl; TPCK) and alanine (Ac-Ala-Ala-Ala-Ala-CH_2CL) can distinguish between trypsin, chymotrypsin- and elastase- like activities respectively by conforming to the active site specificities of these enzymes. More selective chloromethyl ketone inhibitors have been synthesized with 3 or more amino acids to take advantage of further binding specificities.

1.3.2 Cysteine Inhibitors

Endogenous inhibitors, generally known as cystatins, are the ultimate regulators of cysteine proteases. Initially, three distinct inhibitors were found in human tissues: neutral cysteine protease inhibitor (NCPI) isolated from spleen; acid cysteine protease inhibitor (ACPI) isolated from epidermis; and α-cysteine proteinase inhibitor (α-CPI) from serum (Jarvinen 1978). ACPI and NCPI have similar molecular weights of about 12.4 kDa but differ in other properties and have been renamed as stefin (or cystatin) A and stefin (or cystatin) B respectively. The serum proteinase inhibitor is a different type of molecule

with a higher molecular weight (90 kDa and 170 kDa) and has been reclassified as a kininogen. All three inhibitors are group specific against cysteine proteases and do not inhibit serine or aspartic proteases.

Stefin A is predominantly localised in epithelial cells, stefin B is much more generally distributed being also found in lymphocytes, liver (Davies & Barrett 1984), and cultured monocytes and epithelial like cells (Hopsu-Havu *et al.*, 1984). Stefin A binds more tightly to most cysteine proteinases than stefin B and both inhibitors bind reversibly (Lah *et al.*, 1993). Two types of cystatins, C and S, have been found in human saliva. Cystatin C is also abundant in human serum. The cystatins also have a great affinity for cysteine proteases and bind reversibly, they are generally the most effective cysteine protease inhibitors. The kininogens are multi-functional proteins present in plasma, body fluids and certain tissues. They also function as precursors of bradykinin and H-kininogen is a factor in the blood clotting cascade (Barrett *et al.*, 1986).

The mammalian lysosomal cysteine proteinases show acidic pH optimum when assayed *in-vitro*, especially with protein substrates which become unfolded and more susceptible to hydrolysis. Plant cysteine proteases and the calpains are most active at neutral or slightly alkaline pH (Barrett 1986). The specificity sites of papain and other related cysteine proteases contain five to seven extended subsites, the dominant of which binds a phenylalanyl side chain. Cathepsin B accepts a phenyl side chain but also binds as well to an arginyl side chain (Drenth, Kalk, Swen 1976).

Thiol groups are highly reactive and there are numerous compounds which will block the active site of cystein proteases, including organo-mercury compounds such as p-chloromercuribenzoate (PCMB) and chloromercuriphenyl sulphonate (CMPS), alkylating

reagents such as iodoacetate and N ethylmaleimide (NEM), and disulphides such as dipyridyl disulphide (Barrett 1977). As mentioned above, leupeptin and peptidyl chloromethyl ketones inhibit cysteine and serine proteases. More selective than the chloromethyl ketones are the diazomethyl ketones, such as Z-Phe-Ala-CHN_2, which inactivates only cystein proteases (Watanabe, Green, Shaw 1979). The catalytic thiol groups of cysteine proteases can be protected from oxidation by low molecular weight sulphydryl compounds such as cysteine and dithiothreitol (DTT).

1.3.3 Aspartic Inhibitors

Aspartic proteases can act best on peptide bonds between bulky hydrophobic amino acids at pH optima in the range pH 3.5-5.5. The microbial product pepstatin is a powerful inhibitor of aspartic protease and is useful in that it has no or little effect on other proteases (Knight & Barrett 1976).

1.3.4 MMP Inhibitors

Tissue inhibitors of metalloproteases (TIMPS) are a family of low molecular weight inhibitors which form irreversible inactive complexes with activated enzyme. They are widely distributed in tissues and fluids and expressed by many cell types including keratinocytes, fibroblasts, macrophages, and endothelial cells (Birkedal-Hansen 1993).

Two members of TIMP family, TIMP-1 (Mr 28 kDa) and TIMP-2 (Mr 22 kDa), have been identified. TIMPs appear to regulate matrix degradation both by protease elimination and by blockage of autolytic matrix metalloproteases activation. Both TIMP-1 and TIMP-2 form complexes with active collagenases but differ from each other in binding to different parts of the enzyme molecule (Birkedel-Hansel 1993).

1.3.5 Exopeptidase Inhibitors

The classification of exopeptidases has, as outlined in section 1.2.1.5, been based on their substrate and product specificity. Consequently, the action of inhibitors has been of less importance in the categorization of exopeptidases than was the case with endopeptidase. However, there are few class-specific inhibitors of exopeptidases. These include the microbial products bestatin, which inhibits aminopeptidases (Suda *et al.*, 1976), and the diprotins which act against dipeptidyl peptidases (Umezawa *et al.*, 1984). Aminopeptidases are also sensitive to the chloromethyl ketone Leu-CH_2CL (Kettner, Glover, Prescott 1974) and DPP II is inhibited by large cations such as Tris and puromycin. In addition some exopeptidases show inhibitor responses similar to those of the four classes of endopeptidase, serine, cysteine, aspartic and metallo proteases, (McDonald & Berrett 1986).

1.4 Cytokines

The term cytokine is applied to small molecular weight peptides or glycopeptides produced by cells in response to a variety of inducing stimuli. They are secreted by their producer cells and then influence the behaviour of target cells. All cytokines have certain properties in common. Many are produced by multiple cell types such as lymphocytes, monocytes/macrophages, mast cells, eosinophils, even endothelial cells lining blood vessels. Each individual cytokine can have multiple functions depending upon the cell that produces it and the target cell upon which it acts (called pleiotropism). Also, several different cytokines can have the same biologic function (called redundancy). Cytokines can exert their effect through the bloodstream on distant target cells (endocrine), on target cells adjacent to those that produce them (paracrine) or on the same cell that produces the

cytokine (autocrine). Physiologically it appears that most cytokines exert their most important effects in a paracrine and/or autocrine fashion. Physiological roles of cytokines include the control of cell proliferation and cell differentiation, control of host defences against viral, parasitic infections, cytotoxic and phagocytic cells, regulation of haematopoiesis, immune response, inflammatory response and fever, tissue remodelling, bone formation, and wound healing (Clemens 1991).

1.5 Angiogenic Agents

While anti-angiogenic compounds would be useful in treating diseases having uncontrolled angiogenesis, angiogenic compounds are needed in wound healing and tissues damaged by ischemia from heart attacks or stroke (Emanueli & Madeddu 2004).

A number of mediators including growth factors and cytokines have been implicated in angiogenesis. The process of angiogenesis also requires the adhesion of endothelial cells to the ECM components and other endothelial cells therefore, in addition to the above mentioned mediators, ECM components and CAMs may also act as angiogenic mediators (Albelda 1993).

1.5.1 Growth Factors

Several factors that control neovascularisation, including FGF, TGF-ß, and VEGF, are responsible for proliferation, migration and differentiation of endothelial cells to form capillary-like structures. These general stimulators of angiogenesis, produced or secreted by vascular cells, are stored in the extracellular matrix and are released during proteolysis (Bicknell, Lewis, Ferrara 1997).

Heparin binding growth factors consists of aFGF, bFGF, and VEGF. These growth factors are bound to heparin and heparin sulphate in the ECM. The heparin stimulates the angiogenic ability of these growth factors, and their release from the ECM facilitating their interaction with endothelial cells. In addition heparin binding also prevents degradation of these growth factors by proteolytic enzymes, which would otherwise reduce their angiogenic ability (Diaz-Flores, Gutierrez, Varela 1994).

Fibroblast growth factor (FGF)

A variety of tumours have been shown to express FGFs, including the two major prototypes, aFGF and bFGF (Bikfalvi 1995). bFGF is recognised as an important autocrine regulator of endothelial cells. Recent studies have demonstrated that antibodies against bFGF would inhibit angiogenesis and tumour growth *in-vivo* and administration of bFGF would enhance angiogenesis and tumour growth (Gold, Scher, Weinberg 1995). bFGF has also been demonstrated to facilitate every step of angiogenesis in-vitro, while aFGF is known to be angiogenic in *in-vivo* assays (Klagsbrun & D'Amore 1991).

Vascular Endothelial Growth Factor (VEGF)

Identification of VEGF and other growth factors has made it possible to gain information about novel targets for antiangiogenesis. For example, inhibition of VEGF function by neutralising antibodies or mutant receptors has been shown to result in a spectacular tumour regression in animal models (Toi 1995). VEGF exerts its functions via binding to its receptors VEGFR-1 and VEGFR-2, which are predominantly expressed in endothelial cells.

Downregulation of VEGFR-2 mRNA in endothelial cells has been shown to be correlated with the termination of angiogenesis (Skobe *et al*, 1997). VEGF, a heparin-binding protein is an important multifunctional cytokine that promotes vasculogenesis and angiogenesis

(Bicknell, Lewis, Ferrara 1997). It can be produced and secreted by various cell types including many tumour cells (Bikfalvi 1995) and activated macrophages (Bicknell, Lewis, Ferrara 1997). VEGF can promote angiogenesis through a variety of mechanisms. It stimulates the proliferation of endothelial cells and increases their migratory activity, in part by activating genes participating in proteolysis. In many instances these effects are synergistically enhanced by the presence of other angiogenic factors such as bFGF. The property of VEGF as an permeabiliser of many small vessels allows it to influence the production of specific extracellular matrix by promoting the extravasation of plasma fibrinogen leading to fibrin deposition, and ultimately to more extensive angiogenesis, through the participation of invading activated macrophages. VEGF possesses a signal sequence, and is released efficiently from the cells. It is therefore an attractive applicant for a paracrine tumour angiogenesis factor.

Non heparin binding growth factors include TGFα, HGF, EGF, PD-ECGF. These growth factors are mitogens for endothelial cells and have been shown to stimulate angiogenesis in many experimental systems. TGFα and EGF stimulate endothelial proliferation *in-vitro* and angiogenesis *in-vivo* (Folkman & Shing 1992).

PD-ECGF is chemotactic for endothelial cells in-vitro and is also angiogenic in in-vivo assays (Diaz-Flores, Gutierrez & Varela 1994). In addition TGFβ, IGF-1 and PDGF may also play an indirect role in angiogenesis.

TGF β acts as a bifunctional molecule in angiogenesis. It has been concluded that TGF-ß is a positive regulator of angiogenesis. However, it inhibits endothelial cell proliferation *in-vitro*. Hence, it has been concluded that it is an inhibitor at high concentrations and a stimulator at low concentrations. A large number of tumour cells produce TGF-ß, but it is

yet to be established how these factors are activated (Bikfalvi 1995). IGF has been shown to promote endothelial cell chemotaxis and tube formation on endothelial cell culture (Sunderkotter *et al.*, 1991).

1.5.2 Cytokines

Cytokines TNFα, IL-1 β, IL-8, and IL-6 are produced by macrophages and therefore are responsible for macrophage derived angiogenic activity. The role of TNFα and IL-1β in angiogenesis and tumour angiogenesis is the subject of investigation in the present study, described in detail under section 1.8.1 and 1.8.2. IL-1-8 has been shown to stimulate endothelial proliferation and chemotaxis *in-vitro* and induce angiogenesis *in-vivo*. It has also been implicated in tumour induced angiogenesis (Szekanecz *et al.*, 1999). IL-6 has been shown to stimulate the migration but has been shown to inhibit the proliferation of endothelial cells *in-vitro* (Sunderkotter *et al.*, 1994).

1.5.3 Other Factors

Other factors such as angiogenin, angiotropin, HAF, and prostaglandins E1 have also been shown to be angiogenic mediators. Angiotropin and HAF are known to be angiogenic *in-vivo* and have also been shown to stimulate migration of endothelial cells *in-vitro* (Sunderk tter *et al.*, 1994). Whereas angiogenin and prostaglandins E1 do not stimulate endothelial cell proliferation *in-vitro*, they do stimulate angiogenesis *in-vivo* (Diaz-Flores, Gutierrez & varela 1994).

1.5.4 ECM

Adhesion of endothelial cells to ECM stimulates their mitogenesis, proliferation, migration, and chemotaxis. Several components of the ECM can act through other

mechanisms to further promote angiogenesis or inhibit it (Wang, Anderson, Gladson 2005). ECM components comprise mainly of type 1 collagen, type IV collagen, fibronectin, laminin, proteoglycans, thrombospondin 1, vitronectin, and entactin (Diaz-Flores, Gutierrez & Varela 1994). Endothelial cell migration has been shown to be stimulated by type 1 collagen and inhibited by type IV collagen. Fibronectin, laminin, and proteoglycans are involved in the adhesion of endothelial cells to the basement membrane and the underlying ECM. In addition, fibronectin is chemotactic for endothelial cells and promotes the elongation of microvessels in explant cultures *in-vitro*, while laminin and proteoglycans are secreted during endothelial proliferation (Colville-Nash & Scott 1992). Heparin, a proteoglycan as described earlier, is involved in regulation of angiogenic mediator release (Diaz-Flores, Gutierrez, & Varela 1994).

1.5.5 CAM

Attachment of endothelial cells to the ECM is regulated by cell adhesion molecules (Wang, Anderson, Gladson 2005). CAMs include, integrins ($\alpha1\beta1$, and $\alpha2\beta1$), $\alpha4\beta1$ integrins, members of the immunoglobulin superfamily of CAMs, selectins and cadherins (Auerbach & Auerbach 1994).

Integrins are a diverse and large group of heterodimeric glycoproteins. The two subunits, designated as alpha and beta, both participate in binding. Integrins participate in cell-cell adhesion and are of great importance in binding and interactions of cells with components of the extracellular matrix such as fibronectin. Interactions of integrins with the extracellular matrix can have profound effects on cell function, and events such as clustering of integrins activates a number of intracellular signally pathways (Horwitz 1997). The $\alpha1\beta1$ and $\alpha2\beta1$ integrins mediate endothelial adhesion to laminin, types 1 and

IV collagen. A number of integrins mainly β1 and β3 integrins have been implicated in angiogenesis. Endothelial cells may interact with other cells during angiogenesis.

The immunoglobulin (Ig) - like large family of adhesion molecules have components that resemble Ig. The group includes molecules involved in antigen recognition by lymphocytes and other cells (CD3, CD4, CD8). Other subgroups mediate cell adhesion for a variety of different cell types. Adhesion molecules ICAM-1 and ICAM-2 are expressed on epithelial and endothelial cells and recognized by leukocyte integrins. Vascular adhesion molecule VCAM-1 is a ligand for α4 β1 integrin on monocytes and lymphocytes.

Selectins are expressed primarily on leukocytes and endothelial cells and, like integrins, are important in many host defence mechanisms involving those cells. In contrast to other cell adhesion molecules, selectins bind to carbohydrate ligands on cells and the resulting binding forces are relatively weak. For example, selectin-mediated interactions between leukocytes and endothelial cells promote rolling of the leukocytes along the endothelium and integrin-binding allows the leukocytes to be stopped in place (Horwitz 1997).

Calcium-dependent cell adhesion molecules have both adhesion and calcium binding sites on the extracellular domain. They bind to other cadherins on the surface of adjacent cells (Lukáš & Dvořák 2004).

Endothelial CAMs mediating intercellular adhesion include VCAM-1, ICAM-1, CD31, E and P selectins and CD34 (Szekanecz *et al.*, 1999). Alterations in expression and function of cell-cell and cell-matrix adhesion receptors have been identified in the majority of malignant tumours (Pignatelli & Vessey 1994).

Factors	Mode of action
FGF	Stimulates endothelial cell (E.C) proliferation and migration.
TGF β (in vitro)	Inhibits E.C proliferation At high concentrations inhibits E.C cord formation & at lower concentrations stimulates E.C cord formation.
VEGF	Promotes (in vitro) E.C proliferation. Promotes (in vivo) angiogenesis.
Thrombospondin	Antagonises growth factors.
Angiostatin	Endogenous E.C proliferation and migration.
Angiogenin	Angiogenic in vivo but inhibits E.C growth and angiogenesis in vitro.
TNF α (in vitro)	Inhibits E.C proliferation.
TNF α (in vivo)	Stimulates angiogenesis.
IL-1	Inhibits E.C proliferation.
INF α & β	Inhibits angiogenesis. INF α causes downregulation of bFGF mRNA.

(Craft & Harris 1994; Lichtenbeld, van Dam-Mieras, Hillen 1995)

Table 1.3: *Mode of action of some of the angiogenic and antiangiogenic factors.*

1.6 Antiangiogenic Agents

There is ample therapeutic opportunity for the use of antiangiogenic inhibitors in the clinic, as there are several human diseases that are dependent upon angiogenesis. However, no disease has attracted as much attention as a target for antiangiogenic therapy as malignant disorders.

The discovery of the molecular mechanism of physiological and pathological angiogenesis helped to recognise two class of diseases: one where therapeutic angiogenesis can repair the tissue damages (ischemic diseases, arteriosclerosis etc) and the other one where inhibition of pathological angiogenesis can cure the disease or delay its progression (retinopathies, tumour progression, chronic inflammatory processes) (Timar *et al.*, 2001).

Studies have shown the use of angiogenic inhibitors to be as effective agents for blocking tumour-induced angiogenesis and subverting tumour growth and disease dissemination

(Ferrara & Kerbel 2005). One of the unique attractions of targeting tumour angiogenesis is that vascular endothelial cells are a genetically stable population in which acquisition of therapeutic resistance might be less efficient than in genetically unstable tumour cells (Sepp-Lorenzino & Thomas 2002).

Each of the processes involved in angiogenesis, activation of endothelial cells, release of proteases from activated endothelial cells, degradation of the basement membrane surrounding the existing vessel, migration of endothelial cells into the interstitial space, endothelial cell proliferation, and differentiation into mature blood vessels, presents possible targets for therapeutic intervention. Synthetic inhibitors of cell invasion (marimastat, Neovastat, AG-3340), adhesion (Vitaxin), or proliferation (TNP-470, thalidomide, Combretastatin A-4), or compounds that interfere with angiogenic growth factors (interferon-α, suramin, and analogues) or their receptors (SU6668, SU5416), as well as endogenous inhibitors of angiogenesis (endostatin, interleukin-12) are being evaluated in clinical trials against a variety of solid tumours. As basic knowledge about the control of angiogenesis and its role in tumour growth and metastasis increases, it may be possible in the future to develop specific anti-angiogenic agents that offer a potential therapy for cancer and angiogenic diseases (Liekens, De Clercq, Neyts 2001).

1.6.1 Growth factors

TGF β as mentioned previously, section 1.5.1, has a dual role in angiogenesis, depending upon its concentration.

1.6.2 Cytokines

TNFα, IL-1α, IL-1β, and IL-6 as mentioned before have shown to stimulate or inhibit angiogenesis under different circumstances. IL-1α and IL-1β inhibit the growth of

cultured endothelial cells. However, their role *in-vivo* is controversial, as some groups found this cytokine to promote angiogenesis while others have shown to inhibit it (Sunderkotter *et al.*, 1991). Other cytokines IFN γ and IFNα have been shown to inhibit endothelial cell migration and block FGF, VEGF, and ECGF induced endothelial cell proliferation *in-vitro*.

IL-12 has shown to inhibit angiogenesis *in-vivo* via IFN γ mediated pathway (Szekanecz *et al.*, 1999).

1.6.3 Other Factors

Other factors that are antiangiogenic include protamine, PF4, thrombospondin 1, suramin, steroids, protease inhibitors, some antimicrobial agents, angiostatin and angiogenin.

Protamine (Klagsbrun & D'More 1991), PF4 (Daly *et al.*, 2003), Thrombospondin 1 and suramin (Liekens, Clercq, Neyts 2001) block the binding of growth factors to heparin and thus inhibit the actions of growth factors such as VEGF and FGF. Thrombospondin 1 is a potent inhibitor of endothelial proliferation and angiogenesis (Bikfalvi 1995). When cell lines are transformed to a malignant phenotype the production of thrombospondin 1 is downregulated. It has been suggested that control of thrombospondin is associated with the tumour suppressor gene p53 and the mutation of this gene results in decreased production of thrombospondin 1, increased angiogenesis and tumour formation (Grant *et al.*, 1998).

Angiostatic steroids such as MPA inhibit basement membrane degradation, and 2-methoxyestradiol suppresses the proliferation of bFGF stimulated endothelial cells. A variety of antiestrogens including tamoxifen, clomiphene and nafoxidine could also block

angiogenesis *in-vivo.* Cortisone was found to inhibit endothelial cell migration and proliferation (Auerbach & Auerbach 1994).

Protease inhibitors such as TIMP-1 and -2 and PAI-1 and -2 prevent the breakdown of ECM proteins and thereby inhibit angiogenesis (Bicknell, Lewis & Ferrara 1997).

Antimicrobial agents such as minocycline exhibits anti-collagenase activity, and rapamycin inhibits endothelial cell proliferation. Analogues of fumagillin are angiostatic and suppress tumour growth (Szekanecz *et al.*, 1999).

Angiostatin is strongly anti angiogenic *in-vitro*, and can suppress the growth of Lewis lung carcinoma metastasis in tumour bearing mice (Sunassee & Vile 1997). It has also been demonstrated to inhibit bFGF induced endothelial cell proliferation and migration (Calbiochem 1998). .

Angiogenin has been shown to be angiogenic *in-vivo*, but inhibits proliferation of endothelial cells in vitro (Sunassee & Ville 1997). Angiogenin and angiostatin have been shown to work better in combination than either one alone in inducing the regression of tumours in mice (Boehm *et al.*, 1997). Both angiostatin and angiogenin are naturally occurring proteins found in the body.

1.7 Cytokine Therapy

Disorders of growth control, including all cancers, arise from disruption of the signalling pathways which normally control cell growth and differentiation. Many of these pathways are regulated by cytokines, hence attempts have been made to use cytokines to correct or counteract the abnormal behaviour of cancer cells. Furthermore, since immune surveillance is important in the normal suppression of transformed cell growth, stimulation of surveillance by exogenous cytokines might give benefits on top of the direct anti-tumour

effects. Recent years have seen numerous clinical trials of cytokine treatment of cancer, with varying rates of success.

Neutralization of endogenous growth factors and administration of exogenous bioactive cytokines are two distinct biological anti-tumour strategies that show promise for treatment of cancer patients (Shen *et al.*, 2002).

The direct anti-tumour properties of proinflammatory cytokines are generally considered to reside in their ability to inhibit tumour growth or cause cell death (Stoelcker *et al.*, 2000).

1.7.1 TNFα

TNFα is a pleiotropic inflammatory cytokine. It was first isolated by Carswell & co-workers in 1975 in an attempt to identify tumour necrosis factors responsible for necrosis of the sarcoma Meth A (Carswell *et al.*, 1975). It is a stimulating cytokine which influences various types of cells (Nenova & Kovatchev 2000). It was first identified for its anticancer activity (Szatmary 1999).

Human TNF α is a nonglycosylated polypeptide that exists as either a transmembrane or soluble protein. TNF α is produced by many different cell types, primarily by macrophages and monocytes (Eisenstein & Strober 1996). Other sources include stimulated monocytes, fibroblasts, endothelial cells, T-cells and B-lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, and keratinocytes.

TNFα has a very wide range of physiological and pathological effects. These include mediation of inflammation, cellular immune response (Gallea et al., 1995), activation of host defence (Szatmary 1999), activation of neutrophils, upregulation of adhesion molecules, stimulation of several cytokines including IL-1, and intravascular coagulation resulting in thrombosis and arteriosclerosis.

TNFα seems to serve as a mediator in various pathologies. A few such examples include: Septic shock, Cancer, AIDS, Transplantation rejection, Multiple Sclerosis, Diabetes, Rheumatoid arthritis, Trauma, Malaria, Meningitis, Ischemia-Reperfusion Injury, and Adult respiratory distress syndrome. TNFα may be involved in the development of cancers that are associated with chronic inflammation and also promotes the formation of tumours from pre-cancerous cells. There is evidence that TNFα is important for the growth of epithelial tumours (Imperial cancer research fund press release 1999).

It has been identified as an important stimulator of angiogenesis *in-vivo* (Bicknell, Lewis, Ferrara 1997). It is a multifunctional peptide with respect to its angiogenic capability of promoting the expression, synthesis, and secretion of uPA protease by endothelial cells, and by enhancing collagenase synthesis by endothelial cells. *In-vitro*, TNFα stimulates endothelial cell migration and tube formation, but inhibits the proliferation of these cells (Baillie, Winslet, Bradley 1995). It has been demonstrated that TNFα can mediate an indirect metastatic activity *in-vivo* by activating the vascular endothelial cells, resulting in an enhanced retention of the subsequently injected tumour cells. In addition to this indirect effect on the metastatic potential of cancer cells, TNFα has also found to exert a potent direct tumour promoting activity. Nevertheless, TNFα is also known for its anti tumour effects *in-vivo,* including its direct cytostatic and cytotoxic effect on some tumour cells *in-vitro* (Clemens 1991). Treatment of 3LL-s tumour cells with TNFα has been shown to result in a highly tumorigenic and metastatic potential. On the other hand TNFα has shown to posses anti tumour activity (Gallea *et al.*, 1995) by directly killing tumour cells (Eisenstein & Strober 1996). This versatile action could be due to varying concentrations used. For example, low doses of TNFα have been shown to promote angiogenesis, while high doses have been shown to inhibit angiogenesis (Fajardo & Allison 1997).

TNFα induces neutrophil proliferation during inflammation, but it also induces neutrophil apoptosis upon binding to the TNF-R55 receptor (Murray *et al.*, 1997). TNFα is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. The pathological activities of TNFα have attracted much attention. For instance, although TNFα causes necrosis of some types of tumours, it promotes the growth of other types of tumour cells. High levels of TNFα correlate with increased risk of mortality (Rink & Kirchner 1996). If TNFα remains in the body for a long time, it loses its anti tumor activity. This can occur due to polymerization of the cytokine, shedding of TNF receptors by tumor cells, excessive production of anti-TNF antibodies, found in patients with carcinomas or chronic infection, and disruptions in the alpha-2 makroglobulin proteinase system which may deregulate cytokines. Prolonged overproduction of TNFα also results in a condition known as cachexia, which is characterized by anorexia, net catabolism, weight loss and anemia and which occurs in illnesses such as cancer and AIDS. Cachectin and TNFα were once considered different proteins, but in 1985 researchers discovered that the two proteins were homologous (Beutler *et al.*, 1985a).

Since TNFα plays a role in several diseases, a substantial amount of research has been conducted concerning TNFα therapies and anti-TNFα therapies. Because TNFα exhibits anti tumor activity (Orosz *et al.*, 1993), research has been conducted to determine the protein's effectiveness against certain forms of cancers. Utilizing TNFα tumoricidal activities has proved problematic, especially due to the cytotoxin's systematic toxicity. While higher doses of TNFα may exhibit higher cytotoxicity, high doses also lead to systematic toxicity (National Cancer Institute 1995). Some studies involving TNFR-75 and TNFR-55 mutants have suggested that the TNFR-75 receptor plays a role in systematic

toxicity, while TNFR-75 mutants will exhibit cytotoxicity but not systematic toxicity (Van Ostade *et al.*, 1993). Additionally, a mutant form of TNFα which exists only in the transmembrane form acts only by cell-to-cell contact and may result only in cytotoxicity (Perez *et al.*,1990), suggesting that mutant forms of TNFα might effectively be used therapeutically as against specific types of cancers.

1.7.2 IL-1 β

IL-1 β is primarily produced by activated macrophages in response to LPS and TNF. Other sources include osteoblasts, keratinocytes, fibroblasts, monocytes, endothelial cells and PMN leukocytes NK cells, epithelial cells, B lymphocytes, Gliomas, neutrophils, fibroblasts, synovial cells and smooth muscle cells (Clemens 1991).

Most of the functions of TNF α and IL-1 β are similar. IL-1 β has been known to induce B and T cell differentiation, activate natural killer cells, and induce neutrophil release from the bone marrow. It has also been known to induce adhesion molecules such as E-selectin by HUVECs (Markovic *et al.*, 2002).

Clinical trials of IL-1 α and IL-1 β have been completed that assess the toxicities of these cytokines as well as their hematopoietic and anti-tumour effects. Both forms of IL-1 recognise the same cell surface receptors and have similar toxicities and biological activities, both being dose related. IL-1 alone has little anti-tumour activity against melanoma, renal cell carcinomas or other malignancies. However, IL-1 seems to endow certain progenitor cells with responsiveness to other hematopoietic cytokines, including colony-stimulating factors and IL-3 (Clemens 1991).

The use of IL-1 in humans is associated with dose-limiting toxicity, which resembles that of TNFα (Ogilvie *et al*, 1996). IL-1 has been found to be cytotoxic to certain sensitive

tumour cell lines, such as the human melanoma cell line A375. Therefore, it is possible that IL-1 might be used as a therapy in the treatment of cancer (Curtis & Smith 1995) in several different ways: - by directly inhibiting tumour cells. by enhancing the effect of NK cells and cytotoxic T lymphocytes, by augmenting the production of other lymphokines, such as IL-2 and interferon, and synergistically acting with IL-2 and interferon in promoting CTL and NK cell activities (Clemens 1991).

Both TNFα and IL-1 β have been reported to have an anti-tumour effect, they also seem to act as a tumour progression factor regarding the modulation of tumour motility or the augmentation of adhesion molecule expression on the target organs.

Both the cytokines may directly affect the growth and the metastasis of tumour cells. It is therefore important to clarify the direct influence of these cytokines on tumour cells in order to obtain better knowledge of anti-tumour therapy.

1.8 Antiangiogenesis

The knowledge that tumours require the process of angiogenesis for the supply of nutrients, in order for the tumour to grow, offers distinct opportunities for therapy.

Angiogenesis for various types of tumours is a must. Therefore, effective anti-angiogenic therapy might be of great value in general anticancer therapy where conventional anti-cancer therapies are of no value (Rak *et al.*, 2002). This is because different tumours show varying susceptibilities to therapies, such as chemotherapy and radiotherapy. However, it must be taken into consideration that the anti-angiogenic therapy should not interfere with the normal process of angiogenesis in healthy tissues.

Recent studies involving anti-tumour angiogenesis have been of significant value, it deals with the idea of achieving tumour-specific blood clots by molecular engineering. This was

proposed with the thought that an effective way to kill a tumour would be by inducing thrombosis at the tumour site. The molecule of interest was TF which is responsible for the initiation of blood-clotting cascade (Goldenberg 1995). A soluble form of TF was constructed, which, because of its solubility had minimal coagulation properties when administered free in the circulation. An experimental model was provided in which a targeting molecule - the antibody - with specificity for a marker known to be expressed specially on the tumour blood vessel endothelium - the MHC class II molecules - (or VEGF) was linked to a molecule (sTF) which could induce clot formation when immobilized on a cell surface. One such study was done on tumour induced mice, which were treated systemically with the antibody-sTF-complex. It was observed that thrombosis was induced within the growing tumours, and within 72 hours of administration of the complex the entire treated region of the tumour was necrotic. This was compared to the tumour induced mice with no antibody-sTF-complex, which failed to show such anti-tumour affects, moreover, no blood clot was detected in non-tumour tissues in the mice treated with the antibody-sTF-complex. This showed that the proposed work was highly tumour specific and hence, would not interfere with the normal physiology of angiogenesis (Sunassee & Vile 1997).

Therapies that work effectively in arresting the process of tumour induced angiogenesis or even reducing it may cure many patients or allow them to live longer rather than to die from the cancer.

1.9 *In-Vitro* and *in-Vivo* Models to Study Angiogenesis

Angiogenesis can be qualitatively and quantitatively measured in a large variety of in vitro and *in-vivo* model systems. The angiogenic cascade can be dissected in different

sequential steps so that can each can be studied separately *in-vitro*. Research has mainly focused on proliferation and migration of endothelial cells. For human research most laboratories make use of HUVECs. This is the best available source of human endothelium, but the major drawback of these cells is their macrovascular origin, which makes them less suitable for studies on angiogenesis, a microvascular process.

Assays to study proliferation of endothelial cells are based on cell counting or radiolabeled thymidine incorporation, or on colorimetric systems for the measurement of mitochondrial activity by MTT. Also, detection of cell death is a commonly used approach to average cell growth; e.g., apoptosis induction can be studied by detection of subdiploid cells or analysis of DNA degradation profiles or cell morphology (Griffion & Molema 2000).

For analysis of migration of endothelial cells, Boyden chambers are primarily used (Fischer, Stingl, Kirkpatrick 1990). An easier method is the wound assay. This assay system is based on wounding of a confluent monolayer of endothelial cells and measurement of the wound width in time.

Although the advantage of these *in-vitro* assays is clearly the control over the few parameters present, the angiogenic cascade consists of multiple steps. This as a more extended process, can be studied *in-vitro*, too. Most of these assays studying more complex processes of angiogenesis are based on tube formation of long-term cultured endothelial cells in a 3-dimensional semi natural matrix microenvironment. The most commonly used assay system to measure tube formation is the growth factor-induced sprouting of capillary-like structures from a confluent monolayer of endothelial cells grown on a thick gel. These gels can either be composed of a semi natural matrix with or without growth factors (e.g., Matrigel), or be based on collagen or fibrin (Koolwijk *et al.*, 1996).

Besides the advantages that *in-vitro* angiogenesis assays clearly have, the major drawback of all these assays is that they require the endothelial cells to be removed from their natural microenvironment, which may alter their physiologic properties. To study angiogenesis in vivo, the most frequently used assay systems are the chicken chorioallantoic membrane assay (Nguyen, Shing, Folkman 1994), the corneal pocket (Conrad *et al.*, 1994), transparent chamber preparations such as the dorsal skin-fold chamber (Lichtenbeld et al., 1998), the cheek pouch window (Griffion & Molema 2000), and the polymer matrix implants (Plunkett & Hailey 1990). However, *in-vivo* assays also have several disadvantages: the pharmacokinetic properties of the compounds tested, necessary for proper interpretation of results, are often not known and the host will respond non-specifically to the implantation. In this review, these assays will not be discussed in more detail because recently an elegant review on this issue appeared, discussing the pro's and con's of in vivo quantitative angiogenesis assays (Jain *et al.*, 1997).

1.10.0 Detection of Proteases and its Inhibitors in Cell Lines

1.10.1 Biochemical Studies

1.10.1.1Natural Substrates

The detection of protease activities is commonly based on the assessment of the breakdown products of natural or synthetic substrates. In the case of endopeptidases, biochemical assays with a natural substrate usually involve the incubation of the enzyme preparation with a protein containing numerous susceptible bonds under a defined set of conditions. Haemoglobin and casein were used in the past to detect general proteolysis without regard to substrate specificity and their breakdown was subsequently measured spectrophotometrically or by the release of trichloroacetic acid soluble products.

Detection of protease activities has also been carried out by the use of chromogenic and fluorogenic derivatives of proteins such as azocasein, azoalbumin and fluoroscein coupled haemoglobin. These are degraded with release of low molecular weight coloured peptidases which can subsequently be assessed by colorimetric or flurometric procedures (Sarath, Delamotte, Wagner 1989).

1.10.1.2 Synthetic Substrates

Synthetic peptide substrates are widely used to assay the proteolytic activity *in-vitro*. They can be used to investigate the substrate specificity of a given protease, to monitor the enzyme activity during the course of purification or to measure the residual enzyme activity when analysing the kinetics of a reaction between a protease and an inhibitor.

Synthetic substrates consist of a short peptide sequence conjugated to a chromogenic substrates that generate chromogenic compound upon cleavage, examples are peptide 4-nitroanilides (pNA) and peptide thioesters, and fluorogenic substrates that generate a fluorogenic compound upon cleavage. Examples include MCA substrates (peptidyl 4-methyl-7 coumarylamides) and naphtylamide substrates (Gleddie & Michaud 2000). Endopeptidase synthetic substrates have no free amino terminus i.e. the alpha-NH_2 is blocked, whereas aminopeptidase and carboxypeptidase substrates have a free N-terminus and a free carboxy terminus respectively.

1.10.1.3 Biochemical Studies

Visualisation of enzyme activity in cells is based on the principal of conversion of a water-soluble substrate that is specific for the enzyme to be studied leading to water-insoluble and either coloured, electron dense or fluorescent final reaction product. Methods are available for a large number of enzymes (Stoward & Pearse 1991) and methods are

relatively simple, often far more simple then in situ hybridization or immunohistochemical methods. The present study used highly selective fluorogenic substrates Z-Arg-Arg-AMC, for cathepsin B, and Gly-Pro-AMC, for DPP IV enzyme.

Principle of the Assay

This fluorometric assay that was used in the present study was designed for the quantitative *in-vitro* determination of protease activity. For example cathepsin B activity was measured in microplates. The test utilises the ability of cathepsin B to digest the synthetic substrate Z-Arg-Arg AMC (Barrett & Kirschke 1981). Released free AMC is determined fluorometrically at excitation wavelength 360-380 nm and emission wavelength 440-460 nm. The activity of the cathepsin B can be quantified with an AMC standard, or can be displayed as fluorescence units (FU).

1.10.2 Immunological Methods

Immunological methods are widely used to detect pathogens in clinical samples. Accurate diagnosis depends on the affinity and specificity of the antibody preparation used, and high affinity antibodies are essential for the detection of very small amounts of pathogen.

Monoclonal and recombinant antibodies can be produced in potentially unlimited quantities, and the epitopes with which they react can be identified, thus making well characterised preparations of these reagents the preferred choice for inclusion in standard assays. Also, assay specificity can be designed to detect one or more antigens by incorporating several different antibodies.

1.10.2.1 Immunocytochemical Detection

There are many immunoenzymatic staining methods which can be used to localise antigens. The most direct method uses an enzyme labelled primary antibody which reacts with the antigen (Coons, Creech, Jones 1941). This method is rarely used today, because although it can be completed quickly and non specific reactions are limited, little signal amplification is achieved. From this method, a two step indirect method was developed (Nakane & Pierce 1966) where an unconjugated primary antibody binds to the antigen. An enzyme labelled secondary antibody is directed against the primary antibody followed by the substrate chromogen solution. This is more versatile method because a variety of primary antibodies from the same species can be used with the same labelled secondary antibody. The procedure is several times more sensitive than the direct method because several secondary antibodies are likely to react with different epitopes on the primary antibody.

A two step indirect method can be combined with avidin-biotin detection methods to produce considerably greater amplification of reaction product. This method utilises the high affinity of avidin or streptavidin for biotin. Two avidin-biotin methods are in frequent use – the avidin-biotin complex (ABC) (Van der Loos, Das, Houthoff 1987) (Van der Loos, Becker, Van den Oord 1993) and the labelled avidin-biotin technique (LAB) technique (Gown *et al.*, 1986). Both procedures require the use of secondary biotinylated antibody as a link. The sequence of reagent application is primary antibody biotinylated secondary antibody, preformed avidin-biotin complexes (ABC) or enzyme labelled avidin (LAB), and substrate solution. Alkaline phophatase (Mason & Sammons 1978) or horseradish peroxidase (Nakane & Pierce 1966) are commonly used enzyme labels. Sites of antibody binding can be visualised by immunofluorescence techniques (Coons, Creech,

Jones 1941). An antibody is labelled with a fluorescent dye. When antibody binds antigen the reaction can be visualised by activating the fluorescent dye with UV light.

1.10.3 CD 31

CD 31 is one of the adhesion molecules of 130 kDa and belongs to the immunoglobulin (Ig) superfamily. It is present in thrombocytes, lymphocytes, neutrophils, and endothelial cells, in which it concentrates at the intercellular borders of adjacent cells. Its redistribution depends on cytokines, such as INF- and TNF- (Romer *et al.*, 1995). Its ligands include homophilic interactions with itself, v 3 integrins and CD38 (Jackson 2003).

CD 31 has been implicated in various biological functions such as modulation of integrin-mediated cell adhesion, angiogenesis, apoptosis, cell migration, negative regulation of immune signalling, autoimmunity, macrophage phagocytosis, IgE-mediated anaphylaxis, and thrombosis (Jackson 2003). According to several studies, staining of blood vessels with CD31 antibodies has shown to be suitable for the identification of angiogenesis in several types of malignancies such as lung cancer, breast and colorectal cancer. It plays an important role in transendothelial migration of leukocytes without influence on adhesion of these cells to endothelium (Yong *et al.*, 1998).

1.10.4 vWF

Another endothelial cell marker vWF (Werner 1995) was used to identify the cells isolated from human umbilical vein. It is a multimeric plasma glycoprotein with the molecular weight of 270 kDa. This glycoprotein mediates platelet adhesion to injured vessel walls and serves as a carrier and stabiliser for coagulation factor VIII. It is present in endothelial cells, platelets, and megakaryocytes, as well as in numbers of tumours including

hemangiomas, hemangiosarcomas and Kaposi's sarcomas (Ruggeri 2000). Cultured human endothelial cells synthesise vWF (Jaffe, Hoyer, Nachman 1974).

Expression of the von Willebrand Factor gene is tissue specific and confined to endothelial cells and megakaryocytes. vWF is present in plasma, in the Weibel Pallade bodies of endothelial cells, in the alpha-granule in megakaryocytes and platelets derived from them, as well as in the subendothelial matrix of the vessel wall. Von Willebrand Factor serves as a carrier for Factor VIII in plasma, protecting the circulating coagulation coenzyme from proteolytic degradation.

1.11 Viability Assays

Activity assays established the release of proteases, cathepsin B and DPP IV, extracellularly in the two cancer cell lines and HUVECs. However, it was unsure whether the release was due to the cytotoxic effect of the cytokines.

The quantification of cell viability is an important parameter for the description of the status of cell cultures and is a basis for numerous cytotoxicity studies. A variety of techniques have been successfully applied for the evaluation of cell death including microscopy, vital dye uptake or retention, release of cytoplasmic enzymes, measurement of DNA fragmentation or the release of radioisotopic label. The most popular assay is the 51Cr release test, a simple and highly sensitive test.

Several methods have been described in the literature for the quantitation of cell numbers in microplates. The direct cell counting in a haemocytometer, or cell counters, is time consuming, and often not sensitive or accurate enough. The MTT-assay is based on cellular metabolism, is easy, rapid and sensitive but cannot be applied for fixed cells and other methods are hazardous to use.

1.11.1 Cell Growth/Cell Cycle

The cell growth can be divided into three phases:-

1) Lag phase is the initial time period during which the cells are adjusting to their new environment and no growth of the culture is apparent. The length of this phase is dependent upon the growth conditions, and the size and physiological state of the cells. Lag phase is shortest where growth conditions are good and the cell number is large and consists of actively growing (*i.e.* log phase) cells. Poor growth conditions, a small number of cells taken from a previous culture in lag, stationary or death phase result in a longer lag phase.

2) Log phase is the period of maximum growth. This phase of growth is called logarithmic or exponential because the rate of increase in cell number is a multiplicative function of cell number. In this phase one cell divides into two and then four, etc., with each doubling taking the same time.

3) Stationary phase is a plateau stage during which the rate of production of new cells (the birth rate) equals the death rate. In a batch culture cells do not continue to grow at a maximum rate for ever. As the population increases, nutrients are depleted and toxic metabolic products accumulate. These changes result in the slowing and ultimate cessation of growth.

Cell cycle is defined as the period that extends from the time a cell comes into existence, as a result of cell division, until the time that the cell divides to give rise to two daughter cells (Goodman 1994). The cell cycle is divided into four stages: Gap 1, DNA synthesis or S-phase, Gap 2, and mitosis. When modeling the growth of a population of cells, it is commonly useful to assume that every individual cell doubles with every cell cycle, i.e. the daughter cells themselves divide upon completion of the next cell cycle. This is typically

expressed as an exponentially growing population. However, in many circumstances, not all the cells divide, and the rate of population growth can be affected. For example, tumour growth rate would be quite different than that of normal cells (Begg *et al.*, 1985). In cancer cells there is a loss of cell cycle regulation due to genetic changes, which cause the cancer cells to escape from the normal balance of cell growth. Hence, being able to quantify aspects of the cell cycle is important for measuring the growth of tumours under different treatments.

In the first phase (G1) the cell grows and becomes larger. When it has reached a certain size it enters the next phase (S), in which DNA-synthesis takes place. The cell duplicates its hereditary material (DNA-replication) and a copy of each chromosome is formed. During the next phase (G2) the cell checks that DNA-replication is completed and prepares for cell division. The chromosomes are separated (mitosis, M) and the cell divides into two daughter cells. Through this mechanism the daughter cells receive identical chromosomes. After division, the cells are back in G1 and the cell cycle is completed (Leland, Hunt, Nurse 2001).

The duration of the cell cycle varies between different cell types. Cells in the first cell cycle phase (G1) do not always continue through the cycle. Instead they can exit from the cell cycle and enter a resting stage (G0). Often cells in humans enter a G0 (G-zero) stage, essentially a temporary period of non growth. At any given time most human cells are in the G0 stage (Leland, Hunt, Nurse 2001).

For all living eukaryotic organisms it is essential that the different phases of the cell cycle are precisely coordinated. The phases must follow in the correct order, and one phase must be completed before the next phase can begin. Errors in this coordination may lead to chromosomal alterations. The connection between the cell cycle and cancer is obvious:

cell cycle machinery controls cell proliferation, and cancer is a disease of inappropriate cell proliferation. Fundamentally, all cancers permit the existence of too many cells. However, this cell number excess is linked in a vicious cycle with a reduction in sensitivity to signals that normally tell a cell to adhere, differentiate, or die. This combination of altered properties increase the difficulty of deciphering which changes are primarily responsible for causing cancer (Collins, Jacks, Pavletich 1997).

1.11.2 MTT and Other Methods

A variety of refinements and modifications of the MTT assay have been described.

The **MTT** assay is an alternative to the thymidine incorporation test, which measures cell proliferation by determining the amounts of incorporated tritiated thymidine into freshly synthesised DNA. Use of a MTT as an indirect measure of cell number was first reported in the early 1980s (Plumb 2004). It can be used also to determine proliferation of mycoplasm-infected cells, which would interfere with thymidine incorporation measurements. The proliferative profiles of cells as determined by the colorimetric assay, which essentially measures energy metabolism, or radioisotope assay usually do not show large differences.

In general, cells in log phase of growth are exposed to various drugs to assess their cytotoxicity. After removal of the drug, the cells are allowed to proliferate for two to three doubling times in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate. Surviving cell numbers are then determined indirectly by MTT dye reduction.

The level of MTT cleavage by viable cells of various origins is found to be directly proportional to the number of cells but to increase as a non-linear function of time. This

non-linear relationship is related to a time-linear cell death during MTT incubation. The cleavage of MTT by viable cells is found to follow first order kinetics and could be fitted to Michaelis' kinetics (Gerlier & Thomasett 1986).

The Cell Proliferation Reagent **WST-1** has several advantages. In contrast to MTT, which is converted into a water-insoluble formazan and must be solubilised before quantitation, WST-1 yields water-soluble cleavage products, like XTT, which can be measured without an additional solubilisation step. In addition, WST-1 is more stable than XTT. Therefore, WST-1 can be used as a ready-to-use solution and can be stored at 4°C for several weeks without significant degradation. Moreover, WST-1 has a wider linear range and shows accelerated colour development compared to XTT.

The **XTT** assay can be used for the same applications as the MTT assay. The assay is based on the conversion of the yellow tetrazolium salt XTT into an orange formazan dye by metabolically active cells. In contrast to the purple formazan crystals, which are formed in the MTT assay, the XTT formazan dye is soluble in aqueous solutions, and can thus be directly quantitated with an ELISA reader without the need for a solubilisation step.

1.12 Antigen/Antibody Detection

1.12.1 ELISA

Enzyme Linked ImmunoSorbent Assays are designed for detecting and quantitating substances such as peptides, proteins, antibodies and hormones. Other names, such as Enzyme ImmunoAssay (EIA), are also used to describe the same process. In an ELISA, an antigen must be immobilized to a solid surface. The antigen is then complexed with an antibody that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product. The most crucial

element of the detection strategy is a highly specific antibody-antigen interaction (Crowther 1995).

Most commonly, ELISA are performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. It is this binding and immobilisation of reagents that makes ELISAs so easy to design and perform, as first described by Eva Engvall in 1971 (Lequin 2005). Having the reactants of the ELISA immobilised to the microplate surface makes it easy to separate bound from unbound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude preparation.

A detection enzyme may be linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody. It may also be linked to a protein such as streptavidin if the primary antibody is biotin labelled. However, the enzyme is incorporated, the most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Other enzymes have been used as well, but they have not gained widespread acceptance because of limited substrate options. These include ß-galactosidase, acetylcholinesterase and catalase. A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the necessary sensitivity level of the detection and the instrumentation available for detection (spectrophotometer, fluorometer or luminometer).

1.12.2 Types of ELISA

Diagrammatic overview of the four kinds of ELISA can be seen in figure 1.2.

Direct ELISA

Direct ELISA is the most basic of ELISA configurations. It is used to detect an Ag (virus/bacteria/fungus, recombinant peptide/protein, or another Ab) after it has been attached to the solid phase (e.g. a membrane or polystyrene microwell). An Ab, conjugated

with a label e.g. HRPO, AP, FITC is then incubated with the captured antigen. After washing off excess conjugate and incubating with a substrate and chromogen, the presence of an expected colour indicates a specific Ab-Ag interaction. The conjugate could be a commercial preparation specific for the Ag of interest, or an in-house conjugated monoclonal or polyclonal Ab, or even patient serum.

Indirect ELISA

The indirect assay was first described by Weller & Coons in 1954. Once again an Ag is absorbed onto a solid phase. The first, or primary Ab is incubated with the Ag, then the excess is washed off. A second or secondary Ab, the conjugate, is then incubated with the samples. The excess is again removed by washing. For colour to develop, a primary Ab that is specific for the Ag must have been present in the sample (e.g. human serum, CSF or saliva or the supernatant from a hybridoma culture). This indicates a positive reaction. It is important, during assay optimisation, to ensure that the secondary Ab does not bind non-specifically to the Ag preparation or impurities within it, or to the solid phase.

Capture ELISA *(antigen capture)*

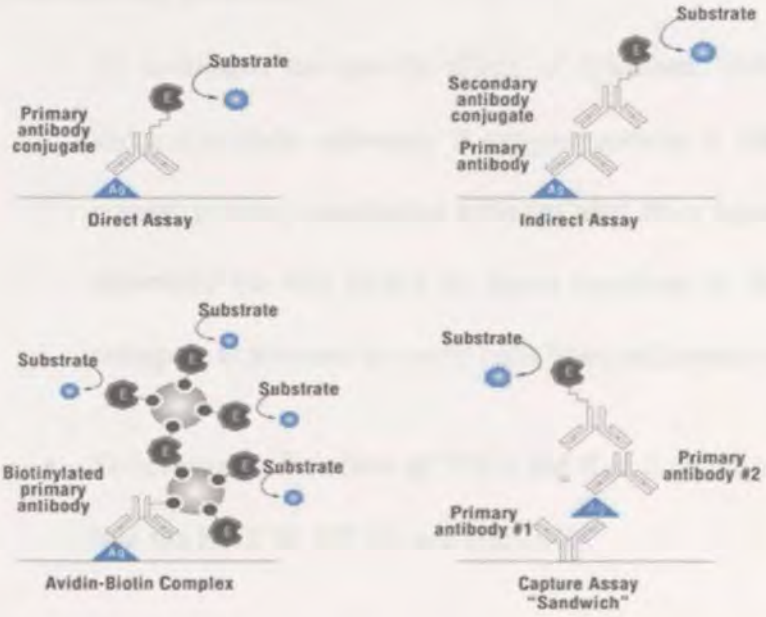
In this, more specific approach, a capturing Ab is absorbed onto the solid phase. The capture antibody may be the reagent to be tested (e.g. the titre of a patient's immune response to a known Ag). However, the Ab may be a standard reagent and the antigen the unknown (as when a patient's serum is being investigated for the presence of a microbial infection).

The same stringent optimisation is required as for Indirect ELISA. This will ensure that the Ab do not cross-react in the absence of Ag, or non-specifically bind to the solid phase. It is also important, when detecting the Ag, to use Ab from different animal species to prevent same-species Ab binding (e.g. a polyclonal rabbit capture Ab will capture a

monoclonal conjugate if it was raised in rabbits. This will produce a positive result in the absence of Ag).

Antibody Capture

The most commonly used ELISA assay format is the sandwich assay. This type of assay is called a "sandwich" assay because the analyte to be measured is bound between two antibodies– the capture antibody and the detection antibody. The sandwich format is used because it is sensitive and robust. In this approach, a capturing Ab is absorbed onto the solid phase. The Ab is designed to capture a class of human Ab e.g. IgG, IgA or IgM. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (detection antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate.



http://www.piercenet.com/Proteomics/browse.cfm?fldID=F88ADEC9-1B43-4585-922E-836FE09D8403

Figure 1.2: *Types of ELISA's - Direct assay, Indirect assay, Antigen capture assay (Avidin-biotin complex), and Capture assay (sandwich).*

1.13 Objective and aims of this PhD thesis.

Since cancers are diseases of disordered cell growth and differentiation, it is not surprising that cytokines are implicated in carcinogenesis, as they play a vital role in control of cell differentiation and proliferation.

Hence, attempts have been made to use cytokines to correct or counteract the abnormal behaviour of cancer cells.

The primary objective of this study was:

To determine the relationship between cytokines and proteases in malignant and normal endothelial cells, and to elucidate the possible involvement of both in tumour angiogenesis.

Therefore the aims were:

- To investigate the specific effects of cytokines, TNFα and IL-1β, on intracellular and extracellular cathepsin B enzyme activity in HRT 18, HT 29 and HUVECs, normal primary endothelial cells isolated from human umbilical vein. This will determine the role played by above cytokines in the regulation of expression of cathepsin B protease in cancer cells lines and normal endothelial cells.

- To investigate the effect of TNFα and IL-1 β on the protein expression of cathepsin B in the HRT 18, HT 29, and HUVECs.

- To investigate the influence of both the cytokines, TNFα and IL-1β, on the intracellular and extracellular activity of DPP IV enzyme, an exopeptidase protease.

- To investigate the effect of TNFα and IL-1 β on the protein expression of DPP IV in HRT 18, HT 29, and HUVECs.

- To elucidate the cytotoxic nature of these cytokines on the three cell lines studied.

- To determine the potency of Mu-Phe-Hph-FMK to inhibit cathepsin B, and of Gly-Pro-Gly-Gly to inhibit DPP IV activity.

- The Long term aim of the project was to investigate the possible use of these cytokines for therapeutic intervention in cancer patients. It was anticipated that the study undertaken would contribute towards providing a novel and more effective therapy in long suffering cancer patients.

2.0 Introduction

In this chapter, the methods used to carry out various experiments in this project are described. Techniques used for the establishment of the cancer cell lines HRT 18 and HT 29, liver cells, thyroid cells, and isolation of endothelial cells from human umbilical cords are described in sections 2.2.1 and 2.2.2. This is then followed by the routine cell culture procedures, including methods used for counting and freezing the cells for subsequent use. Section 2.2.6 provides a description for the immunocytochemical protocol used for the staining of normal endothelial cells, which was carried out to verify the characteristics of the cells. Verification was conducted by staining the endothelial cells by two endothelial markers (CD 31 and vWF).

2.1 Materials

All materials used in this study are listed in Appendix 1.

In order to begin any experimental studies it was necessary to establish the five cell types namely, HRT 18, HT 29, normal human umbilical vein endothelial cells, human liver and thyroid cells in culture. Human liver and thyroid cells were grown to be used as positives for cathepsin B and DPP IV enzyme respectively. Cell lines HRT 18, HT 29, liver cells and thyroid cells were purchased from TCS cell works (TCS Cell Works, Milton Keynes,U.K.) as a frozen aliquot of 10^6 cells/ml. The cells were thawed as described in section 2.2.4. Finally, following thawing of the cells, they were established in culture using the method described in section 2.2.1. Human umbilical cords were obtained from St. George's Hospital upon ethical approval and patients consent.

Following criteria was applied for the selection of umbilical cords for this study:-

a) Only cords that were not injured were used in the experiments. Any cord with visible sign of damage was discarded.

b) The cords that were used were from women aged between 25 to 35, who had normal deliveries.

2.2 Cell culture

All routine handling of cultures, (Freshney 1994) including the setting up of the experiments and media preparation was carried out under sterile conditions in a class [11] Gelman (BH48) laminar air flow cabinet. Plastic tissue culture flasks and media components usually arrived pre-sterilised from the manufacturer, otherwise sterilisation of solutions was carried out using a 0.20μm pore filter (Sartorius). Forceps, scissors, eppendorfs and microtips were autoclaved for 20-30 minutes at 121°C and 15 psi to ensure sterility.

2.2.1 Cell lines

Tumour cell lines HRT 18 and HT29, liver cells, thyroid cells, and normal human umbilical endothelial cells were grown in culture in RPMI 1640, MEM, EMEM, RPMI 1640, and Iscove's growth media respectively (refer to appendix 2 for media preparations). Once confluent, the cells were gently washed with sterile PBS buffer after which, 5 mls of Trypsin-EDTA was added to the flask and incubated at 37°C and 5% CO_2 for 3 minutes. Trypsin is a cell dissociation medium that detaches the cells adhered at the bottom of the flask by digesting the adhesion proteins. Following trypsinisation, the trypsin/cell

suspension was suspended in 5 mls of growth media (depending on the cells e.g. for HRT 18 cells RPMI 1640 growth media was used) and centrifuged at 1000 RPM for 5 minutes. The supernatant was discarded and 5 mls of fresh media was added to the cell pellet. The cell suspension was then transferred to a 25cm^2 tissue culture flask (if a T75cm^2 flask was used the cell pellet was suspended in 10 mls of growth media) and incubated at 37°C in an atmosphere of 5% CO_2/95% air and high humidity. The cells were washed with warm PBS and fed with growth media every two days. The cells needed to be subcultered i.e. split, in general cell culture term passaged, before they became fully confluent (while there was still some growing space available), this keeps the cells in the active log phase of growth since cells, if left too long at confluence, may not grow well after subculturing. The cells were split at a ratio of 1:3 (1 flask into 3 flasks) each containing 2 x 10^5 cells in 5 mls of growth media cultured in a T25cm^2 flask (Freshney 1994).

For the following experiments to be consistent it was made sure that the number of cells per flask or chamber slide was uniform (10^5 cells/ml). For this purpose a cell count was carried out before cells were transferred to a culture flask or a chamber slide for experimentation. The procedure that was used for counting the cells is described in section 2.2.3. The cell counting for each cell line was carried out three times for two aliquots and the average was recorded.

2.2.2 Isolation and Culture of Human Umbilical Vein Endothelial Cells (HUVEC).

Human umbilical cord veins were utilised as a source of endothelial cells (HUVEC) in these experiments. The cells were harvested from umbilical cords by the method described by Jaffe *et al* (1973). Fresh human umbilical cords (n=50) were collected from the

maternity department at St. George's Hospital, following ethical approval and patients consent. All umbilical cords used were considered normal by the obstetric staff at St George's Hospital.

A sterile technique was employed in all manipulations of the cord. The cord was severed from the placenta soon after birth (by the nurse at the maternity department, St. George's Hospital), placed in a sterile container containing Hanks buffer, and kept at 4°C until processing. The storage time for the cords did not exceed 48 hours.

The isolation of endothelial cells from the cord was carried out under sterile conditions, in a laminar air flow cabinet. The cord was wiped with 75% ethanol and checked for clamp marks and needle punctures. If a cord was unsuitable for experimental purposes, i.e. had injuries or puncture marks, the cord was then discarded (this excluded the 50 cords used). The ends of suitable cords were cut and a 14 gauge i.v. cannula was gently pushed into both sides of the cord. A string was tied at either end of the cord to secure the cannula in place. Hanks buffer was gently flushed through the vein, using a syringe, until the effluent was clear and the remaining fluid was gently squeezed out. The cord was then filled with collagenase solution, (for detaching endothelial cells) by a syringe, and red bungs were then put at each end of the cord to make sure that the solution did not leak out. The cord containing collagenase solution was placed in a petri dish containing 20 mls of Hanks. The petri dish was left in the incubator for 20 minutes. Cells were flushed out of the cord, into a sterile universal, using Hanks/FCS solution, and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 5 ml of Iscoves growth medium. The cell suspension was transferred into a T25cm^2 flask and incubated. The cells were allowed to grow for 2 days, after which the old media was aspirated and fresh

warm media added. After 5-10 days the cells were passaged in T25cm^2 culture flasks for use in the studies (the cells were fed every 2 days with the media described in appendix 2).

HUVECs were maintained in Iscove's media was supplemented with 20% FCS, 2% heparin, 3mg/ml ECGS, 1% streptomycin and penicillin and 1% fungizone at 37°C in an atmosphere of 5% CO_2/95% air and high humidity. Confluent cells were trypsinized and subcultured at a ratio of 1:3 (1 flask of T25cm^2 was split into 3 T25cm^2 flasks). Primary cells such as endothelial cells isolated from human umbilical cord may be capable of one or two divisions in culture, and given the right conditions can survive for some time, but they do not continue to grow and eventually senesce and die. For this reason the cultured endothelial cells were used in their 3rd passage.

To ensure that the cells obtained by the above procedure were definitely endothelial in origin, they were examined morphologically (figure 3.6 a and b) and by immunocytochemistry (figure 3.7 – figure 3.12).

2.2.3 Cell Count

2.2.3.1 Using a Coulter Counter

Confluent cells were passaged as described earlier (section 2.2.1). After centrifugation, the supernatant was discarded and 4 ml of corresponding media was added to the cell pellet and mixed thoroughly. 0.5 ml of cells was added to 19.5 ml of isotonic II solution aliquoted in a small plastic bottle. The bottle containing the cells and Isotonic II was then placed in an automatic coulter counting machine and the number of cells was recorded in triplicate. 2 sets of samples were taken from each cell line, and each sample was measured thrice. The average of the samples was recorded.

Figure 2.0: A Coulter Counter is a device, which counts cells as they float in a liquid by passing them through a small slit. The device does this by measuring the change in electrical conductivity at the slit when a cell passes through it.

http://www-personal.umich.edu/~pattylen/lab/counter.doc

2.2.3.2 Using Haemocytometer

Initial cell counting was achieved by the use of an automatic counter (figure 2.0) at the Royal London Hospital at Whitechapel. However, counting of the cells was later achieved by the use of a haemocytometer at Kingston University. This was due to not having access to the equipment at the Royal London Hospital for completion of the experiments.

However, the number of cells counted, prior to each experiment, by the two different methods were consistent and comparable.

Cells were counted using a Neubauer standard haemocytometer chamber (Scientific, Loughborough, U.K.) This is a specialised microscope glass slide on which grids have been engraved. There are 4 large 1mm squares on each corner and one large 1mm square in the middle as shown above. When a special coverslip is correctly placed over the central region of the haemocytometer the height between each square and the coverslip is 0.1 mm, giving a volume of $0.1mm^3 = 10^{-4}$ ml in each square (figure 2.1).

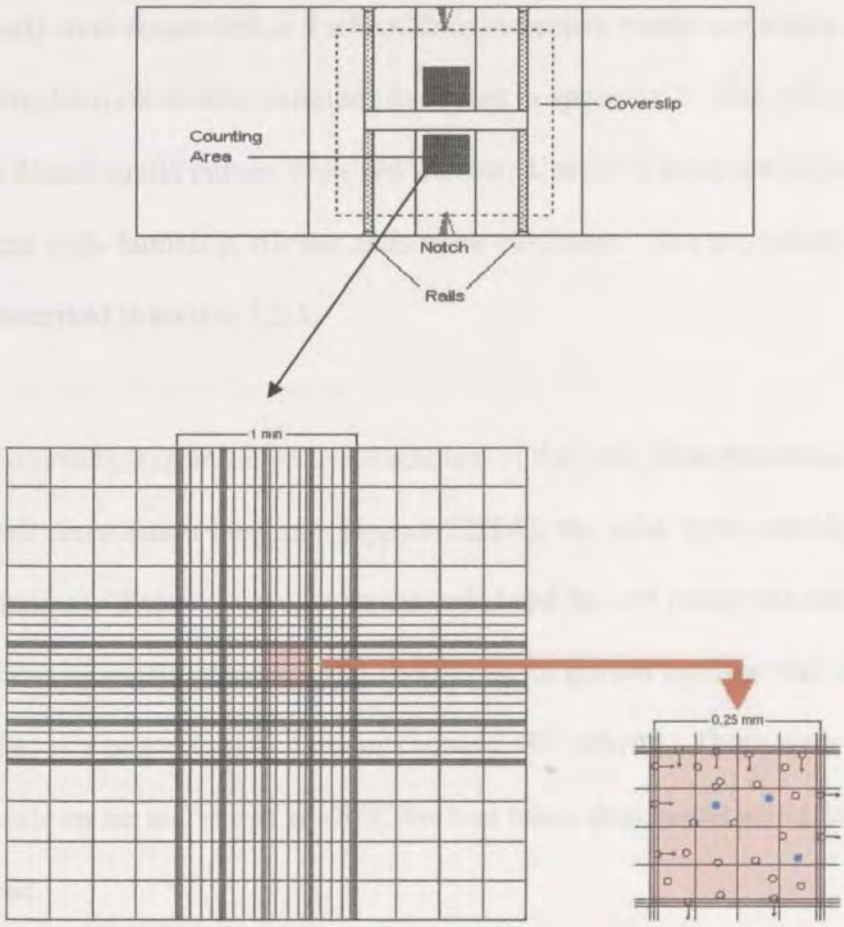
Following centrifugation, at 1000 RPM for 5 minutes, the cell pellet was suspended in 2 mls of their corresponding growth media (see appendix 3). 40μl of the cell suspension was added to an eppendorf containing 40μl of trypan blue (0.25% in PBS). Trypan blue is a

stain that distinguishes viable cells from nonviable cells, only non-viable cells absorb the dye and appear blue. 10µl of the cell/trypan blue mixture was then added to the haemocytometer, and viable cells were counted using a light microscope at 100 magnification.

The total number of cells per ml was calculated as follows: -

Cell number = (cell count/number of squares) x 10^4 x dilution factor

Viability = 100 x (number of viable cells / total number of cells)



Modified from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

Figure 2.1: *Haemocytometer*

2.2.4 Thawing of the cells, Cell Freezing and Reconstitution Procedure

HRT 18, HT 29, liver cells, and thyroid cell lines were purchased as a frozen aliquot of 10^6 cells/ml. Upon arrival of the cells, the cells were immediately thawed by placing the vial containing the frozen cell pellet in a 37 °C water-bath for 1 minute or until the cells had been thawed. Once thawed, the cell suspension was added to 5 mls of their respective media (appendix 3), which had been pre-warmed at 37 °C using a water-bath. The media containing the cells was then centrifuged at 1000 RPM for 5 minutes to remove any traces of DMSO from the cells. The supernatant was discarded and the pellet of both the cancer cells were suspended in 5 mls of their respective media containing FCS and penicillin and streptomycin at concentrations described in appendix 3. The cells were then transferred to a $25cm^2$ sterile culture flask and incubated, at 37°C in an atmosphere of 5% CO_2/95% air and high humidity, till the cells were confluent. The procedure for post confluency is described in section 2.2.1.

Following trypsinisation or detachment of the cells, from the tissue culture flask, using the cell dissociation medium (Trypsin-EDTA), the cells were centrifuged at 800 RPM for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in pre-cooled freezing media containing 50 % FBS, 30 % growth medium and 20 % DMSO, placed on ice, at a concentration of approximately 10^6 cells/ml. These were then placed in freezing vials on ice and stored at -70°C for four hours then immersed in liquid nitrogen till further use.

2.2.5 Determination of Growth Curve for all the three Cell Types

It was important to construct a growth curve (shape of a graph of viable cell number versus time), so that the growth characteristics of the cells, being cultured, could be assessed.

Therefore, in order to determine the growth characteristics of HRT 18, HT 29, liver cells, thyroid cells, and HUVECs, cells were grown in their corresponding media, following the protocol described in section 2.2.1.

The culture doubling time, the time when the number of cells at log phase doubles, once calculated for a cell line, allows prediction of the likely cell concentration at any time in future. Cells should be subcultured before they reach a concentration at which their growth slows, i.e. the end of their log phase, which can be seen clearly in the graphs above for each of the cells investigated.

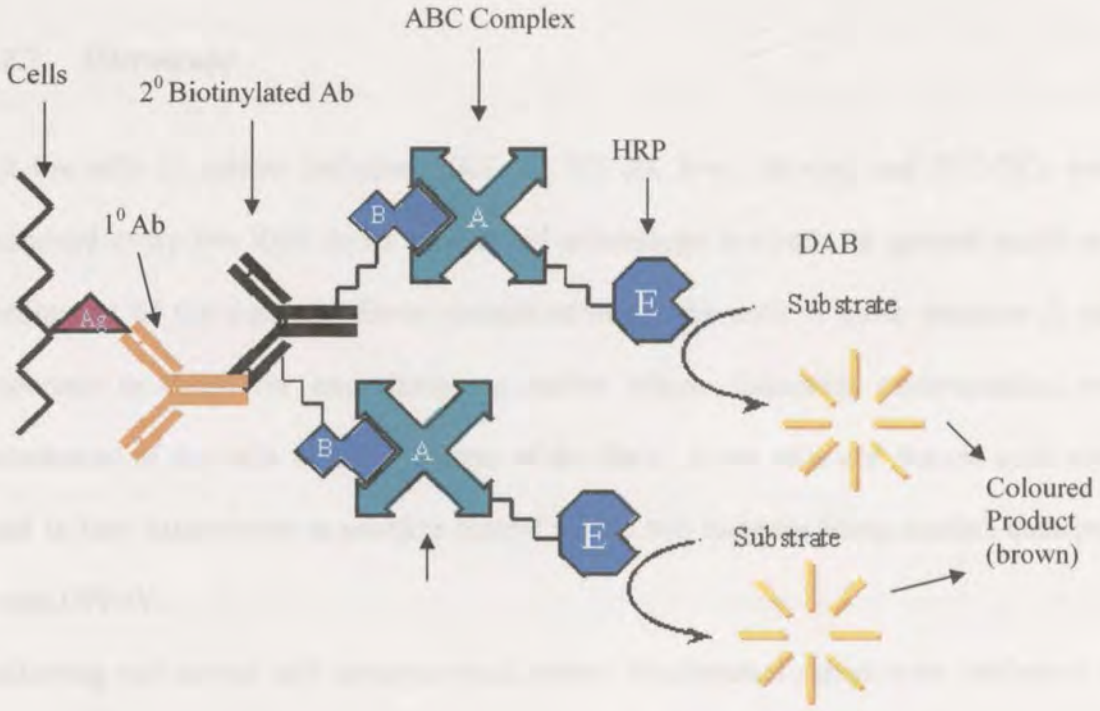
Cells were seeded at a concentration of 2×10^5 cells per $25cm^2$ sterile tissue culture flasks. The flasks were then incubated at 37°C in an atmosphere of 5% CO_2/95% air and high humidity. Cell counts (in triplicate for two aliquots) were performed for individual flasks after trypsinization at 24-hour intervals for 6 days. Growth curve was then constructed for each cell type.

Viability studies were done by Trypan blue dye exclusion method and the cells/$25cm^2$ flask was counted. All cell counts were repeated three times (for two aliquots) and the average of the reading was noted for accuracy.

2.2.6 Immunocytochemistry on HUVECs (CD31 and vWF)

Immunocytochemistry was carried out in order to stain endothelial cells isolated from human umbilical cord. This was necessary since isolation of endothelial cells from human vein, following digestion by collagenase, often leads to fibroblast contamination (Jaffe *et al.*, 1973). vWF and CD 31 are established endothelial cell markers (Appleton, Attanoos, Jasani 1996) and were used to stain cells that were harvested from umbilical cords.

Endothelial cells that had been isolated from human umbilical vein (at their 2nd passage) were seeded onto sterile chamber slides at a concentration of 10^5 cells/ml and placed in a humidified incubator at 37^0C. Once confluent the slides were rinsed with PBS twice for 2 minutes and then immersed in pre-cooled acetone:methanol for 10-15 minutes. The cells were washed once again with PBS. The slides were then laid flat in a humidity chamber and 500-800 μl of primary CD31 and/or vWF antibody (see appendix 4) was pipetted over the cells. The slides were left in the incubator at 37^0C for 1 hour. Following this, the cells were washed with PBS and the procedure repeated with secondary antibody. Secondary antibody that was used for CD31 analysis was biotinylated goat anti-mouse IgG and the secondary antibody that was used for vWF analysis was biotinylated goat anti-rabbit IgG. Cells with secondary antibody were incubated for 30 minutes at 37^0C. The slides were then washed once again and few drops of ABC reagent was added following which the slides were incubated for further 30 minutes. After the incubation period a few drops of DAB was added to the cells and left for 1-2 minutes after which the cells were washed and left to air dry for 5-10 minutes. The slides were then mounted with a coverslip using DPX mountant and viewed under a light microscope. The antibodies used and the dilution factors are listed in appendix 4. The immunocytochemistry of HUVECs was carried out three times for each marker (CD31 and vWF). This procedure was repeated each time endothelial cells were harvested from the cord and used for further experimentation.



Modified from http://www.piercenet.com/presentations/nonrad/sld017.htm

Figure 2.2: *An immunocytochemical ABC reaction.*

Figure 2.2 shows how a primary antibody reacts with the immobilised antigen to form an antigen-antibody complex. A second, biotinylated antibody, specific for primary antibody reacts with the complex. Streptavidin conjugated to Horseradish Peroxidase (ABC reagent used in this experiment) reacts with this complex immobilising the peroxidase at the site of the antigen. Finally, substrate, DAB, is added causing a coloured precipitate to form on the slide at the location of the antigen. Therefore, visualisation is based on enzymatic conversion of a chromogenic substrate DAB into a coloured brown precipitate by horseradish peroxidase (HRP) at the sites of antigen localisation. This can be viewed using a light microscope.

2.2.7 Microscopy

All the cells in culture including HRT 18, HT 29, liver, thyroid, and HUVECs were examined every two days under an inverted microscope to check for general health and confluency of the culture. Since contamination of the cells is quite common it was important to check for any change in media colour, indicating contamination, and detachment of the cells from the bottom of the flask. Liver cells and thyroid cells were used in later experiments as positive control for the two enzymes being studied, cathepsin B and DPP IV.

Following cell culture and immunocytochemistry, biochemical assays were performed to examine the intracellular and extracellular activities of cathepsin B and DPP IV protease under the influence of cytokines such as TNFα and IL-1β, in the three cell types namely HRT 18, HT 29 and normal endothelial cells isolated.

2.3 Activity Assay

The purpose of this experiment was to evaluate the influence of cytokines on protease activity in cancer cells and normal cells. The two cytokines studied were TNFα and IL-1β, and the two proteases investigated were cathepsin B endopeptidase and DPP IV exopeptidase. Before investigating the influence of these cytokines on proteases it was necessary to carry out preliminary experiments on the three cell types, HRT 18, HT 29, and HUVECs, to assess the optimal time and best suited medium required to achieve optimal enzyme activity.

Dose response experiments, described under section 2.3.3, were carried out in order to investigate the influence of various doses (5, 10, 50, 100, 250 ng/ml) of TNFα and IL-1 β on the three cell types, HRT 18, HT 29 and HUVECs. The intracellular and extracellular

enzyme activity for both the enzymes was measured with the help of a cytoflour machine, which is a computerised fluorescent plate reader. The dose response assay was carried out in triplicate, and each time the average of 12 samples was taken.

In brief, once confluency was achieved for the HRT 18, HT 29 and HUVECs, the cells were trypsinised, counted and seeded onto 96 well plates, at a density of 10^5 cells/ml. Cells were grown in their corresponding growth medium, which is listed in appendix 2.

Choice of Substrate

Protease activity in the cell lines studied was investigated using synthetic peptide substrates containing amino acid sequence that have proved suitable for detection of various enzymes.

The two highly specific substrates used for cathepsin B and DPP IV enzymes were Z-Arg-Arg-AMC and Gly-Pro-AMC respectively. The activity assay carried out was based on the AMC leaving group that fluoresces upon enzyme specific peptidase activity. Chapter 1, introduction (section 1.10.1.1 and 1.10.1.2), gives a more detailed account of the various substrates which are generally used in protease activity assays.

Principle of the Assay

The fluorometric assay used has been designed for the quantitative *in-vitro* determination of protease activity in microplates. The test utilises the ability of cathepsin B and DPP IV to digest the synthetic substrates Z-Arg-Arg AMC and Gly-Pro-AMC. Released, free AMC is determined fluorometrically at excitation wavelength 360-380 nm and emission wavelength 440-460 nm. The activity of the two enzymes under investigation can be quantified with an AMC standard, or can be displayed as fluorescence units (FU).

Cathepsin B activity was measured as described by Barrett & Kirschke (1981) and DPP IV activity according to Hopsu-Havu & Glenner (1966). Highly specific cathepsin B inhibitor, CA-074 (irreversible inhibitor) and a DPP IV inhibitor, Diprotin A, were used as negative standards in the activity assay that was carried out. All the experiments were carried out in triplicate and the average of the 12 samples was then taken.

Calculations

The data obtained can be displayed in three ways.

1. As fluorescence units: The fluorescence value of all samples was obtained by subtracting the value of the blank, and then by calculating the mean fluorescence value for each sample in triplicate.

2. As % activity: % increase/decrease in enzyme activity can be measured by calculating mean fluorescence value for each sample then dividing the fluorescence value of the variable (sample) by fluorescence value of the control and then multiplying the value by 100.

3. As μmole of free AMC/mg total protein/time (min.): This can be achieved by calculating the mean fluorescence value as stated above. Thereafter, a graph can be plotted correlating the mean fluorescence values of the AMC standards (y-axis) to their concentration in μmole (x-axis). The cathepsin B activity of the unknown samples can then be interpolated from the standard curve. The amount (μmole) of free AMC per mg of total protein and time unit can then be calculated.

   In this study, results were expressed in fluorescence units, and as % activity.

2.3.1 Time course response

Once confluent, HRT 18, HT 29 and HUVECs were grown in 96 well plates and incubated for 48 hours with six different media. Intracellular and extracellular activity of the two enzymes was measured, as described under section 2.4, by a cytoflour machine every 6 hours. The incubation period that gave the optimal enzyme activity was used for subsequent experiments. The experiment was carried out in triplicate and an average of 12 samples was taken.

2.3.2 Choice of Media

HRT 18, HT 29 and HUVECs were seeded on to 96 well plates in 6 different media, PBS, Isotonic, HBSS (all supplemented with 0.3% BSA), MEM, DMEM and RPMI 1640 (without glutamine), for 48 hours. Intracellular and extracellular activity of the enzymes was measured as described in section 2.4.2 and 2.4.1 respectively, by a cytoflour machine, every 6 hours. The media that gave the optimal enzyme activity was used for subsequent experiments. The experiment was carried out in triplicates and an average of 12 samples was taken each time.

2.3.3 Dose response

Both cancer cell lines and HUVECs were grown on 96 well plates at a density of 10^5 cells/ml. Once the cells reached confluency they were treated with TNFα and IL-1β at concentrations 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml and 250ng/ml. The treated cells were incubated for the time period that had been shown to give the optimal enzyme activity.
The treatment of the cells with cytokines was carried out in the media that was shown to give an optimal enzyme activity. Following incubation, the intracellular and extracellular activity of both the enzymes was measured. The procedure was carried out in triplicate,

and each time an average of the 12 samples (96 well plate) was recorded and finally overall average was calculated.

2.4 Analysing 96 well plates

2.4.1 Extracellular activity

Once the cells had been incubated for the required amount of time, 50 μl of cell supernatant was removed from each well and transferred to a sterile 96-well plate. Care was taken in transferring the supernatant as it was possible to dislodge the cells while pipetting the supernatant. 10 μl of fluorogenic substrate Z-Arg-Arg-AMC (1 mM), 135 μl buffer (0.1M MES pH 5.5 with 0.1% v/v Triton X-100), and 5 μl DTT (100 mM) was added to the 96 well plate containing 50 μl of supernatant in order to measure cathepsin B activity. However, to measure DPP IV activity 10 μl of Gly-Pro-AMC substrate (1 mM) and 140 μl buffer (0.1M Tris-HCL pH 8.0 with 0.1% v/v Triton X-100) was added to the 50 μl supernatant. The plate was then left to stand at room temperature for 2 hours, after which it was read using a cytoflour machine. An outlay of the procedure used can be seen in figure 2.3.

2.4.2 Intracellular activity

To measure intracellular activity the remaining solution left in the 96-well plate was removed. In order to measure intracellular cathepsin B activity, 10 μl of Z-Arg-Arg-AMC substrate (1 mM), 185 μl buffer (0.1M MES pH 5.5 with 0.1% v/v Triton X-100), and 5 μl DTT (100 mM) was added to the wells which contained cells only. To measure intracellular DPP IV activity 10 μl of Gly-Pro-AMC substrate (1 mM) and 190 μl buffer (0.1M Tris-HCL pH 8.0 with 0.1% v/v Triton X-100) was added to the wells containing

cells. The plates were left for 2 hours following which the enzyme activity was measured. Both the intracellular and extracellular procedures are outlined in appendix 3. For both intracellular and extracellular activity of the enzymes, the specificity of the reaction was verified by incubation in the presence of inhibitor specific for cathepsin B and DPP IV. 10 μM CA-074, prepared in DMSO, was used as a selective cathepsin B inhibitor, and Diprotin A (10 μM) inhibitor was used for DPPIV enzyme (concentration of DMSO was limited to 0.1%).

Adherent cells (HRT 18, HT 29, and HUVEC's)

Cells were plated onto 96 well plates

Treatments (cytokines) were added to the confluent cells

Reagents were added to the two plates to measure protease activity

Extracellular

Intracellular

Incubate at room temperature for 2 hours

Fluorescence was recorded at excitation 355 and emission 460nm.

Cytoflour Machine (to measure the fluorescence)

http://www.promega.com/applications/cellprolif/images/celltiterblue/3888MA11_2A.gif

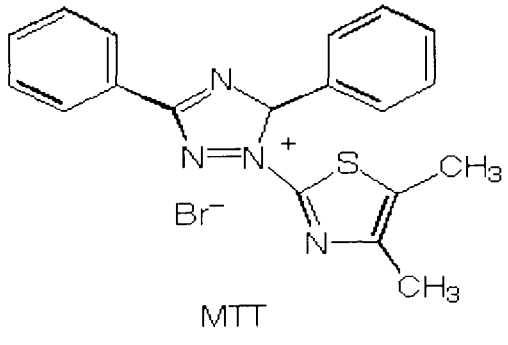
Figure 2.3: *An overview of the procedure used to determine protease activity.*

Once the activity assays were performed to determine the expression of intracellular and extracellular enzyme activity in cytokine treated cells, further experiments were carried out. Viability assay, MTT, was performed to determine the effect of the cytokines on the cells following treatment with TNF α and IL-1β. The MTT assay was carried out in triplicates and each time an average of 3 samples was taken.

2.5 Viability Assay

Cell viability assays are used as a quantitative parameter to assess the cytotoxicity of exogenous substrates or drugs. There are several methods that can be used to quantitate viability of cells. The present study used MTT assay as means to assess the viability of cells following exposure to cytokines.

2.5.1 MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay



http://www.biotium.com/structures/10059small.gif

Figure 2.4: *Structure of MTT*

This study was carried out to assess the viability of cells that had been subjected to treatment with various doses of cytokines, TNF α and IL-1β. MTT assay is a quantitative colorimetric method to determine cell proliferation. It evaluated the effect of cytokines, TNF α and IL-1 β on the growth of the three cell lines studied, HRT 18, HT 29 and HUVECs.

The MTT test, standard test for cytotoxicity, (Hansen, Nielsen, Berg 1989) was undertaken according to the method described by Mosmann (1983). This test gives a precise indication of mitochondrial function, and hence viability of the cell.

2.5.2 Determination of standards for viability test for HRT 18, HT 29, and HUVECs

The two carcinoma cell lines and normal endothelial cells were trypsinised, as discussed in section 2.2.1. The number of cells were counted for all the three cell types and seeded onto 96 well plates at varying concentrations as shown in the table below (table 2.0).

	1	2	3 Number of cells plated	4	5 Duplicate	6	7 Triplicate	8	9	10	11	12
A	*Blank*		2.3×10^5 200µls cell suspension	100 µls	2.3×10^5 200µls cell suspension	100 µls	2.3×10^5 200µls cell suspension	100 µls				
B			1.2×10^5 100µls media	100 µls	1.2×10^5 100µls media	100 µls	1.2×10^5 100µls media	100 µls				
C	*Blank*		5.8×10^4 100µls media	100 µls	5.8×10^4 100µls media	100 µls	5.8×10^4 100µls media	100 µls				
D			2.9×10^4 100µls media	100 µls	2.9×10^4 100µls media	100 µls	2.9×10^4 100µls media	100 µls				
E	*Blank*		1.5×10^4 100µls media	100 µls	1.5×10^4 100µls media	100 µls	1.5×10^4 100µls media	100 µls				
F			7.3×10^3 100µls media	100 µls	7.3×10^3 100µls media	100 µls	7.3×10^3 100µls media	100 µls				
G			2.3×10^3 100µls media	100 µls	2.3×10^3 100µls media	100 µls	2.3×10^3 100µls media	100 µls				
H			2.3×10^3 100µls media	100 µls	2.3×10^3 100µls media	100 µls	2.3×10^3 100µls media	100 µls				
			100µls		100µ ls		100µls					

Table 2.0: *The table above, representing a 96 well plate, gives an overview of the dilutions for HRT 18 cells, as an example. Each well consisted of 100 µls of solution.*

All the experimental work was carried out under sterile conditions, in a class [11] Gelman (BH48) laminar air flow cabinet. MTT stock solution was prepared as 5mg/ml MTT in PBS. The solution was then filtered through a 0.2 μm filter and stored at 2-8°C or else at -20°C for extended storage.

MTT solution was added to the wells being assayed and incubated at 37°C for 2 hrs.

After the MTT containing medium was removed, the cells were washed with phosphate buffered saline (PBS) and the formazan product dissolved with 0.1 N HCL Isopropyl alcohol, following which the absorbance was measured. The absorbance for the standards (dilutions) for each of the cell type, HRT 18, HT 29 and HUVECs, was carried out in triplicate to get a more accurate value.

For each cell type the linear relationship between cell number and signal produced was established, to allow an accurate quantification of changes in the rate of cell proliferation.

2.5.3 Determination of cell viability of treated HRT 18, HT 29, and HUVECs

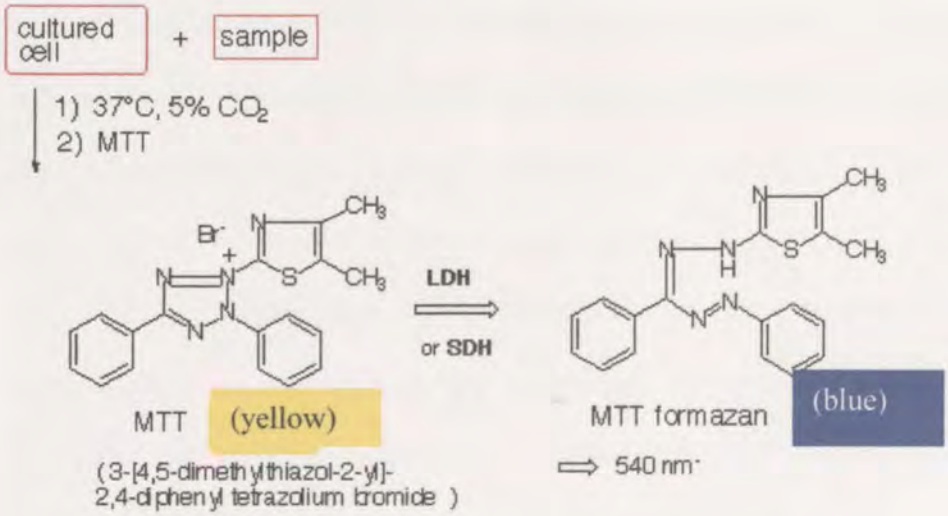
MTT assays were carried out to examine the viability of the three cell lines, HRT 18, HT 29 and endothelial cells, under the effect of different concentrations (5ng/ml, 10ng/ml, 50ng/ml 100ng/ml, and 250ngml) of cytokines, TNFα and IL-1 β.

The cells were plated onto 96 well plates (100μl) at a density of 10^5 cells/ml, and incubated for 2 hrs at 37 °C. Following this, the cells were incubated with or without cytokines at varying concentrations. 10μl of MTT (3-4,5-dimethylthiazol 2,5-diphenyl tetrazolium bromide) (5mg/ml Sigma, UK) was then added to each well and incubated at 37°C for 2 hours. The reagent diffuses into the cells and is cleaved to formazan. The resulting deep blue crystals were dissolved in 0.1 N HCL Isopropyl alcohol, and the absorbance measured

using a scanning multiwell spectrophotometer (Elisa reader 2000) at wavelength 570nm (figure 2.5). This procedure was carried out in triplicate and the average was recorded.

Figure 2.5: *MTT stain was quantified using a plate reader (spectrophotometer). http://www.anticancer.com/HDRA_reader.jpg*

Comparison of absorbance of a treated cell value to an untreated control provides a relative increase in cellular proliferative activity. The MTT reaction can be seen in the diagram below. The blue dye generated, as shown in the figure 2.6 can be quantified by a scanning multiwell spectrophotometer (ELISA reader) by measuring the absorbance of the dye solution at appropriate wavelengths.



Modified from http://www.dojindo.com/newimages/MTT-RS.jpg

Figure 2.6: *Formation of insoluble blue formazan reaction product following, cleavage of the yellow MTT tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] by living cells.*

All the measurements for determination of cytokine (TNF α and IL-1β) cytotoxicity in HRT 18 cells, HT 29 cells and HUVECs, using the MTT assay, were performed in triplicate, to ensure accuracy of the results.

2.5.3.1 MTT Calculation

Once the mean and standard error for each sample was calculated, the percentage survival was calculated as shown below :-

$$\% \text{ survival} = \frac{\text{Absorbance of the cells exposed to cytokines X 100}}{\text{Absorbance of the control cells}}$$

The activity assay described in section 2.4 investigated the effect of two cytokines, TNFα and IL-1β, on intracellular and extracellular activity of cathepsin B and DPP IV enzymes in two carcinoma cell lines, HRT 18 and HT 29, and also in normal endothelial cells derived from human vein umbilical cord. Further experiments with ELISA determined the effect of the cytokines named above on the intracellular and extracellular protein levels of the enzymes cathepsin B and DPP IV.

2.6 RNA, DNA and Protein isolation of HRT 18, HT 29 and Endothelial cells.

2.6.1 Sample preparation

Tri-reagent purchased from Sigma (Poole, Dorset,U.K.) was used for the simultaneous isolation of RNA, DNA and protein from HRT 18, HT 29 and endothelial cells, following treatment with the cytokines TNFα and IL-1 β, and from untreated liver and thyroid cells. RNA, DNA and protein was also isolated from untreated HRT 18, HT 29 and endothelial cells as controls. The cells when confluent were lysed directly on a 25 cm^2 flask by using 1ml of Tri-reagent/10 cm^2 (2.5 ml). The cell lysate was passed several times through a pipette to form a homogenous lysate, which was then placed into a sterile DEPC treated eppendorf. The samples were allowed to stand at room temperature for 5 minutes, to allow complete dissociation of nucleoprotein complexes, after which 0.2 ml of chloroform/ml of Tri-reagent was added to the eppendorfs. The samples were covered tightly and shaken vigorously for 15 seconds and allowed to stand at room temperature for 2-15 minutes. The resulting mixture was then centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation the mixture separated into three phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA).

2.6.1.1 RNA Isolation

The aqueous phase was transferred to a fresh eppendorf and 0.5 ml of isopropanol/ml of Tri-reagent used in the sample preparation was added and mixed. The samples were allowed to stand at room temperature for 5-10 minutes and thereafter centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and bottom of the eppendorf. The supernatant containing the interphase and organic phase was left on ice for subsequent isolation of the DNA and proteins.

The supernatant was discarded and the RNA pellet was washed by adding 1 ml of 75% ethanol/ml of Tri-reagent used in sample preparation. Samples were then vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C. The RNA pellet was allowed to air dry for 5-10 minutes whilst making sure that the pellet was not dried completely. 100 µls of TE buffer was added to the RNA pellet. The samples were mixed thoroughly by repeated pipetting with a micropipette in order to facilitate dissolution. The RNA was then quantified by measuring optical density at 260 nm in a spectrophotometer, a reading of 1.0 OD equal to 40 µg/ml of RNA. RT-PCR was then carried out. However, no bands for the samples were detected.

2.6.1.2 DNA Isolation

The remaining aqueous phase overlying the interphase was removed and discarded. To precipitate the DNA from the interphase and organic phase 0.3 ml of 100% ethanol/ml of Tri-reagent used in sample preparation was added, mixed and allowed to stand at room temperature for 2-3 minutes. The samples were then centrifuged at 2,000 x g for 5 minutes at 4°C. The supernatant was removed and used for protein isolation. DNA pellet was discarded since it was not required for the experiments in this project.

2.6.1.3 Protein Isolation

Protein was precipitated from the phenol-ethanol supernatant by adding 1.5 ml of isopropanol/ml of Tri-reagent used in sample preparation. The samples were allowed to stand at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet washed 3 times in wash buffer (0.3 M guanidine hydrochloride/95% ethanol solution), using 2 ml of wash buffer/ml of Tri-reagent used in sample preparation. During each wash, samples were stored in wash buffer for 20 minutes at room temperature and then centrifuged at 7,500 x g for 5 minutes at 4°C. Following the 3 washes, 2 ml of 100% ethanol was added to the protein pellet, vortexed and allowed to stand at room temperature for 20 minutes. The samples were then centrifuged at 7,500 x g for 5 minutes at 4°C. The protein pellet was allowed to dry for 5-10 minutes, after which it was dissolved in 1% SDS (400 µls). Any insoluble material was removed by centrifugation at 10,000 x g for 10 minutes at 4°C. The resulting supernatant was then transferred to a fresh eppendorf and stored at -20°C for experimentation.

Before any experiments could be carried out total protein concentration in the isolates was determined, by Lowry protein determination.

2.6.2 Lowry Protein Determination

Protein levels were determined as described by Lowry *et al.* (1951). Protein samples (40 µl) were diluted with 0.5 M sodium hydroxide (960 µl). Bovine serum albumin (500 µg/ml of 0.5 m sodium hydroxide), in the range 0-150 µg was used as protein standard. 5 ml of freshly prepared Lowry solution [2% sodium potassium tartrate (2 ml), 2% copper sulphate (2 ml) and 2% sodium carbonate (200 ml)] was added to the samples and the standards. Tubes were then vortexed and left to incubate at room temperature for 10

minutes. 0.5 ml Folin-Ciocalteau phenol reagent (diluted 1:1 with distilled water) was added and the tubes were immediately vortexed. Tubes were incubated for a further 30 minutes at room temperature and absorbance was read at 720 nm.

2.7 ELISA (Enzyme Linked Immunoabsorbant Assay)

As described in section 2.6.1.3, protein isolated from the cell lines HRT 18, HT 29, liver, thyroid, and endothelial cells (stimulated and non-stimulated) was assayed using specific ELISA to detect the protein concentrations of cathepsin B and DPP IV in the extracts (intracellular) and in the media (extracellular) of all cell lines.

Principle of ELISA

Enzyme Linked Immunosorbent Assay is a useful and powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine and culture supernatant. It has become an important technique in diagnostics such as for initial screening for HIV and in cancer research.

ELISA requires coating antibody or antigen onto a polystyrene 96 well microtitre plate optimised for protein binding. In the following experiment the type of ELISA used was sandwich ELISA. It measures the amount of antigens, present in the samples, controls or calibrators, between two layers of antibodies. The antigens to be measured must contain at least two antigenic sites, as two antibodies act in the sandwich. The substance to be measured in the sample is firstly bound by primary antibodies absorbed onto the microtitre well and then to the secondary antibodies (Law, 1996). As described previously under section 1.12.1 a detection enzyme is either linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody or linked to a protein such as streptavidin if the primary antibody is biotin labelled. The amount of

protein present in the sample is therefore directly proportional to HRP activity which upon addition of the appropriate substrate (TMB) produces blue colour when oxidised with hydrogen peroxide, catalysed by HRP. The colour then changes to yellow upon addition of acid (stop solution). The colour intensity is then measured using a spectrophotometer at a wavelength of 450 nm.

A standard curve is incorporated into a sandwich ELISA assay by making serial dilutions of a standard protein solution of known concentration. Standard curves are generally plotted as the standard protein concentration (typically ng or pg of protein/ml) versus the corresponding mean absorbance value of replicates. The concentrations of the specific protein in samples can be interpolated from the standard curve. Generally, it is useful to perform a dilution series of the unknown samples this is to ensure that the absorbance falls within the linear portion of the standard curve.

2.7.1 Cathepsin B

Dilutions (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) were prepared for total protein that was isolated from the five cell lines, HRT 18, HT 29, liver, thyroid, and HUVECs [stimulated (intracellular and extracellular), unstimulated (intracellular and extracellular)] as stated in section 2.6.1.3. This was carried out in order to find the suitable dilution for carrying out the experiment. All the experiments were carried out in triplicate to ensure accuracy.

The cathepsin B concentration of the sample and control was determined from the calibration curve obtained by plotting the cathepsin B concentration of the calibrators versus the absorbance at 450 nm. A commercially available cathepsin B enzyme-linked immunosorbent assay (ELISA) kit was used and calibrated with known concentrations of human cathepsin B ranging from 500 to 7.8 ng/ml.

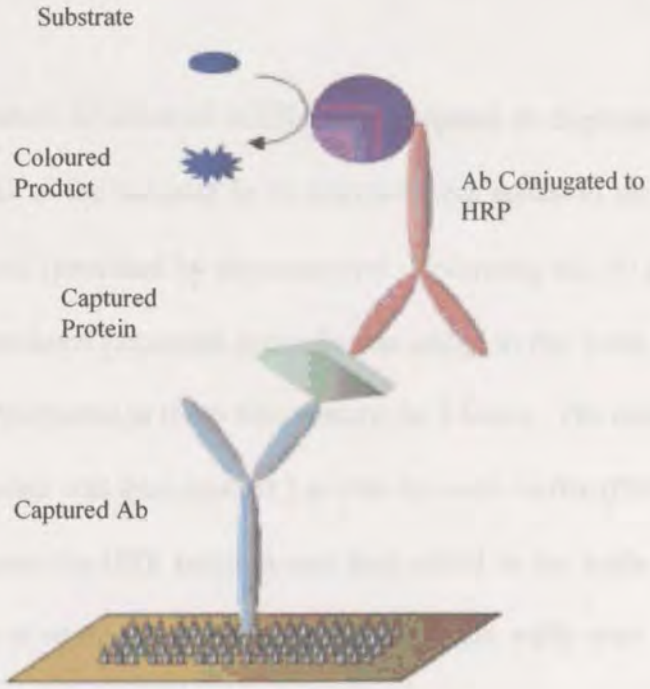
Multiwell plates supplied by the IDS (U.K.) were pre-coated with rabbit polyclonal antibodies specific for human cathepsin B.

100 μl of calibrators, controls and diluted samples (the appropriate dilution factor was obtained prior to carrying out the experiment as mentioned above) were pipetted into selected wells. For blanks 100 μl of dilution buffer was used. During this first incubation period of 2 hours at 37°C, antigens present in the calibrators, controls and samples were bound to the antibodies at the solid phase. Any unbound material was removed by washing the wells with wash buffer comprising of PBS with 1% Tween 20.

Following washing 100 μl of sheep anti-human cathepsin B antibodies, conjugated with horseradish peroxidase was added into each well. The plate was incubated for further 2 hours at 37°C. This resulted in the formation of a sandwich complex:

Immobilised rabbit anti-HCTSB IgG + CTSB + sheep anti-HCTSB IgG-HRP.

Wells were once again washed. After the wells had been washed, 200 μl of TMB (3,3',5,5' - tetramethylbenzidine and hydrogen peroxide) was added to each well. The plate was incubated for 15 minutes at room temperature in the dark. The enzymatic reaction was then terminated with the addition of 50μl/well of stopping solution (2 M H_2SO_4). The experiment was carried out in triplicate and the average was calculated. Absorbance at 450nm was measured using an ELISA microplate reader (*Bio-Rad*, Model 550).



http://www.uq.edu.au/vdu/ELISA.htm

Figure 2.7: *A sandwich ELISA showing a capturing antibody, pre-coated on microwell plate, and a captured protein. This complex is detected by an antibody conjugated enzyme (HRP) which is detected by a substrate (TMB) resulting in a coloured product which can be measured by the use of an ELISA plate reader.*

2.7.2 DPP IV

As described in section 2.7.1, similarly the appropriate dilution for the total protein to be used to detect DPP IV was examined.

For the detection of DPP IV protein in the cells (intracellular) and the supernatants (extracellular) the commercially available sCD26 ELISA kit was used. Monoclonal antibody (murine) to human sCD26 pre-coated 96 well plate was used in order to determine the protein concentration within the cytokine treated cells, HRT 18, HT 29, and HUVECs, and also in positive control cells (liver for cathepsin B and thyroid for DPP IV).

Standard dilutions of sCD26 were prepared in duplicate ranging from 500 to 15.6 ng/ml. 20 μl of the samples to be analysed was added to rest of the well containing 80 μls of diluent (provided by the company). Following this 50 μl of biotin conjugated anti-sCD26 monoclonal (mureine) antibody was added to the wells. The microwell plate was covered and incubated at room temperature for 3 hours. The microwell plate was then emptied and the plate was then washed 3 x with the wash buffer (PBS with 1% TWEEN 20). 100 μl of streptavidin-HRP solution was then added to the wells and the plate was incubated for 1 hour at room temperature. Once again the wells were emptied and then washed 3 times with the wash buffer. 100μl of TMB substrate solution was then added to all the wells and incubated at room temperature for 10 minutes. The reaction was stopped after 10 minutes by addition of 100 μl stop solution (1 M phosphoric acid). DPPIV protein analysis was also carried out in triplicate and the average was recorded. ELISA plate reader was thereafter used to measure the colour intensity at 450 nm.

2.8 Inhibitor Experiments

In addition to determining the activities of the two enzymes, an endopeptidase cathepsin B and exopeptidase DPP IV, under the influence of various doses of cytokines (TNF α and IL-1β) further characterisation of enzyme activities was obtained by testing sensitivity of these enzymes to specific inhibitors. The inhibitors used were Mu-Phe-Hph-FMK inhibitor for cathepsin B and Gly-Pro-Gly-Gly for DPP IV enzyme. Inhibitor studies for both the enzymes, cathepsin B and DPP IV were carried out in triplicate and each time the average of 12 samples was taken to ensure accuracy.

2.8.1 Measurement of Enzyme activity

Enzymatic activity of the cells was measured by using 10 μl Z-Arg-Arg-AMC (1 mM) for cathepsin B as substrate and 10 μl of Gly-Pro-AMC substrate (1 mM) for DPP IV. Assays were performed at 25°C in 185 μl of 0.1M MES pH 5.5 with 0.1% v/v Triton X-100, and 5 μl DTT (100 mM) for cathepsin B and, 190 μl of 0.1M Tris-HCL pH 8.0 with 0.1% v/v Triton X-100 for DPP IV, in a fluorometer at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Fluorescence measurements were carried out on a cytoflour machine (described under section 2.4). An outline of the procedure used is summarised in figure 2.8.

2.8.1.1 Cathepsin B Inhibitor

Cathepsin B inhibitor, FMK, and their dilutions were prepared in dimethylsulfoxide and the untreated reactions were run with an equal volume of dimethylsulfoxide. For inhibition

assays, the reaction mixture for detecting cathepsin B activity were incubated for 15 minutes, and also for 30 minutes (individually) with 10 – 100 μM concentrations of FMK. The enzyme activity of cathepsin B under the influence of the inhibitor FMK at various concentrations was determined in all the three cell lines, namely HRT 18, HT 29 and HUVECs.

The assay procedure for the inhibitor was the same as described above in section 2.8.1, except that the inhibitors were incorporated into the assay buffer at the desired concentration and the samples were left to equilibrate for 10 minutes at room temperature before adding the substrates. All assays were carried out in triplicate and the percentage remaining was determined by comparison with controls containing no inhibitors. The suppressive ability of the inhibitor was then measured using a cytoflour machine that measured the fluorescence. The percentage inhibition of cathepsin B with FMK is explained in chapter 3.

2.8.1.2 DPP IV Inhibitor

Gly-Pro-Gly-Gly an inhibitor of DPP IV was used in order to assess its ability to inhibit DPP IV activity. The inhibitor and their dilutions were prepared in dimethylsulfoxide and the untreated reactions were run with an equal volume of dimethylsulfoxide. Once HRT 18, HT 29 and HUVECs were grown to confluency on 96 well plates, inhibitor diprotin was added to the wells at varying concentrations, 10 – 100 mM, for 15 and 30 minutes. Control wells consisted of no inhibitor, and/or DMSO (which was used to dissolve inhibitor), and for positive control DPP IV protein was used. All assays were carried out in triplicate and the percentage remaining was determined by comparison with controls containing no inhibitors. Cytoflour machine was used to assess the activity of DPP IV.

Adherent cells (HRT 18, HT 29, and HUVEC's)

Cells were plated onto 96 well plates and incubated till cells were confluent

Respective inhibitors were added for cathepsin B and DPP IV

Reagents were added to the two plates to measure protease activity

Intracellular

Extracellular

Incubate at room temperature for 2 hours

Fluorescence was recorded at an excitation wavelength of 355 nm and an emission wavelength of 460 nm

Cytoflour Machine (to measure the fluorescence)

Figure 2.8: *An overview of the procedure carried out for inhibitor studies for both the enzymes cathepsin B and DPP IV in the three cell lines under investigation.*

2.9 Statistical Analysis

In all the experiments conducted, where necessary statistical analysis was carried out. Results were tested for normality using Kolmogorov-Smirnov Goodness-of-Fit Test for Gaussian (normal) distribution of data. Results were subjected to ANOVA with Tukey's multiple comparison test. Tukey's allows for comparison of "treatment" groups with each other. Significance levels were set at $p < 0.05$ and $p < 0.005$. All the graphs presented are represented with standard error bars.

3.0 Growth Curves for HRT 18, HT 29, Liver cells, Thyroid cells, and HUVECs

Experiments were carried out to investigate the pattern of growth of the cancer cells and normal endothelial cells, and to determine the differences in the rate of growth of the cell types studied namely HRT 18, HT 29, liver cells, thyroid cells, and HUVECs. For this purpose *in-vitro* growth rate of each cell type was studied, and their growth curves were plotted. All the three cell types showed a varied growth pattern in terms of their doubling time. Graph below shows the growth curve for HRT 18, HT 29, liver cells, thyroid cells, and HUVECs with the following phases: - Lag phase, log phase and plateau phase (each phase is described in section 1.11.1).

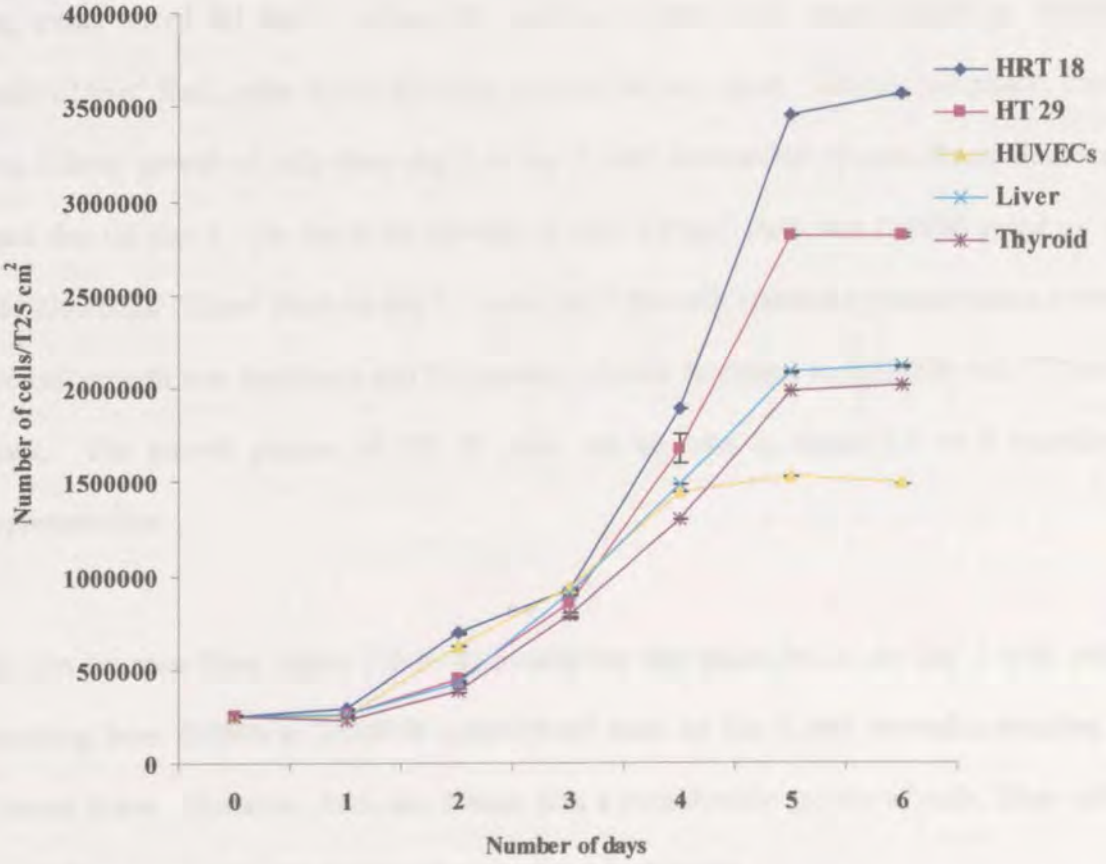


Figure 3.0: *Growth curves for the five cell lines, HRT 18, HT 29, Liver cells, Thyroid cells, and HUVECs,*

In HRT 18 cancer cells, as shown above in the graph (figure 3.0), the log phase (period of maximum growth) began at day 1 till day 5. At day two the number of cells counted was 289000 cells/T25cm^2 flask, reaching up to 3450000 cells/T25cm^2 flask by day 5. After the growth peak at day 5, the cells reached the plateau/stationary phase, where the rate of production of new cells (the birth rate) was equal to the death rate. The number of cells doubled on day 4 (from 926667 cells/T25cm^2 flask on day 3 to 1883333 cells/T25cm^2 flask on day 4) and day 5 (from 1883333 cells/T25cm^2 flask on day 4 to 3450000 cells/T25cm^2 flask on day 5).

In HT 29 cancer cells, a similar growth pattern was observed as was for HRT 18 cells. The lag phase lasted till day 1, where the number of cells went from 250000 to 260000 cells/T25cm^2 flask, after which the cells reached the log phase. During this phase, there was a steep growth of cells from day 2 to day 5, with the number of cells almost doubling each day till day 5. On day 2 the number of cells/T25cm^2 flask was 450000 going up to 2810000 cells/T25cm^2 flask by day 5. After day 5 the cells reached a plateau phase, where the cell growth was stationary and the number of cells decreased to 2809000 cells/T25cm^2 flask. The growth pattern of HT 29 cells can be seen in figure 3.0 as a graphical representation.

As can be seen from figure 3.0 in liver cells the log phase began on day 1 with cells reaching from 256800 to 2080078 cells/T25cm^2 flask on day 5, and thereafter reaching a plateau phase. However, from day 2 there was a considerable growth of cells. Liver cells multiplied at a slower rate compared to the two cancer types.

Growth curve patterns for thyroid cells was similar to that of liver cells. The lag phase of the thyroid cells lasted till day 1, with cell number increasing considerably from day 2 to day 5 (386999 to 1980000 cells/T25cm^2). 95% cell confluency was achieved by day 6 (2012229 cells/T25cm^2).

HUVECs grew exponentially from day 1 (248000) until day 4 (1437500), after which time proliferation slowed reaching a stationary phase presumably caused by cell quiescence as monolayers approached confluency.

The graphical representations of growth patterns of the cell types, HRT 18, HT 29, liver cells, thyroid cells, and HUVECs, gives a clear indication of the growth rate of these cells.HRT 18 cell line seemed to grow at an increased rate in comparison to HT 29 cell line. The cell doubling time for HRT 18 was 34 hrs, whereas for HT 29 cells were 53 hrs. Cell doubling time for liver cells and thyroid cells was 56 hrs and 62 hrs respectively and the plateau phase for both of these cell lines was achieved after 120 hrs (5 days), similar to the cancer cell lines. HUVECs however had a cell doubling time of 39 hrs but at 96 hrs reached the plateau phase, while both HRT 18 and HT 29 cells reached plateau phase at 120 hrs (5 days). Figure 3.0 shows growth pattern for all the five cell types (HRT 18, HT 29, Liver cells, Thyroid cells, and HUVECs).

3.1 Morphology of Tumour and primary endothelial cells

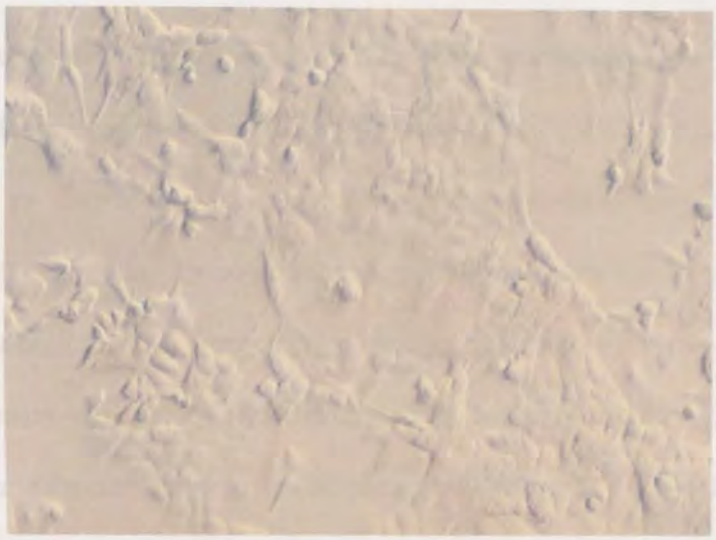
All the five cell types adhered to the base of the flask in which they were grown. The two cancer cell lines formed a 95 % confluency (coverage of the flask) in 4 - 5 days, with liver cells achieving 95% confluency at day 6, which was similar to thyroid cells. Whereas, the

confluency of human umbilical endothelial cells was achieved within 7 days. Growth pattern for the cell lines used can be seen in figure 3.0.

When examined under the light microscope, HRT 18 and HT 29 cells appeared to be of different sizes and were crowded together in a disorderly fashion. The two cancer cell types did not maintain contact inhibition *in-vitro*. These cells adhered to the base of the flask and were piled up on top of one another.

Light microscopy of endothelial cells isolated from human umbilical vein showed adhesion of the cells to the T25 tissue culture flask after 24 hours in culture. A confluent monolayer of the cell was achieved between days 5-7. Endothelial cells grew as monolayers of closely opposed, polygonal large cells as shown in figure 3.1 (a) and (b).

(a)



(b)

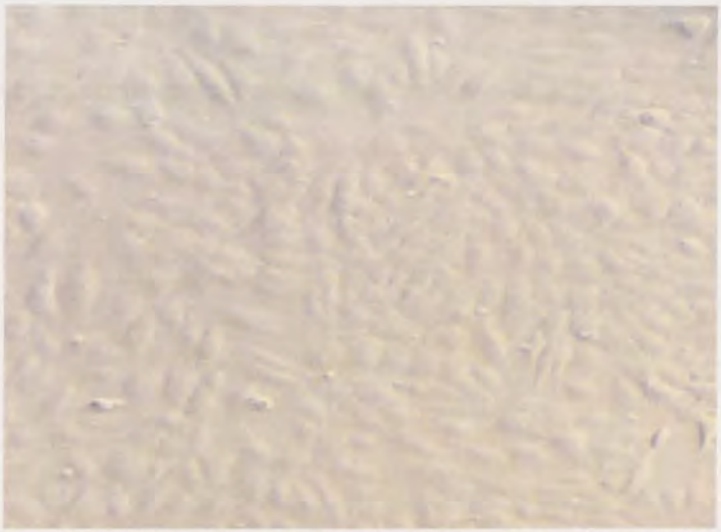


Figure 3.1: *Light microscopy of primary monolayer of endothelial cells isolated from human umbilical vein.* ***(a)*** *50% confluent,* ***(b)*** *95% confluent. Original Magnification (x 100)*

3.1.1 Immumnocytochemical staining of HUVECs

Following isolation, endothelial cells were grown on chamber slides in their first passage. The slides were stained by CD31 and vWF antibodies using a standard immunocytological technique to examine the presence of these endothelial cell markers.

Immunocytochemistry of HUVECs using endothelial cell markers, CD31 or platelet endothelial adhesion molecule (PECAM-1) and vWF, resulted in positive staining of the cells as shown in figure 3.2 and 3.5 (a) and (b) respectively, identifying the cells as endothelial cells. Endothelial cell staining displayed characteristic features of elongated granules, known as Weibel Palade bodies, throughout cytoplasm. Staining of the cells was necessary as isolating endothelial cells often leads to contamination by fibroblasts, which appear as overlapping layers of parallel arrays of slender, spindle-shaped cells when seen under a light microscope. By carrying out specific test for identifying endothelial cells it was ensured that the subsequent experiments were carried out on endothelial cells only. However, no fibroblast contamination was noted in any of the experiments conducted.

(a)

(b)

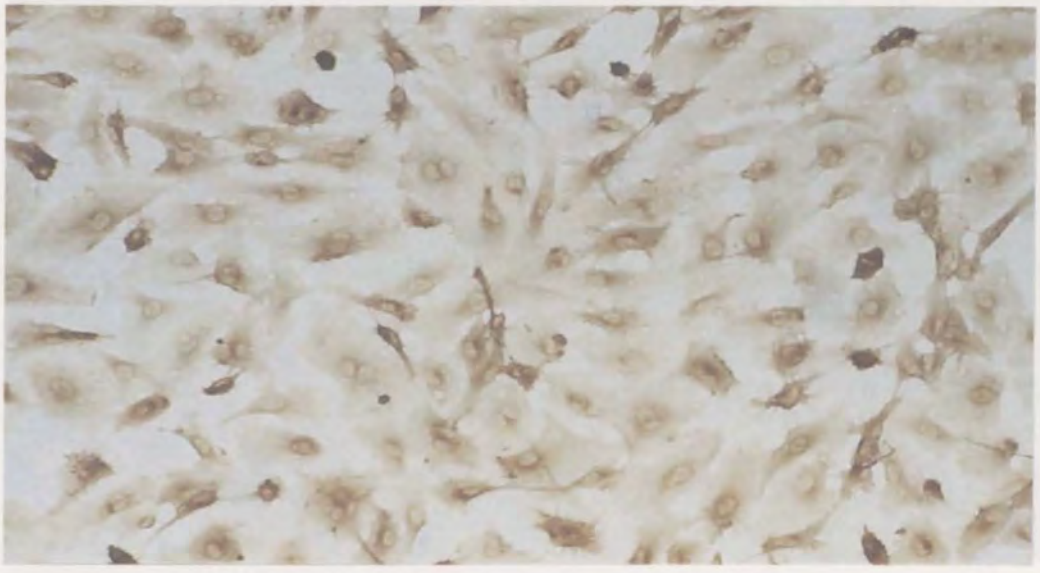


Figure 3.2 (a) and (b): *Light micrograph of endothelial cells stained with CD31 x 10 (100 magnification)*

(a)

(b)

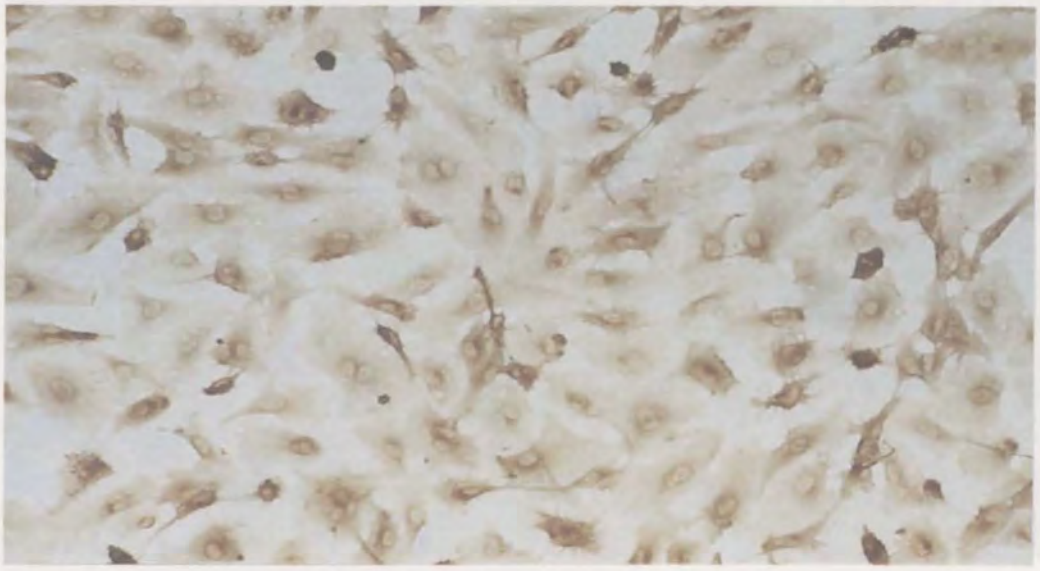


Figure 3.2 (a) and (b): *Light micrograph of endothelial cells stained with CD31 x 10 (100 magnification)*

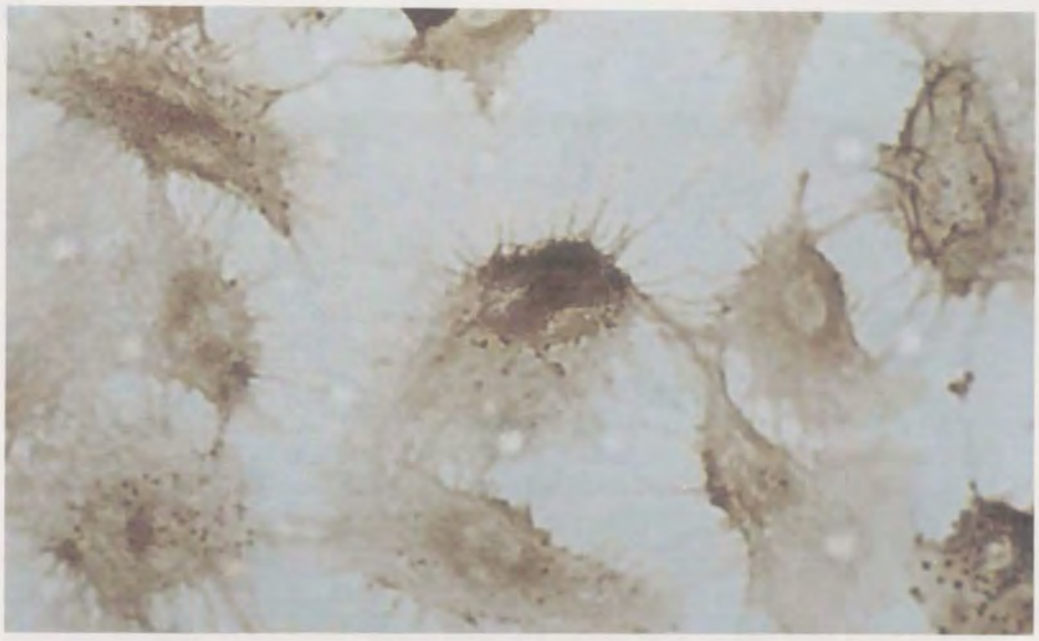
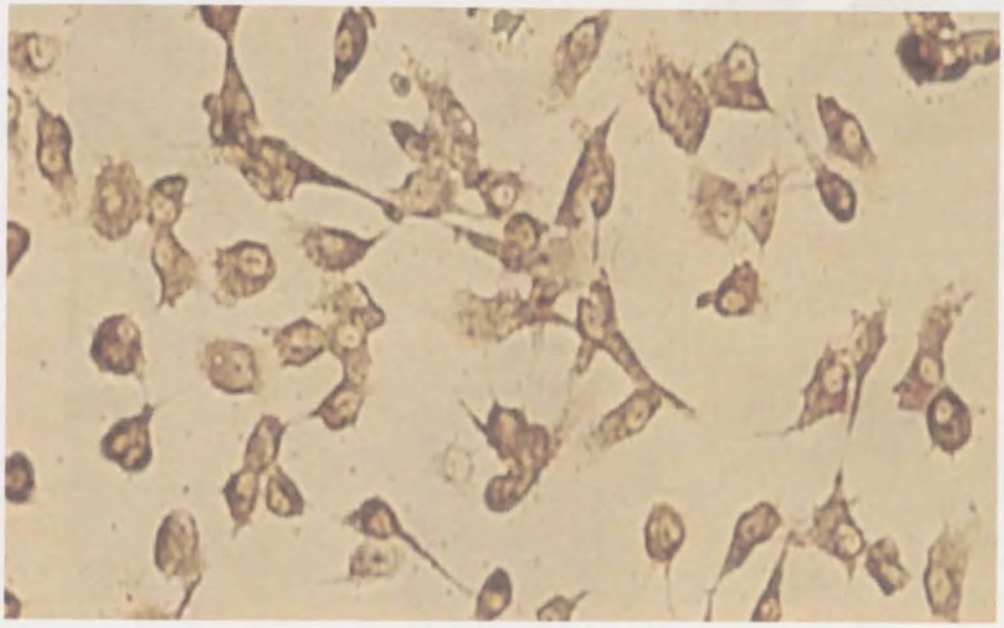


Figure 3.3: *Light micrograph of endothelial cells stained with CD31 x 40 (400 magnification)*

Figure 3.4: *Light micrograph of endothelial cells stained for CD31 negative control x 10 (100 magnification)*

(a)



(b)

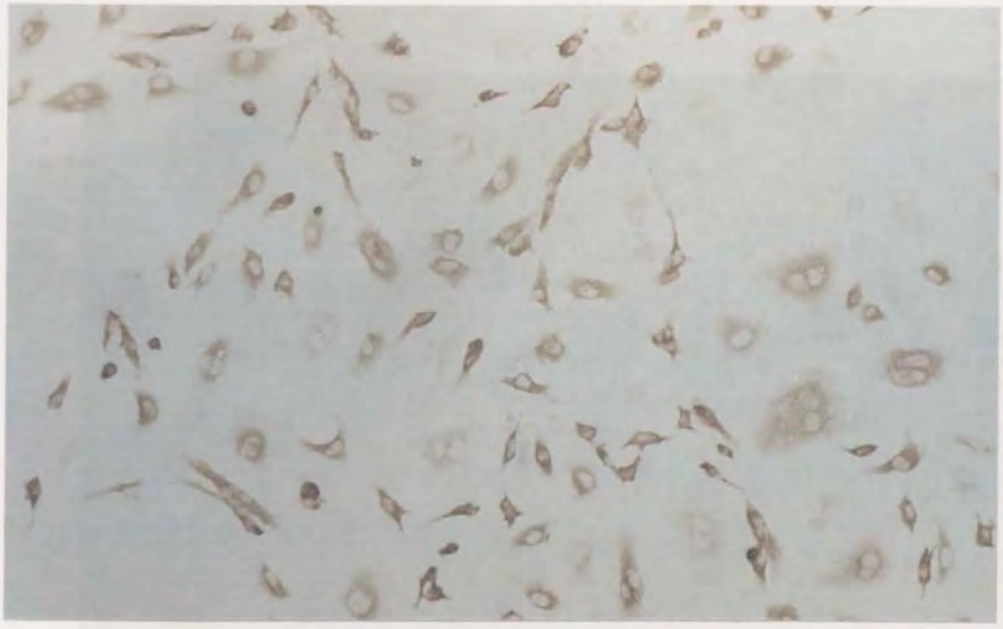


Figure 3.5 (a) and (b): *Light micrograph of endothelial cells stained with vWF x 10 (100 magnification)*

Figure 3.6: *Light micrograph of endothelial cells stained with vWF x 40 (400 magnification)*

Figure 3.7: *Light micrograph of endothelial cells stained with vWF negative control x 10 (100 magnification)*

Monoclonal antibody to CD31 is very specific for the staining of vascular endothelial cells in normal and cancerous tissue. Figure 3.2 (a), (b) and 3.3 shows staining of endothelial cells with Mouse anti-CD31 in comparison to figure 3.4, negative control cells where there is no staining. vWF another endothelial cell marker can be seen in Figure 3.5 (a), (b) and 3.6 which shows Rabbit anti-vWF staining of HUVECs in comparison to figure 3.7, negative control where there is no staining of the cells.

Examination of cells stained with the two most widely accepted cell markers for the study of angiogenesis and neovascularization, CD31 antibodies and vWF, it was clear that the endothelial cells derived from human vein umbilical cords were normal endothelial cells. It confirmed that further experiments were carried out solely on normal endothelial cells.

3.2 Biochemical Studies/Activity assay

Intracellular and extracellular cathepsin B and DPP IV enzyme activity was measured in three cell lines namely, HRT 18, HT 29 and HUVECs. Synthetic peptide substrates were used in order to monitor the enzyme activity of cathepsin B and DPP IV. The substrates consisted of a peptide sequence in which the acyl group is conjugated to a flourogenic group (7-amino-4 methyl coumarin). AMC was released in the solution upon cleavage with the enzyme. The amount of fluorescence detected as the flourogenic compound cleaved off indicated the amount of enzyme activity, which was measured using a cytoflour machine.

The following results show the best-suited media and incubation period for each of the enzymes in different cells lines, and the effect of cytokines on the activity of these enzymes both intracellularly and extracellularly.

3.2.1 Time course response

HRT 18, HT 29, and endothelial cells (HUVECs) were incubated in 6 different media over a range of incubation period following which, cathepsin B and DPPIV activity was measured in all the three cell lines. Cathepsin B showed optimal activity after 30 hours incubation in MEM media in HRT 18 cells, while in HT 29 cells the time at which there was maximal activity was at 18 hours. In HUVECs the maximal cathepsin B activity was observed at 12 hour incubation. Optimal activity for DPP IV was noted at 12 hours in HRT 18 cells and at 42 hrs in HT 29 cells. Furthermore, HUVECs showed to have a maximal DPP IV activity at 48 hrs. The above incubation periods for the optimal protease activity can be seen in table 3.0 (a) and (b). Bar graph representations for the time course and choice of media for both the enzymes can be seen in figure 3.13 - fig.3.18.

3.2.2 Choice of Media

HRT 18, HT 29, and endothelial cells were incubated in 6 different media, PBS, Isotonic, HBSS (all supplemented with 0.3% BSA), MEM, DMEM and RPMI 1640 (without glutamine), for 48 hours. The activity of cathepsin B and DPP IV enzyme was measured every 6 hours for 48 hours. The media that gave the optimal enzyme activity for both the enzymes was noted. The media that was chosen for cathepsin B activity measurements in subsequent experiments for the two tumour cell lines and endothelial cells was MEM, as this media provided the highest cathepsin B activity, although the incubation time varied, as shown in table 3.0(a). However, the media that resulted in maximal DPP IV activity in HRT 18, HT 29, and HUVECs was PBS, RPMI, and Hanks respectively, as can be seen in table 3.0(b).

Fig. 3.8

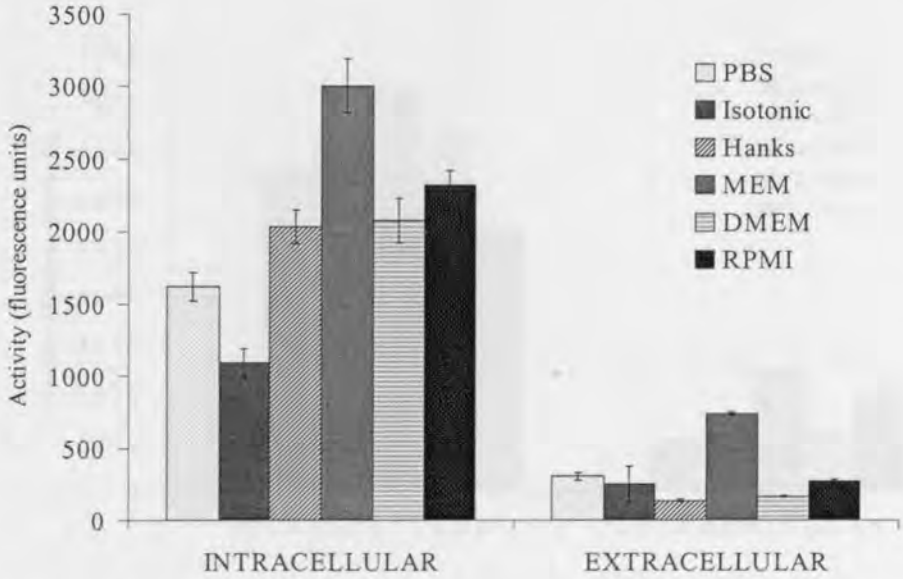


Figure 3.8: *Intracellular and extracellular cathepsin B activity in HRT 18 cells following 30 hour incubation in 6 different media. (The error bars represent an average of 16 samples, which was repeated thrice).*

Fig. 3.9

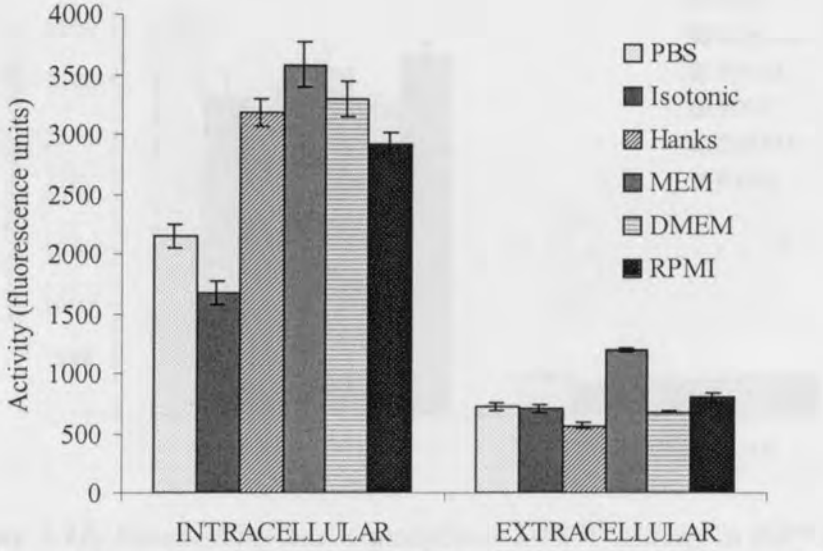


Figure 3.9: *Intracellular and extracellular cathepsin B activity in HT 29 cells following 18 hour incubation in 6 different media.*

Fig. 3.10

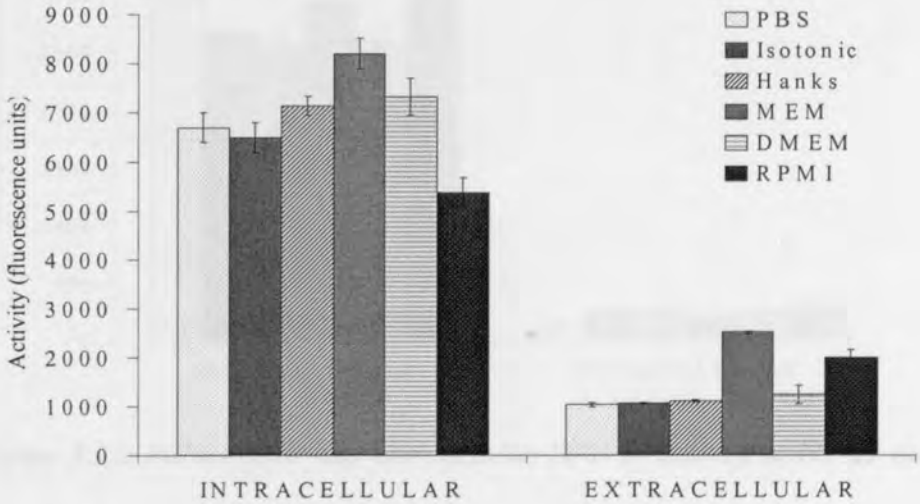


Figure 3.10: *Intracellular and extracellular cathepsin B activity in endothelial cells following 12 hour incubation in 6 different media.*

Fig. 3.11

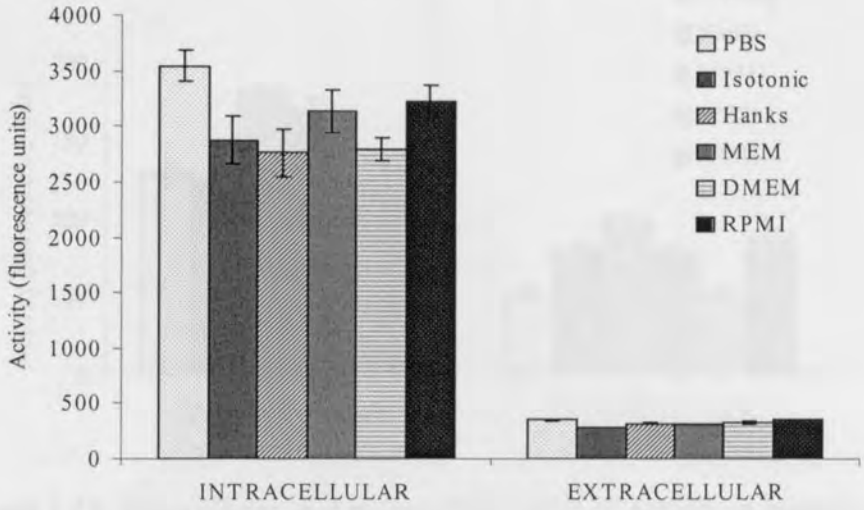


Figure 3.11: *Intracellular and extracellular DPPIV activity in HRT 18 cells following 12 hour incubation in 6 different media.*

Fig. 3.12

Figure 3.12: *Intracellular and extracellular DPP IV activity in HT 29 cells following 42 hour incubation in 6 different media.*

Fig. 3.13

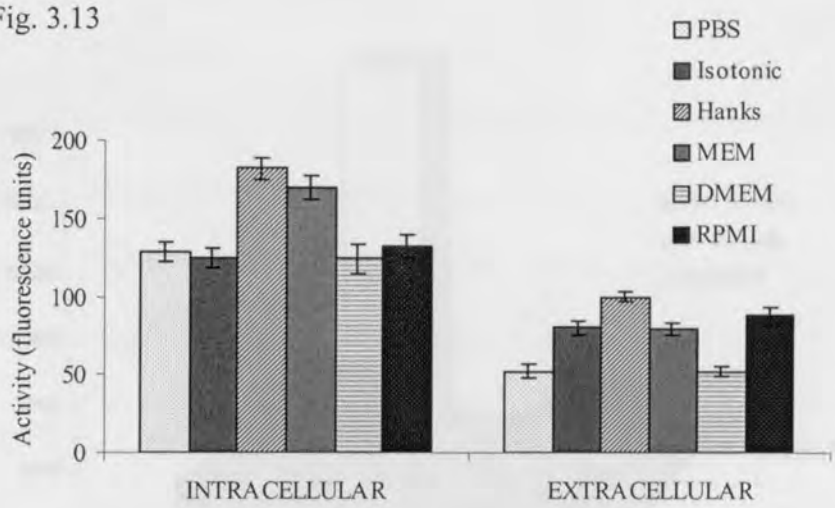


***Figure 3.13**: Intracellular and extracellular DPP IV activity in endothelial cells following 48 hour incubation in 6 different media.*

In brief, the best-suited medium and optimal incubation period for both the enzymes used in the consecutive experiments are outlined in table 3.0 (a) and (b).

(a)

Cell lines	*Media*	*Incubation period*
HRT 18	MEM	30 hours
HT 29	MEM	18 hours
E.C.	MEM	12 hours

(b)

Cell lines	*Media*	*Incubation period*
HRT 18	PBS	12 hours
HT 29	RPMI	42 hours
E.C.	Hanks	48 hours

***Table 3.0:** The media and incubation period that gave the highest measurement of enzyme activity in the three cell lines. **(a)** Cathepsin B activity, **(b)** DPP IV activity.*

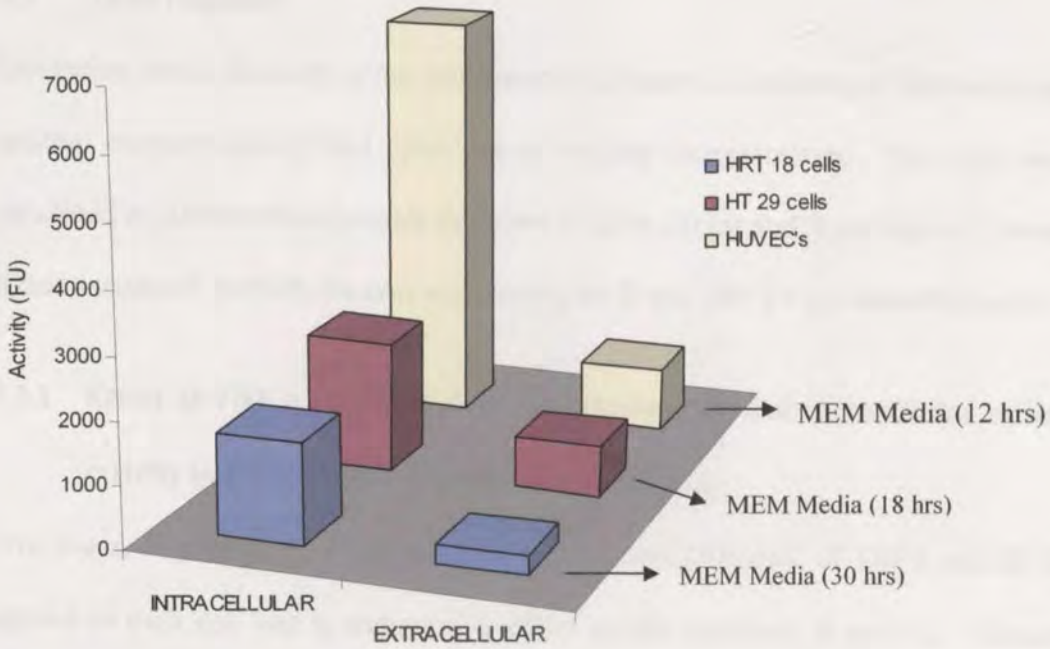


Figure 3.14: *A bar graph representation of optimal cathepsin B activity that was achieved by incubation of the cells, HRT 18, HT 29 and HUVECs, in specific media and time required for optimal enzyme activity.*

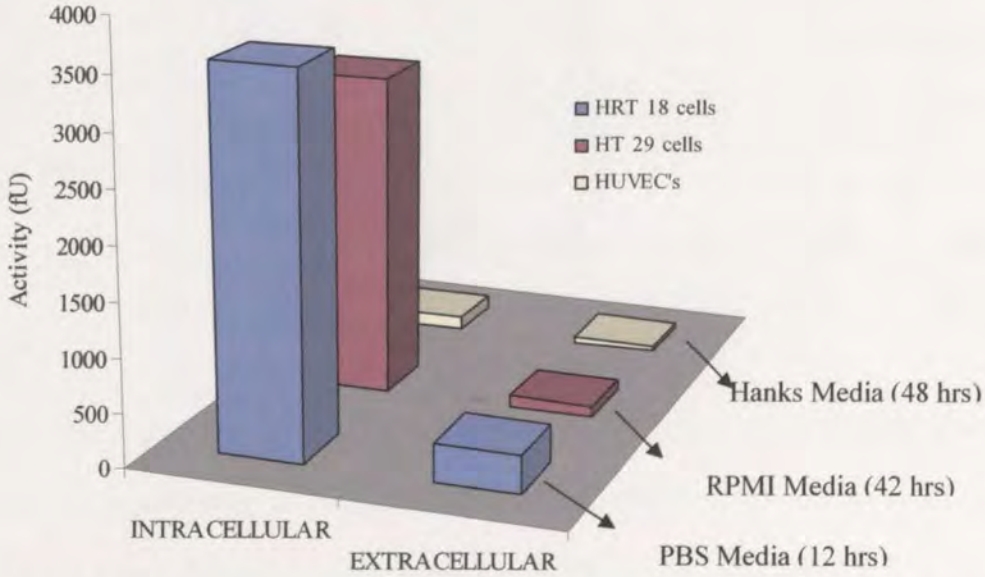


Figure 3.15: *A bar graph representation of optimal DPP IV activity that was achieved by incubation of the cells, HRT 18, HT 29 and HUVECs, in specific media and time required for optimal enzyme activity.*

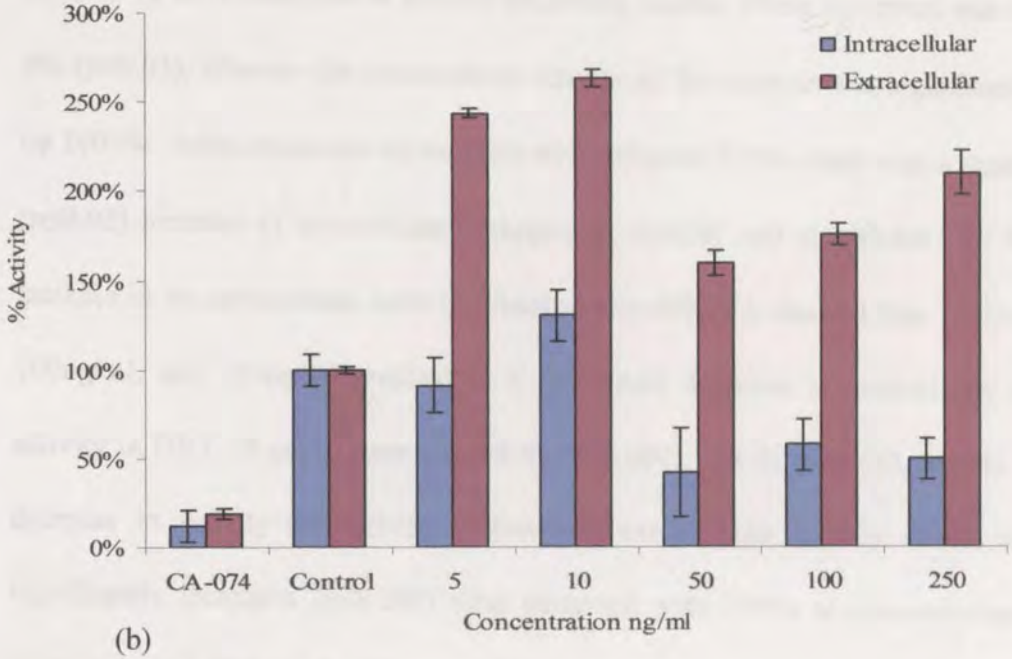
3.3 Dose response

Incubation media for each of the cell lines was prepared, consisting of the media that gave optimal enzyme activity and cytokines at varying concentrations. The cells were then incubated at different time periods as shown in table 3.0 (a) and (b) or figure 3.14 and 3.15. Results obtained for both the enzymes, cathepsin B and DPP IV are described below.

3.3.1 Effect of TNF α and IL-1 β on the intracellular and extracellular cathepsin B activity in HRT 18, HT 29 and endothelial cells.

Five doses, 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml and 250ng/ml, of TNFα and IL-1β were applied to each cell line to measure its effect on the cathepsin B activity. However, no significant dose response was apparent. Figure 3.16 – Figure 3.18, represents the % increase/or decrease in intracellular and extracellular cathepsin B activity, following treatment with cytokines TNFα and IL-1β, at various concentrations.

(a)



(b)

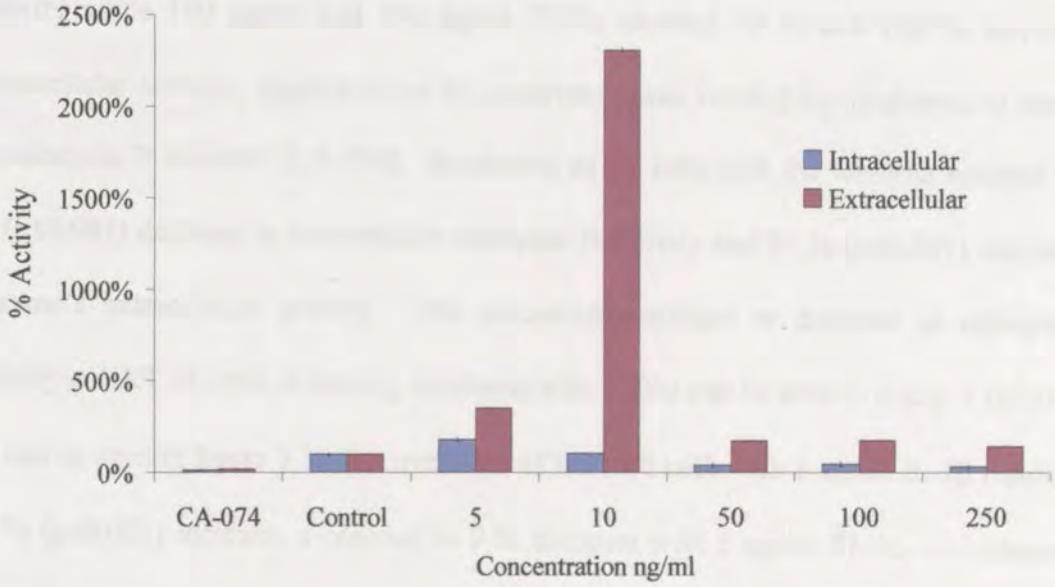


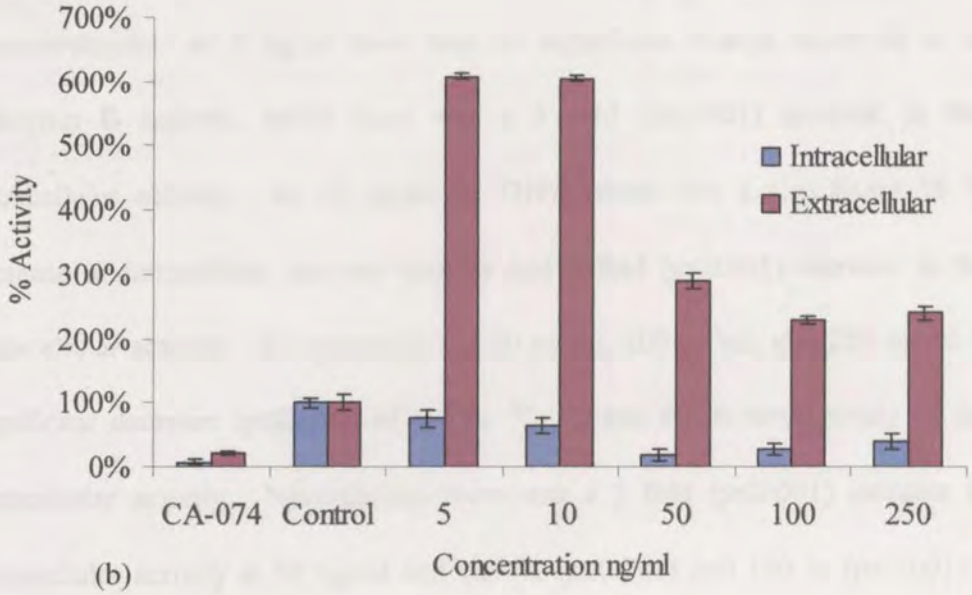
Figure 3.16: *% increase/decrease in intracellular and extracellular cathepsin B activity in HRT 18 cells following 30 hrs stimulation with cytokines, at concentrations 5ng/ml, 10ng/ml 50ng/ml, 100ng/ml and 250ng/ml in MEM media.* ***(a)*** *TNFα,* ***(b)*** *IL-1 β.(p values of intracellular and extracellular cathepsin B activity are compared with the control in all subsequent experiments).*

In HRT 18 cells cathepsin B activity following 5ng/ml TNFα treatment was decreased by 9% ($p \leq 0.05$), whereas the extracellular activity of the enzyme was significantly increased by 100 %. After treatment of the cells with 10ng/ml TNFα there was a significant 30 % ($p \leq 0.05$) increase in intracellular cathepsin B activity and significant 100 % ($p \leq 0.001$) increase in its extracellular activity. Analysis by ANOVA showed that TNFα at 50ng/ml, 100ng/ml, and 250ng/ml resulted in a significant decrease in intracellular cathepsin B activity in HRT 18 cells, there was 58 % ($p \leq 0.001$), 43 % ($p \leq 0.01$), and 51 % ($p \leq 0.01$) decrease in activity respectively. However, extracellular activity of the enzyme was significantly increased ($p \leq 0.001$) after treatment with TNFα at concentrations 50ng/ml, 100ng/ml, and 250ng/ml. 50 ng/ml TNFα resulted in a 60 % increase in cathepsin B activity while 100 ng/ml and 250 ng/ml TNFα showed 75 % and 100 % increase in extracellular activity. Specificity of the experiment was verified by incubation of the cells in cathepsin B inhibitor (CA-074). Incubation of the cells with the inhibitor resulted in 88 % ($p \leq 0.001$) decrease in intracellular cathepsin B activity and 81 % ($p \leq 0.001$) decrease in enzyme's extracellular activity. The percentage increase or decrease in cathepsin B activity in HRT 18 cells, following treatment with TNFα can be seen in figure 3.16 (a).

As can be seen in figure 3.16 (b), treatment of HRT 18 cells with 5 ng/ml IL-1β resulted in 80 % ($p \leq 0.001$) increase, a contrast to 9 % decrease with 5 ng/ml TNFα, in cathepsin B activity intracellularly and a double fold ($p \leq 0.001$) increase in its extracellular activity. Addition of 10 ng/ml of IL-1β to the cells resulted in a significant 10 fold ($p \leq 0.001$) increase in extracellular cathepsin B enzyme activity, however no significant change in intracellular enzyme activity was noted. At IL-1β concentrations 50 ng/ml, 100 ng/ml, and 250 ng/ml, similar pattern of dose response was observed as was for TNFα at these concentrations. There was a significant 60 % ($p \leq 0.001$) decrease in intracellular enzyme

activity at 50 ng/ml, and 71 % ($p \leq 0.001$) increase in activity extracellularly. 54 % ($p \leq 0.001$) and 69 % ($p \leq 0.001$) significant decrease in intracellular cathepsin B activity and 74 % ($p \leq 0.001$) and 42 % ($p \leq 0.001$) increase in extracellular activity was noted with 100 ng/ml and 250 ng/ml IL-1β respectively.

(a)



(b)

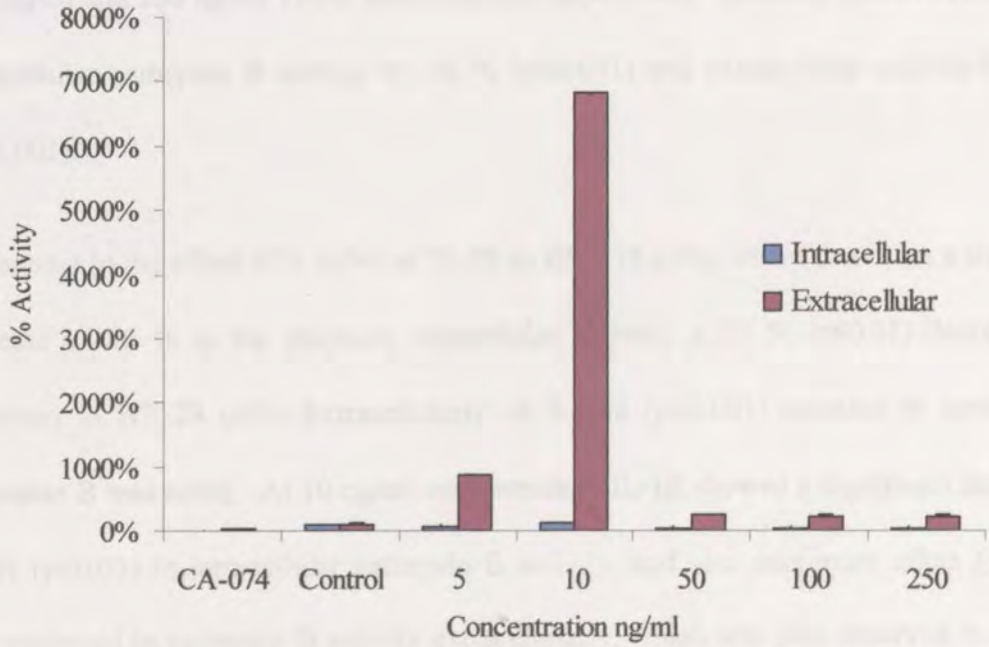


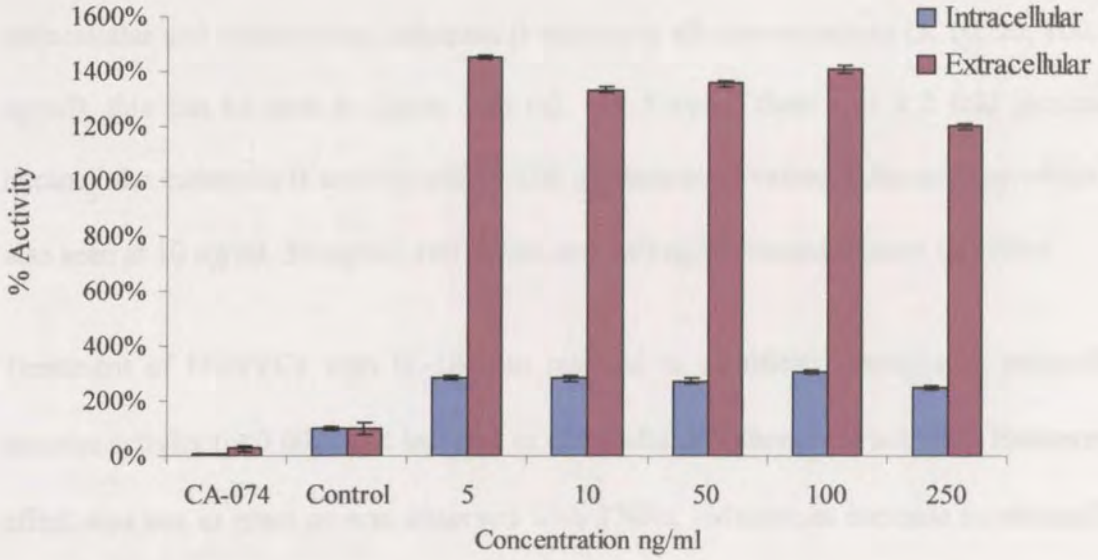
***Figure 3.17:** % increase/decrease in intracellular and extracellular cathepsin B activity in HT 29 cells following 18 hrs stimulation with cytokines, at concentrations 5ng/ml, 10ng/ml 50ng/ml, 100ng/ml and 250ng/ml in MEM media. . **(a)** TNF α, **(b)** IL-1 β.*

In HT 29 cells TNFα resulted in decrease in intracellular cathepsin B activity at certain concentrations. At 5 ng/ml there was no significant change observed in intracellular cathepsin B activity, while there was a 5 fold ($p \leq 0.001$) increase in the enzymes extracellular activity. At 10 ng/ml of TNFα, there was a significant 36 % ($p \leq 0.01$) decrease in intracellular enzyme activity and 5 fold ($p \leq 0.001$) increase in the enzymes extracellular activity. At concentrations 50 ng/ml, 100 ng/ml, and 250 ng/ml there was a significant decrease ($p \leq 0.001$) of 81 %, 72 %, and 60 % respectively in the enzymes intracellular activity. Nevertheless there was a 2 fold ($p \leq 0.001$) increase in enzymes extracellular activity at 50 ng/ml and 129 % ($p \leq 0.001$) and 140 % ($p \leq 0.001$) increase at 100 ng/ml and 250 ng/ml TNFα concentration respectively. Inhibitor CA-074 reduced the intracellular cathepsin B activity by 92 % ($p \leq 0.001$) and extracellular activity by 80 % ($p \leq 0.001$).

In contrast to the effect of 5 ng/ml of IL-1β in HRT 18 cells, where there was a significant increase of 80 % in the enzymes intracellular activity, a 29 % ($p \leq 0.01$) decrease was observed in HT 29 cells. Extracellularly an 8 fold ($p \leq 0.001$) increase in extracellular cathepsin B was noted. At 10 ng/ml concentration, IL-1β showed a significant increase of 20 % ($p \leq 0.05$) in intracellular cathepsin B activity, and also maximum affect ($p \leq 0.001$) was observed in cathepsin B activity extracellularly, which was also observed in HRT 18 cells. At concentrations 50ng/ml, 100ng/ml and 250 ng/ml the pattern of dose response was similar to that observed in HRT 18 cells. Intracellular cathepsin B activity was significantly decreased ($p \leq 0.001$) by 61%, 67% and 64% at concentrations 50ng/ml, 100ng/ml and 250 ng/ml, while there was a two fold increase in the enzymes extracellular activity at 50ng/ml ($p \leq 0.001$), 100ng/ml ($p \leq 0.001$) and 250 ng/ml ($p \leq 0.01$). Specificity of

the enzyme activity was measured by incubation of the cells with an inhibitor specific for cathepsin B (CA-074). 92 % and 80 % decrease in enzyme activity was observed in cathepsin B activity with inhibitor. The effects of both the cytokines TNF α and IL-1β on HT 29 cells can be seen in figure 3.17 (a) and (b).

(a)



(b)

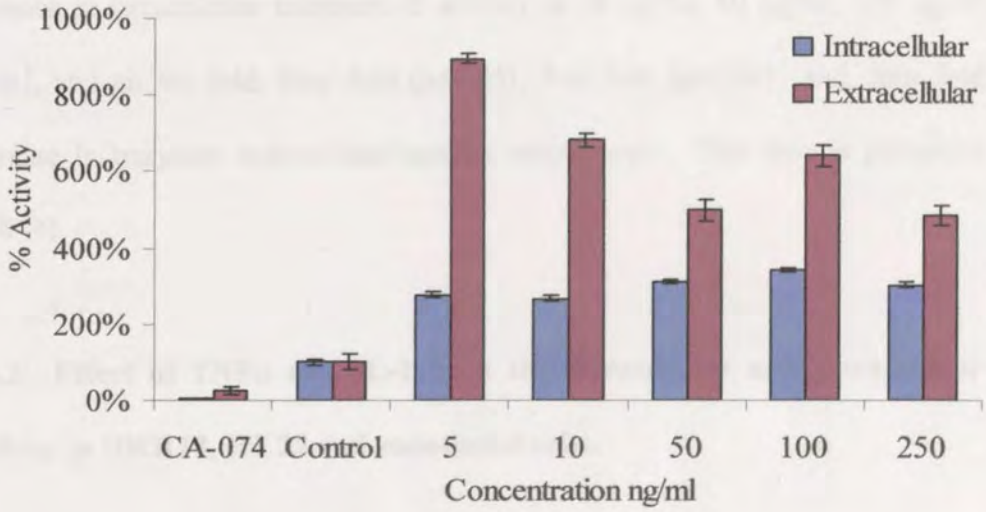


Figure 3.18: *% increase/decrease in intracellular and extracellular cathepsin B activity in HUVECs following 12 hrs stimulation with cytokines, at concentrations 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml and 250ng/ml in MEM media.* ***(a)*** *TNF α,* ***(b)*** *IL-1 β.*

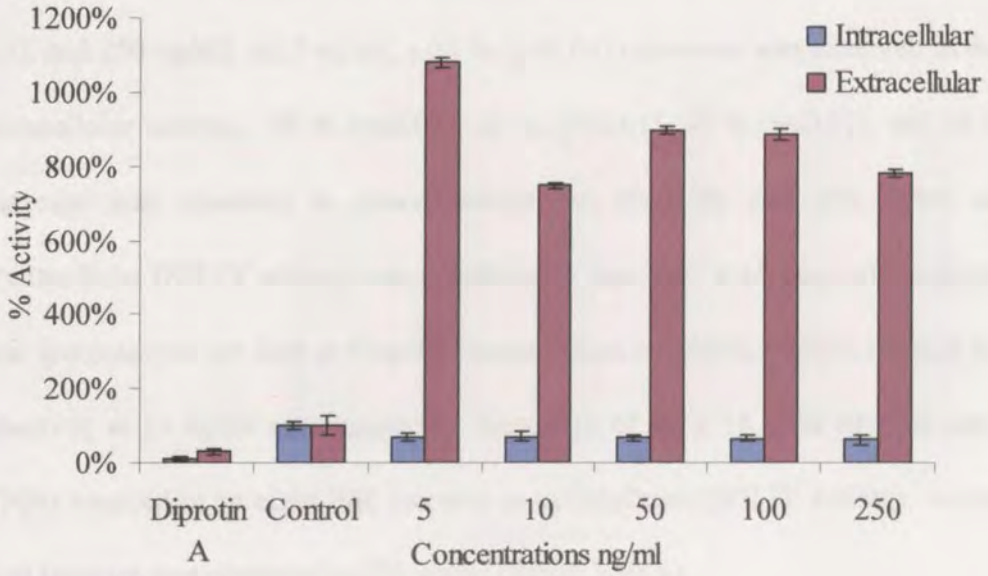
Treatment of HUVECs with TNFα resulted in a significant increase ($p \leq 0.001$) in both, intracellular and extracellular cathepsin B activity at all concentrations (5, 10, 50, 100, 250 ng/ml), this can be seen in figure 3.18 (a). At 5 ng/ml there was a 2 fold increase in intracellular cathepsin B activity and 10 fold increase in its extracellular activity which was also seen at 10 ng/ml, 50 ng/ml, 100 ng/ml, and 250 ng/ml concentrations of TNFα.

Treatment of HUVECs with IL-1β also resulted in significant increase in intracellular enzyme activity ($p \leq 0.001$) and increase in extracellular cathepsin B activity. However the effect was not as great as was observed with TNFα. Maximum increase in extracellular cathepsin B activity was achieved at 5 ng/ml concentration (eight fold increase), while a two fold increase was observed in enzymes intracellular activity. There was a two fold increase in intracellular cathepsin B activity at 10 ng/ml, 50 ng/ml, 100 ng/ml, and 250 ng/ml, and an six fold, four fold ($p \leq 0.05$), five fold ($p \leq 0.01$) and, four fold ($p \leq 0.01$) increase in enzymes extracellular activity respectively. This data is presented in figure 3.18 (b).

3.3.2 Effect of TNFα and IL-1 β on the intracellular and extracellular DPP IV activity in HRT 18, HT 29 and endothelial cells.

Five doses, 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml and 250ng/ml, of TNF α and IL-1β were applied to each cell line to measure its effect on the DPP IV B activity. However, no significant dose response was apparent. Figure 3.19 – Figure 3.21 represents the % decrease/or increase in the enzyme activity (intracellular and extracellular) following treatment of the cell lines with cytokines, under investigation.

(a)



(b)

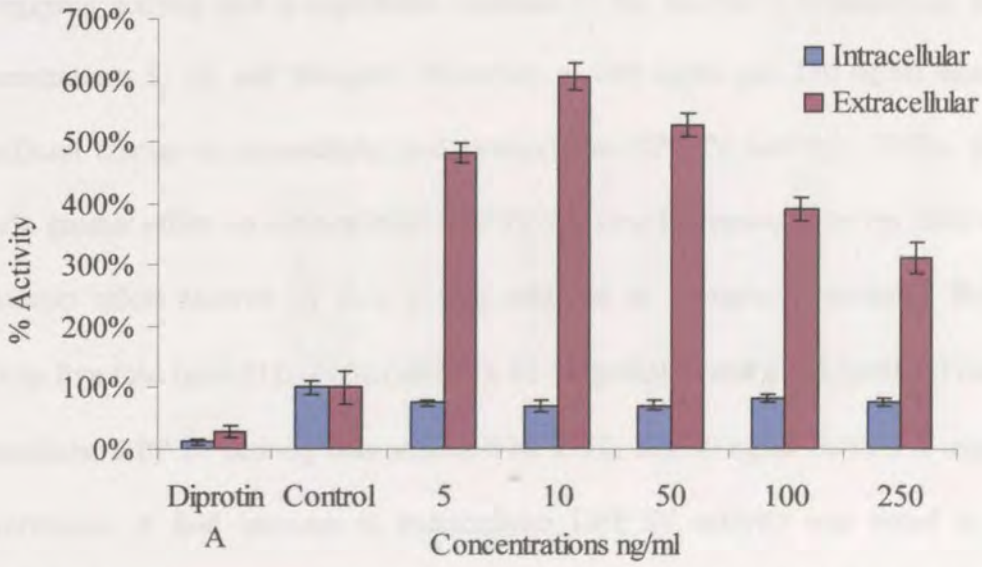
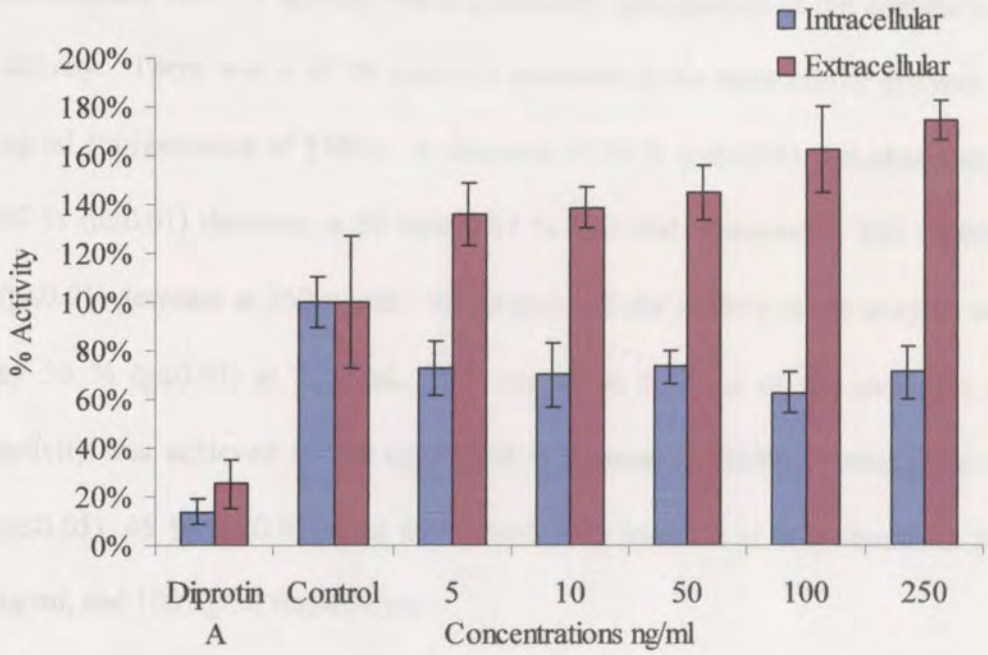


Figure 3.19: *% increase/decrease in intracellular and extracellular DPP IV activity in HRT 18 cells following 12 hrs stimulation with cytokines, at concentrations 5ng/ml, 10ng/ml 50ng/ml, 100ng/ml and 250ng/ml in PBS (0.3% BSA) media.* ***(a)*** *TNF α,* ***(b)*** *IL-1 β.(p values of intracellular and extracellular DPP IV activity are compared with the control in all the experiments).*

Treatment of HRT 18 cells with TNFα resulted in significant decrease in intracellular DPP IV activity and significant increase in its extracellular activity, at concentrations 5, 10, 50, 100, and 250 ng/ml. At 5 ng/ml, a 31 % ($p \leq 0.001$) decrease was observed in the enzyme's intracellular activity. 29 % ($p \leq 0.01$), 33 % ($p \leq 0.01$), 35 % ($p \leq 0.01$), and 39 % ($p \leq 0.01$) decrease was observed at concentrations 10, 50, 100, and 250 ng/ml respectively. Extracellular DPP IV activity was significantly increased at all concnetraions ($p \leq 0.001$). It was increased by ten fold at 5 ng/ml concentration of TNFα, while a six fold increase was observed at 10 ng/ml concentration. Treatment of HRT 18 cells with 50 and 100 ng/ml TNFα resulted in an eight fold increase in extracellular DPP IV activity, whereas a seven fold increase was observed at 250 ng/ml (figure 3.19, a).

Addition of IL-1 β to HRT 18 cells resulted in a significant decrease in intracellular DPP IV enzyme activity and a significant increase in the enzyme's extracellular activity at concentrations 5, 10, and 50ng/ml. However, at 100 ng/ml and 250 ng/ml there was no significant change in intracellular and extracellular DPP IV activity. TNFα showed to have a greater effect on extracellular DPP IV activity, increasing it to ten fold, while the maximum effect showed by IL-1 β was achieved at 10 ng/ml, increasing the enzyme activity five fold ($p \leq 0.01$). 24 % ($p \leq 0.05$), 31 % ($p \leq 0.05$), and 29 % ($p \leq 0.05$) decrease in intracellular DPP IV activity was achieved by 5, 10, and 50 ng/ml of IL-1 β respectively. Nevertheless, 4 fold increase in extracellular DPP IV activity was noted at 5 ng/ml ($p \leq 0.001$) and 50 ng/ml ($p \leq 0.01$) of IL-1β, and a five fold increase ($p \leq 0.01$) at 10 ng/ml. This data is represented as a bar graph in figure 3.19 (b). Diprotin A, inhibitor of DPP IV, reduced its activity by 89 % (intracellular) and 71 % (extracellular) ($p \leq 0.001$).

(a)



(b)

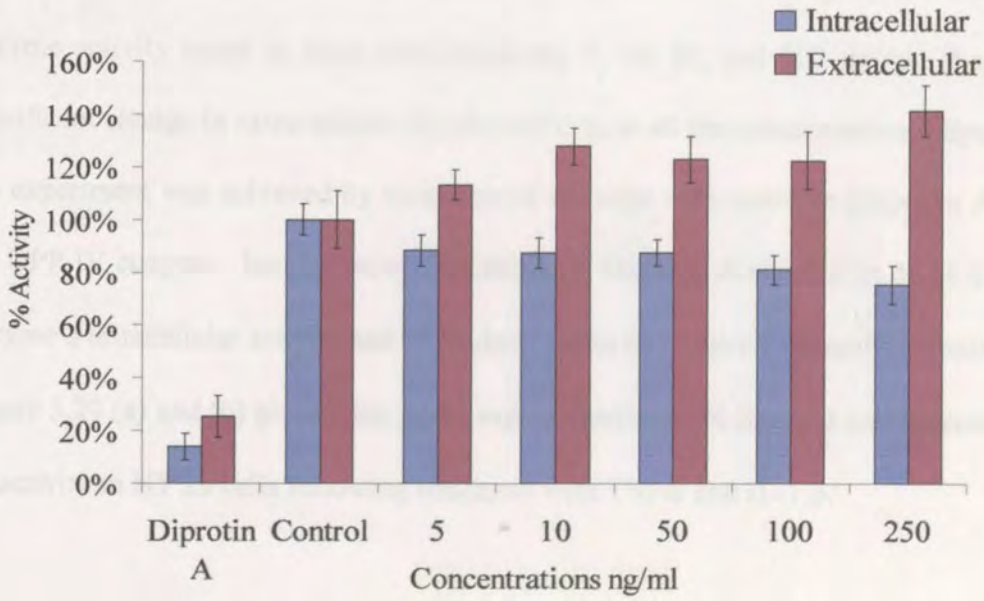


Figure 3.20: *% increase/decrease in intracellular and extracellular DPP IV activity in HT 29 cells following 42 hrs stimulation with cytokines, at concentrations 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml and 250ng/ml in RPMI media.* ***(a)*** *TNFα,* ***(b)*** *IL-1 β.*

Incubation of HT 29 cells with TNFα resulted in a significant downregulation of intracellular DPP IV activity and a significant upregulation of the enzyme's extracellular activity. There was a 27 % ($p \leq 0.01$) decrease in the intracellular enzyme activity at 5 ng/ml concentration of TNFα. A decrease of 30 % ($p \leq 0.001$) was observed at 10 ng/ml, 27 % ($p \leq 0.01$) decrease at 50 ng/ml, 37 % ($p \leq 0.001$) decrease at 100 ng/ml and a 29 % ($p \leq 0.01$) decrease at 250 ng/ml. While extracellular activity of the enzyme was increased by 36 % ($p \leq 0.05$) at 5 ng/ml. The maximum increase of the enzyme's extracellular activity was achieved at 250 ng/ml (74 % increase ($p \leq 0.001$), while there was a 39 % ($p \leq 0.05$), 45 % ($p \leq 0.01$), and 62 % ($p \leq 0.001$) increase at concentrations 10 ng/ml, 50 ng/ml, and 100 ng/ml respectively.

IL-1 β treatment of HT 29 cells resulted in a significant decrease of 25 % in intracellular DPP IV activity at 250 ng/ml ($p \leq 0.001$) whereas, there was no significant change in the enzyme activity noted at other concentrations, 5, 10, 50, and 100 ng/ml. There was no significant change in extracellular enzyme activity, at all the concentrations. Specificity of the experiment was achieved by treatment of the cells with inhibitor (Diprotin A) specific for DPP IV enzyme. Incubation of the cells with Diprotin A resulted in 86 % decrease in enzyme's intracellular activity and 75 % decrease in its extracellular activity ($p \leq 0.001$).

Figure 3.20 (a) and (b) gives a bar graph representation of % increase and decrease in DPP IV activity in HT 29 cells following treatment with TNFα and IL-1 β.

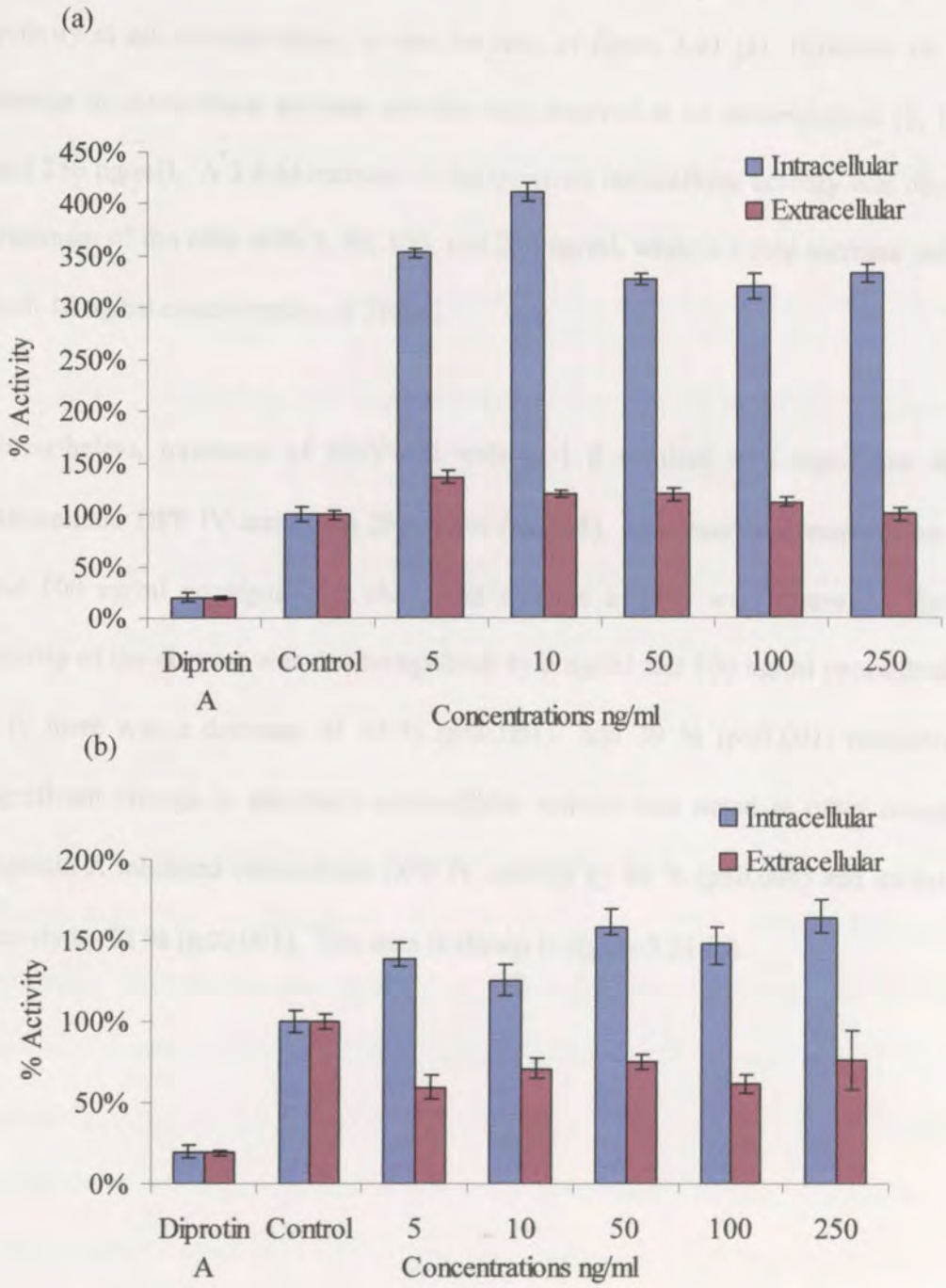


Figure 3.21: *% increase/decrease in intracellular and extracellular DPP IV activity in HUVECs following 48 hrs stimulation with cytokines, at concentrations 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml and 250ng/ml in Hanks (0.3% BSA) media.* ***(a)*** *TNF α,* ***(b)*** *IL-1 β.*

In HUVECs TNFα resulted a significant increase ($p \leq 0.001$) in intracellular DPP IV activity at all concentrations, as can be seen in figure 3.21 (a), However no significant change in extracellular enzyme activity was observed at all concentration (5, 10, 50, 100 and 250 ng/ml). A 2 fold increase in the enzymes intracellular activity was observed with treatment of the cells with 5, 50, 100, and 250 ng/ml, while a 3 fold increase was achieved with 10 ng/ml concentration of TNFα.

Nevertheless, treatment of HUVECs with IL-1 β resulted in a significant increase in intracellular DPP IV activity at 250 ng/ml ($p \leq 0.05$). However, at concentration 5, 10, 50, and 100 ng/ml no significant change in enzyme activity was observed. Extracellular activity of the enzyme was down-regulated by 5 ng/ml and 100 ng/ml concentration of IL-1 β, there was a decrease of 40 % ($p \leq 0.001$) and 39 % ($p \leq 0.001$) respectively. No significant change in enzyme's extracellular activity was noted at other concentrations. Diprotin A inhibited intracellular DPP IV activity by 80 % ($p \leq 0.001$) and its extracellular activity by 81 % ($p \leq 0.001$). The data is shown in figure 3.21 (b).

3.4 MTT assay to Measure Cell viability of HRT 18, HT 29, and HUVECs Subjected to Cytokines TNF α and IL-1 β.

Cells, HRT 18, HT 29 and HUVECs that had been treated with cytokines, TNFα and IL-1 β at concentrations 5 ng/ml, 10 ng/ml, 50 ng/ml, 100ng/ml, and 250 ng/ml, were subjected to MTT solution in order to determine their viability after the cytokine treatments.

3.4.1 MTT Assay for Cell Viability of HRT 18, HT 29, and HUVECs, treated with Cytokines, TNF α and IL-1 β at various concentrations.

The MTT test was carried out to investigate the effect of various concentrations of the two cytokines, TNFα and IL-1 β, on the two cancer cell lines and HUVECs.

Graphs below give an overview of % increase/or decrease in cell number, when exposed to TNFα and IL-1 β.

A standard curve was obtained for all the three cell lines under investigation, HRT 18, HT 29 and HUVECs, as described under section 2.5.2. The corresponding media and incubation time which was used in subsequent experiments with cathepsin B activity were obtained from activity experiments, as described in section 3.3.1 and 3.3.2.

Standard curves were performed in order to understand the cell viability of the cells studied i.e. the rate at which, or the time it took the cells to reach the stationary phase. Using the standard curve it could be deduced how long it takes for a cell line to grow or to proceed from one phase to the other.

As the number of cells increased, as shown in the standard curve, there was an increase in absorbance. Viability of the cells HRT 18 (figure 3.22 a, and 3.23 a) and HT 29 (figure 3.24 a, and 3.25 a) decreased at 300000 cells/flask, leading to a stationary phase, whereby

the cells did not continue to grow. This, was in contrast to HUVECs where the stationary phase was reached at 100000 cells/flask (3.36 a, and 3.37 a).

3.4.1.1 MTT assay for cell viability of HRT 18 cells under the influence of TNF α and IL-1 β

Fig. 3.22 (a)

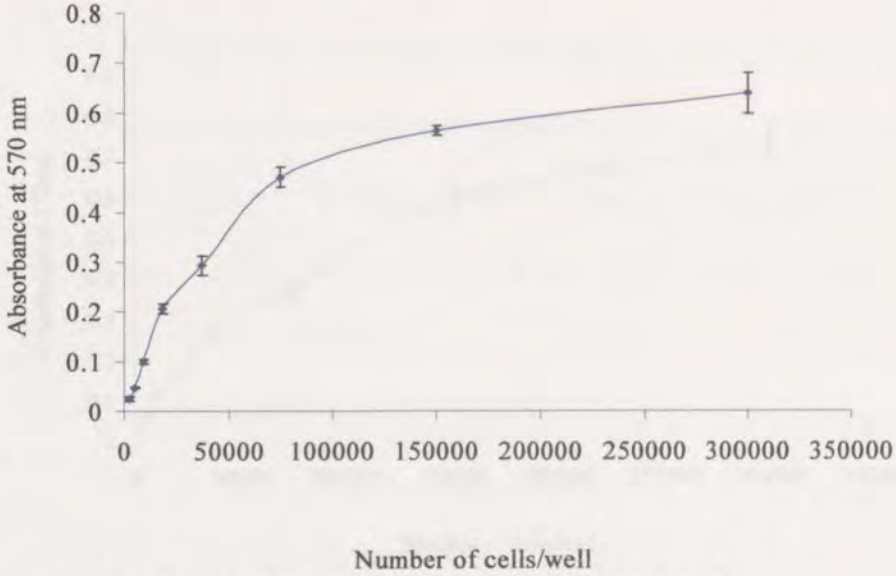


Figure 3.22 (a): *MTT assay for HRT 18 standard in MEM*

Fig. 3.22 (b)

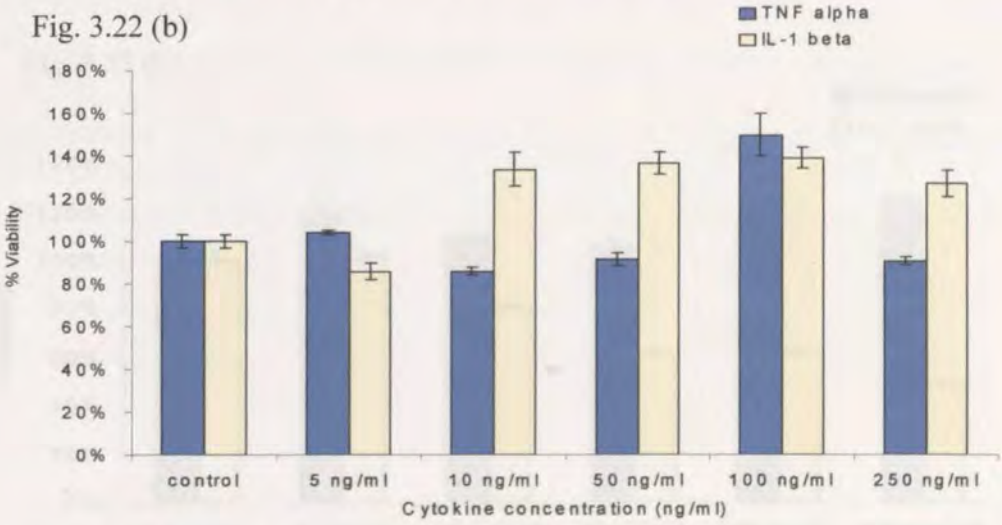


Figure 3.22 (b): *% viability of HRT 18 cells in MEM following 30 hour incubation with cytokines TNFα and IL-1 β, showing optimal conditions for cathepsin B activity (p values of different concentrations of TNF α and IL-1 β are compared with the control in all the experiments).*

Cytokine Conc. μg/ml	0	5	10	50	100	250
% viability TNF α	100	4↑	14↓	9↓	49↑	10↓
% viability IL-1 β	100	14↓	33↑	37↑	39↑	27↑

Table 3.1: *The % (↑) increase/decrease (↓) in cell viability calculated for HRT 18 cells, treated with TNF α and IL-1 β*

Fig. 3.23 (a)

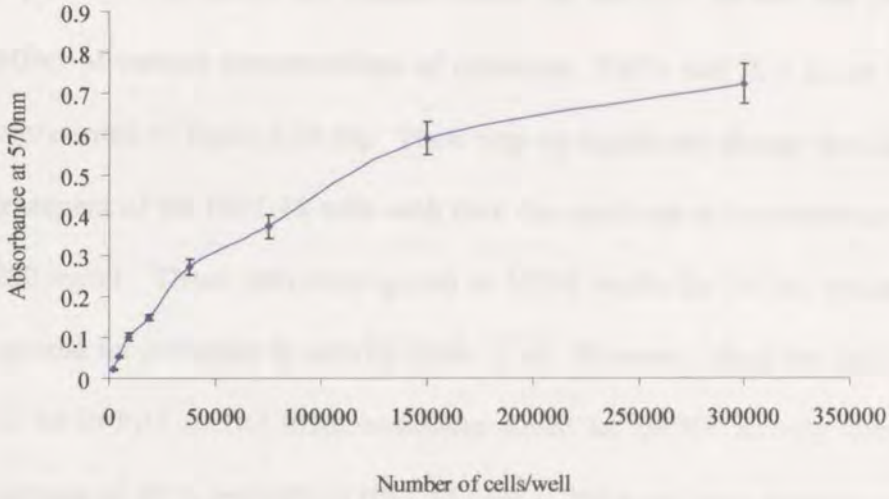


Figure 3.23 (a): *MTT assay for HRT 18 cells standard in PBS (0.3% BSA)*

Fig. 3.23 (b)

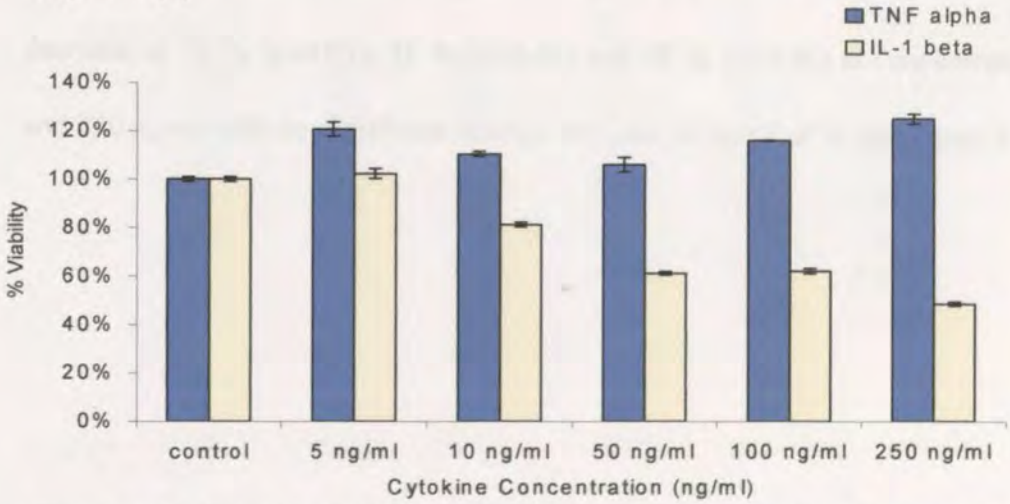


Figure 3.23 (b): *% viability of HRT 18 cells in PBS following 12 hour incubation with cytokines TNFα and IL-1 β, showing optimal conditions for DPP IV activity.*

Cytokine Conc. μg/ml	0	5	10	50	100	250
% viability TNF α	100	21↑	10↑	6↑	16↑	25↑
% viability IL-1 β	100	2↑	19↓	39↓	38↓	52↓

Table 3.2: *The % increase/decrease in cell viability calculated for HRT 18 cells, treated with TNF α and IL-1 β, at 12 hour incubation in PBS.*

Figure 3.22 (a) shows the standard curve for the HRT 18 cell line in MEM media. The effect of various concentrations of cytokines, TNFα and IL-1 β, on the HRT 18 cells is represented in figure 3.22 (b). There was no significant change in cell viability following treatment of the HRT 18 cells with both the cytokines at concentrations 5,10, 50, 100,and 250 ng/ml. These cells were grown in MEM media for 30 hrs, which are the conditions optimal for cathepsin B activity (table 3, a). However, when the cells were incubated for 12 hrs in PBS (0.3%) BSA, conditions suited for DPPIV activity, there was a significant increase of 25 % ($p \leq 0.05$) in HRT 18 cells at 250 ng/ml concentration of TNFα. However, there was no significant change in cell viability at concentration of 5, 10, 50, and 100 ng/ml. Whereas, IL-1 β had a significant decrease in viable cell population. There was a decrease of 39 % ($p \leq 0.01$), 38 % ($p \leq 0.01$) and 52 % ($p \leq 0.05$) at concentrations 50, 100, and 250 ng/ml with no significant change, at 5 and 10 ng/ml of IL-1β (figure 3.23, b).

3.4.1.2 MTT assay for cell viability of HT 29 cells under the influence of TNF α and IL-1 β

Fig. 3.24 (a)

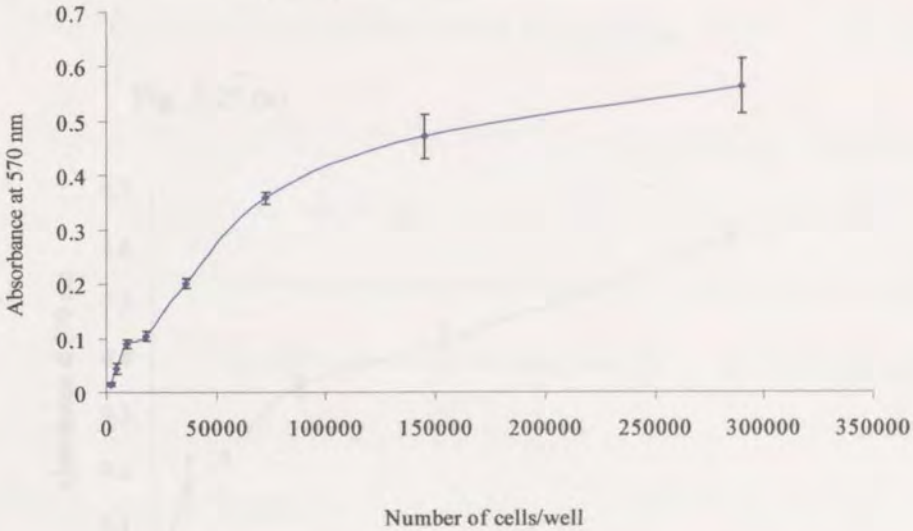


Figure 3.24 (a): *MTT assay for HT 29 cells standard in MEM media*

Fig. 3.24 (b)

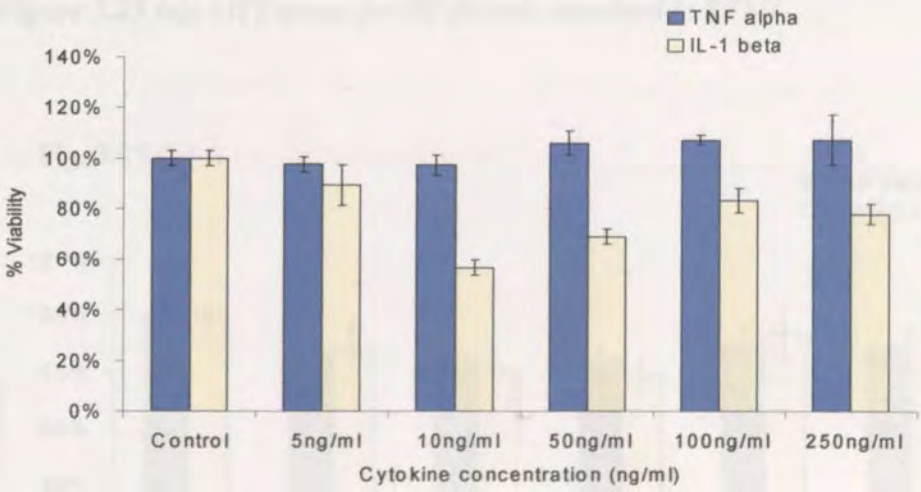


Figure 3.24 (b): *% viability of HT 29 in MEM following 18 hour incubation with cytokines TNFα and IL-1 β, showing optimal conditions for cathepsin B activity.*

Cytokine Conc. μg/ml	0	5	10	50	100	250
% viability TNF α	100	2↓	3↓	6↑	7↑	7↑
% viability IL-1 β	100	11↓	43↓	31↓	17↓	22↓

Table 3.3: *The % increase/decrease in cell viability calculated for HT 29 cells, treated with TNF α and IL-1 β, for 18 hours in MEM media.*

Fig. 3.25 (a)

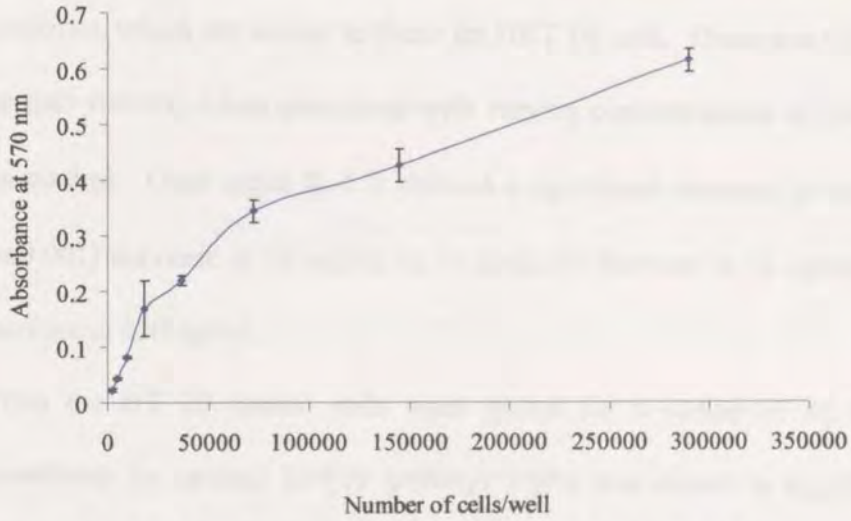


Figure 3.25 (a): *MTT assay for HT 29 cells standard in RPMI*

Fig. 3.25 (b)

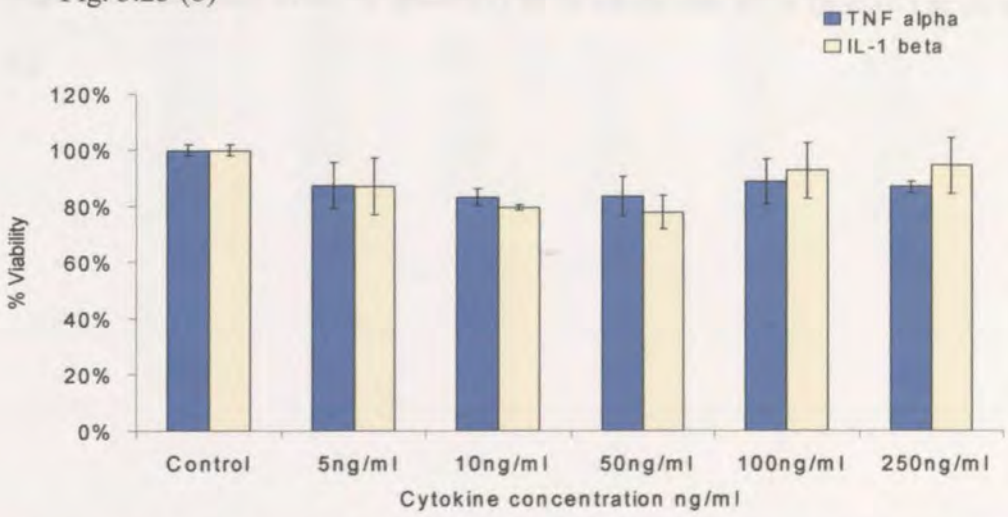


Figure 3.25 (b): *% viability of HT 29 cells in RPMI following 42 hour incubation with cytokines TNFα and IL-1 β, showing optimal conditions for DPP IV activity.*

Cytokine Conc. μg/ml	0	5	10	50	100	250
% viability TNF α	100	13↓	17↓	17↓	12↓	13↓
% viability IL-1 β	100	13↓	21↓	22↓	8↓	6↓

Table 3.4: *The % increase/decrease in cell viability calculated for HT 29 cells, treated with TNF α and IL-1 β, for42 hours in RPMI media.*

Figure 3.24 (b) shows the results for HT 29 cells following 18 hr stimulation with the two cytokines, which are similar to those for HRT 18 cells. There was no significant change in the cell viability when stimulated with varying concentrations of TNFα in comparison to the control. Once again IL-1 β showed a significant decrease in viable cells, with 43 % ($p \leq 0.001$) decrease at 10 ng/ml, 31 % ($p \leq 0.01$) decrease at 50 ng/ml, and 22 % ($p \leq 0.05$) decrease at 250 ng/ml.

When the HT 29 treated cells were grown for a period of 42 hrs in RPMI media (conditions for optimal DPPIV activity) TNFα was shown to significantly decrease cell viability, at concentrations, 10, 50, and 250ng/ml by 17 % ($p \leq 0.01$), 17 % ($p \leq 0.05$), and 13 % ($p \leq 0.01$) respectively. IL-1 β was also shown to decrease the cell number. There was a significant decrease of 21 % ($p \leq 0.001$) at 10 ng/ml and 22 % ($p \leq 0.01$) at 50 ng/ml (3.25, b).

3.4.1.3 MTT assay for cell viability HUVECs under the influence of TNF α and IL-1 β

Fig. 3.26 (a)

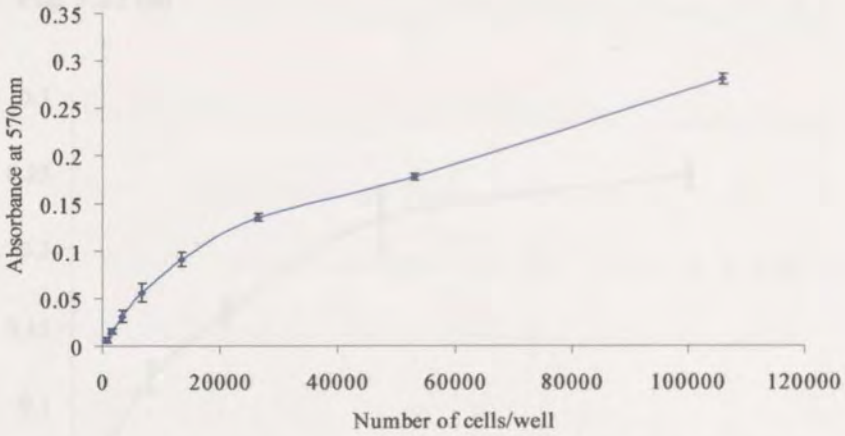


Figure 3.26 (a): *MTT assay for HUVECs standard in MEM media*

Fig. 3.26 (b)

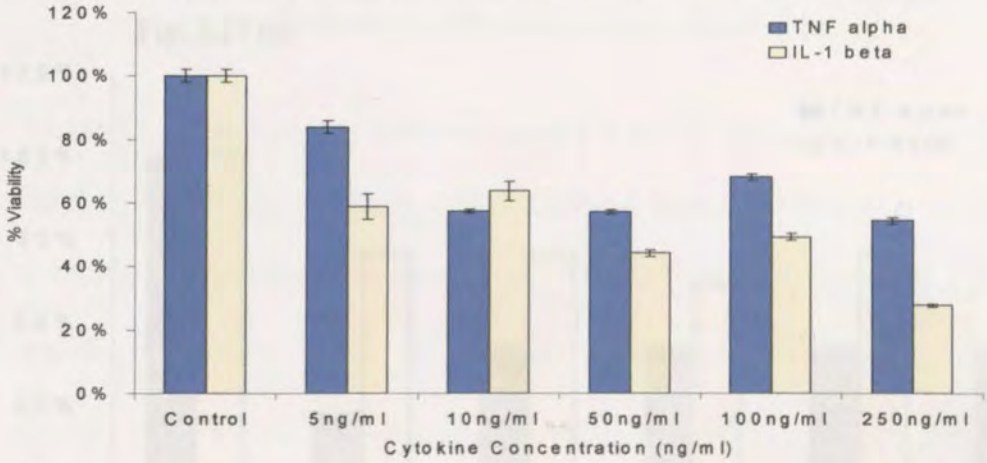


Figure 3.26 (b): *% viability of HUVECs in MEM following 12 hour incubation with cytokines TNF α and IL-1 β, showing optimal conditions for cathepsin B activity.*

Cytokine Conc. μg/ml	0	5	10	50	100	250
% viability TNF α	100	16↓	43↓	43↓	32↓	46↓
% viability IL-1 β	100	41↓	36↓	56↓	51↓	72↓

Table 3.5: *The % increase/decrease in cell viability calculated for HUVECs, treated with TNF α and IL-1 ß. for 12 hour in MEM media.*

Fig. 3.27 (a)

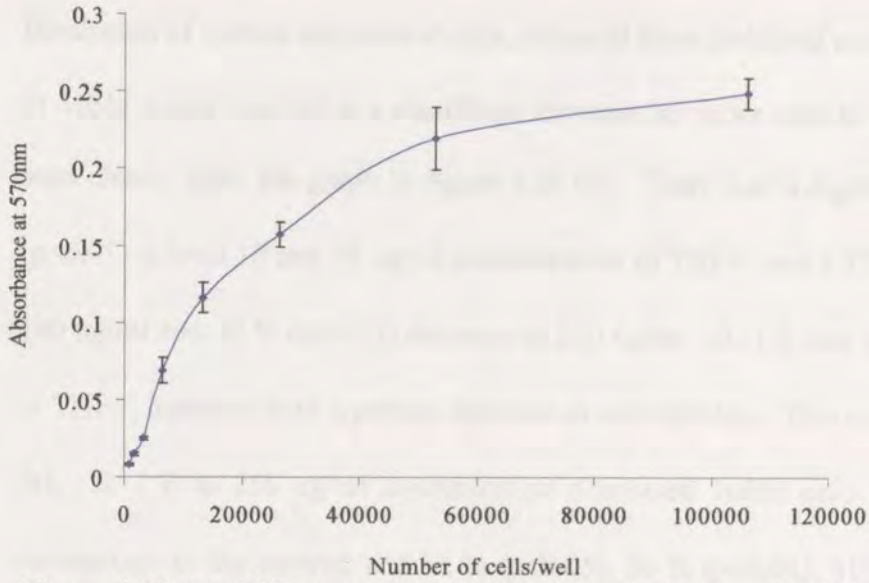


Figure 3.27 (a): *MTT assay HUVECs standard in Hanks with 0.3% BSA*

Fig. 3.27 (b)

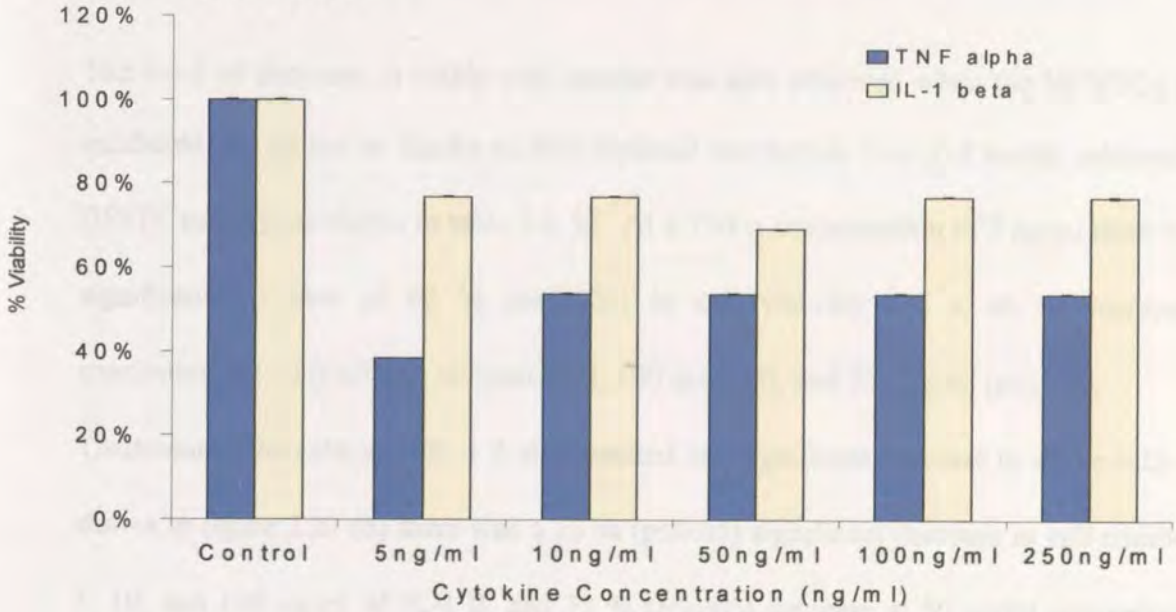


Figure 3.27 (b): *% viability of HUVECs in Hanks (0.3% BSA) following 48 hour incubation with cytokines TNFα and IL-1 β, showing optimal conditions for DPP IV activity.*

Cytokine Conc. μg/ml	0	5	10	50	100	250
% survival TNF α	100	62↓	46↓	46↓	46↓	46↓
% survival IL-1 β	100	23↓	23↓	31↓	23↓	23↓

Table 3.6: *The % increase/decrease in cell viability calculated for endothelial (HUVECs) cells, treated with TNFα and IL-1 β, for 48 hours in Hanks (0.3 % BSA).*

Incubation of normal endothelial cells, obtained from umbilical cord, with TNFα for 12 hrs in MEM media resulted in a significant decrease in viable cells in the culture. This can be seen clearly from the graph in figure 3.26 (b). There was a significant decrease of 43 % ($p \leq 0.01$) at both 10 and 50 ng/ml concentration of TNFα, and a 32 % ($p \leq 0.05$) decrease at 100 ng/ml and 46 % ($p \leq 0.01$) decrease at 250 ng/ml. IL-1 β also showed a similar pattern to TNFα, however with a greater decrease in cell viability. This can be seen in figure 3.26 (b). IL-1 β at 250 ng/ml concentration decreased viable cells by 72 % ($p \leq 0.001$) in comparison to the control, and 36 % ($p \leq 0.05$), 56 % ($p \leq 0.01$), 51 % ($p \leq 0.01$) decrease in cell number at concentrations 10, 50, and 100 ng/ml respectively.

The trend of decrease in viable cell number was also observed when the HUVECs were incubated for 48 hrs in Hanks (0.3%) (optimal incubation time and media achieved for DPPIV activity, as shown in table 3.0, b). At a TNFα concentration of 5 ng/ml there was a significant decrease of 62 % ($p \leq 0.001$) in cell viability and a 46 % decrease at concentrations 10 ($p \leq 0.01$), 50 ($p \leq 0.001$), 100 ($p \leq 0.05$), and 250 ng/ml ($p \leq 0.01$).

Treatment of the cells with IL-1 β also resulted in a significant decrease in viable cells. As shown in figure 3.27 (b) there was a 23 % ($p \leq 0.05$) significant decrease in cell number at 5, 10, and 100 ng/ml of IL-1 β, and 31 % ($p \leq 0.05$) decrease at 50 ng/ml concentration. However, no significant change was observed at 250 ng/ml concentration of IL-1β.

3.5 ELISA (Enzyme Linked Immunoabsorbent Assay)

Following MTT assay, which examined the viability of HRT 18, HT 29 and HUVECs upon treatment with TNFα and IL-1β, ELISA experiments were performed in order to determine cathepsin B and DPP IV protein content following treatment with the cytokines, named above. The sandwich ELISA measured the amount of antigen (for cathepsin B and DPP IV) between two layers of antibodies.

A standard curve was obtained by preparing and assaying a set of known samples at range of concentrations. Following which, samples with unknown protein concentration were assayed, then for each measurement obtained, the standard curve was used to find the corresponding substance concentration which would determine the protein concentration of the proteases. A software package (Microplate Manager Bio-Rad Laboratories, Inc) was employed, that created the standard curve and interpolated unknown concentrations of protein of choice from it. The graphs representing standard curves can be seen below (figure 3.28 – figure 3.31).

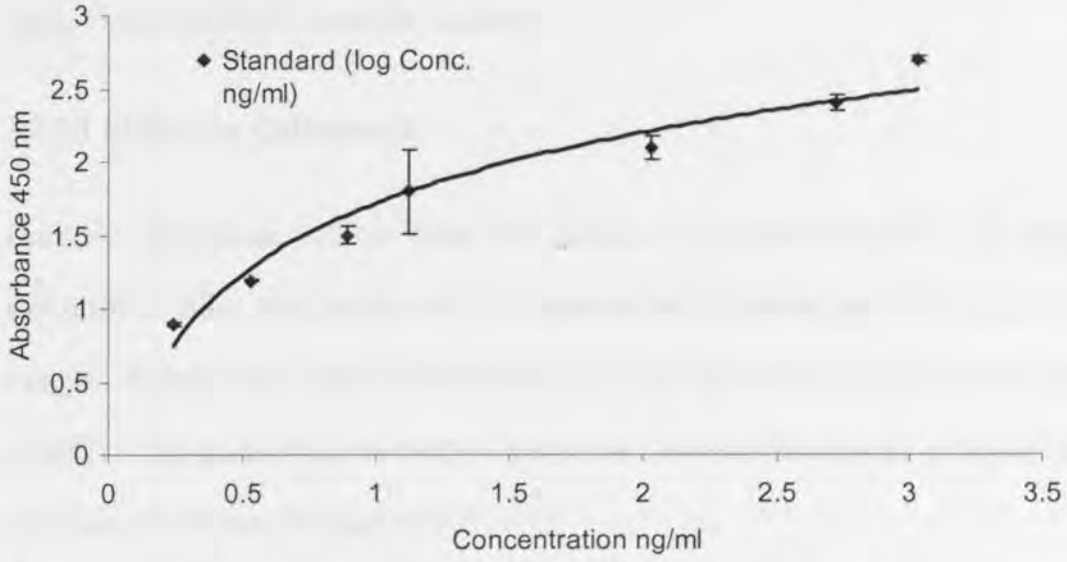


Figure 3.28: *Graph representing ELISA standard curve for cathepsin B to determine the appropriate dilution.*

3.5.1 Determination of Dilution of Cathepsin B Protein for ELISA

From the values obtained by plotting the standard curve for cathepsin B (figure 3.28), the concentration of cathepsin B protein in the sample, that was isolated using Tri reagent, was calculated. A series of dilutions were prepared (1/1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64), to determine the dilution range appropriate for determining the protease concentration. The dilution that was suitable for determination of cathepsin B protein from the samples was 1/2. This dilution was used to assay the presence of cathepsin B protein in cell lines, HRT 18, HT 29 and HUVECs, that had been subjected to 5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml and 250 ng/ml of TNFα and IL-1 β, and also in liver cells (as positive control). At 1/2 dilution the concentration of cathepsin B protein was 237.696 ng/ml in intracellular HRT 18 control cells, 241.856 ng/ml in intracellular HT 29 control cells and 173.324 in intracellular HUVECs control cells. In HRT 18 extracellular control cells the protein concentration at 1/2 dilution was 0.002 ng/ml, in HT 29 extracellular control cells was 0.191 ng/ml, and in HUVECs control cells was 0.209 ng/ml. The protein concentrations for the other dilutions of the samples are listed in appendix 5. All the experiments were carried out in triplicate to ensure accuracy.

3.5.1.1 ELISA for Cathepsin B

Once the appropriate dilution factor was obtained for protein sample to be assayed for cathepsin B, Elisa was carried out to determine the concentration of cathepsin B in the samples derived from cell lines HRT 18, HT 29, HUVECs, and liver cells (positive control). The standard curve used to determine the concentration the cathepsin B in the cell lines can be seen in figure 3.29.

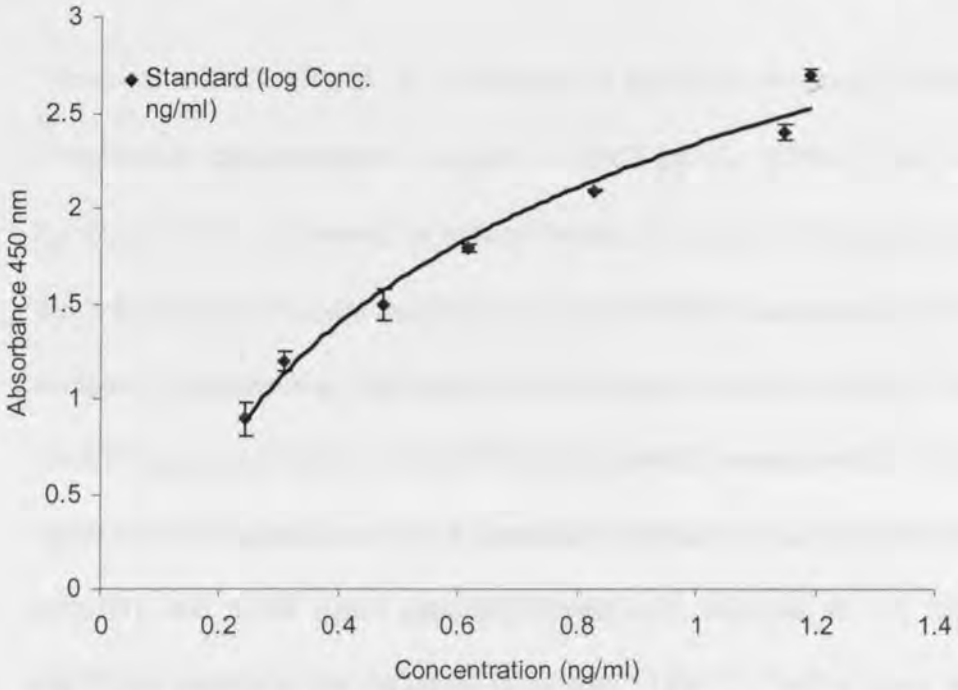


Figure 3.29: *Logarithmic graph representing standard curve for cathepsin B to determine the protein concentration in HRT 18, HT 29, HUVECs, and Liver cells.*

3.5.1.1.1Determination of Cathepsin B Protein Concentration in HRT 18 Cells, Following Stimulation with TNF α and IL-1 β

Following treatment of HRT cells with cytokine TNFα, there was a significant increase in intracellular cathepsin B protein (measured by ELISA). The highest intracellular cathepsin B concentration (210.988 ng/ml, $p \leq 0.001$) was achieved at 10 ng/ml of TNFα, while at 5 ng/ml, 50 ng/ml, 100 ng/ml and 250 ng/ml the concentration of cathepsin B was 189.090 ng/ml ($p \leq 0.001$), 168.987 ng/ml ($p \leq 0.01$), 148.246 ng/ml ($p \leq 0.05$) and 152.980 ng/ml ($p \leq 0.01$) respectively. This was in comparison to control cells where the concentration of cathepsin B was measured to be 140.145 ng/ml. Extracellular cathepsin B activity in HRT 18 cells following 5 ng/ml TNFα resulted in a significant increase in cathepsin B protein (12.999 ng/ml, $p \leq 0.01$) in comparison to control cells (9.054 ng/ml), however at 10 ng/ml, 50 ng/ml and 100 ng/ml there was no significant change in cathepsin B protein. 250 ng/ml of TNFα resulted in an increase in extracellular cathepsin B protein (12.176 ng/ml, ($p \leq 0.01$).

Treatment of HRT 18 with IL-1 β resulted in significant increase in intracellular cathepsin B protein at concentrations 5 ng/ml (288.987 ng/ml, $p \leq 0.001$) and 10 ng/ml (195.960 ng/ml, $p \leq 0.001$). However, at concentrations 50 ng/ml, 100 ng/ml and 250 ng/ml there was a significant decrease ($p \leq 0.001$) in the intracellular cathepsin B protein. Extracellular cathepsin B protein was also significantly elevated at concentrations 5 ng/ml and 10 ng/ml (14.879 ng/ml, $p \leq 0.001$, and 28.897 ng/ml, $p \leq 0.001$ respectively). At concentrations 50 ng/ml and 250 ng/ml there was a significant decrease in cathepsin B protein, 8.196 ng/ml ($p \leq 0.05$), and 8.188 ng/ml ($p \leq 0.05$) respectively, whereas at 100 ng/ml there was no significant change in the cathepsin B protein. Table 3.7 below gives an overview of the intracellular and extracellualar cathepsin B protein concentration upon treatment of HRT 18 cells with various concentrations of TNFα and IL-1 β.

	Intracellular Cathepsin B in HRT 18 cells	Extracellular Cathepsin B in HRT 18 cells		Intracellular Cathepsin B in HRT 18 cells	Extracellular Cathepsin B in HRT 18 cells
Dilution 1/2	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ½	Average Concentration ng/ml	Average Concentration ng/ml
Control	140.145	9.054	Control	140.145	9.054
TNFα 5ng/ml	189.090	12.999	IL-1β 5ng/ml	288.987	14.879
TNFα 10ng/ml	210.988	9.087	IL-1β 10ng/ml	195.960	28.897
TNFα 50ng/ml	168.987	9	IL1β 50ng/ml	102.321	8.196
TNFα 100ng/ml	148.246	10	IL-1β 100ng/ml	108.679	9.097
TNFα 250ng/ml	152.980	12.176	IL-1β 250ng/ml	90.066	8.188

Table 3.7: *Intracellular and extracellular cathepsin B protein in HRT 18 cells following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

3.5.1.1.2Determination of Cathepsin B Protein Concentration in HT 29 Cells, Following Stimulation with TNF α and IL-1 β

Treatment of HT 29 cells with TNFα showed a completely opposite pattern to treatment of HRT 18 cells with the cytokine, since the treatment of the former resulted in a significant decrease in intracellular cathepsin B protein. Compared to control cells, where the cathepsin B protein concentration was 587.649 ng/ml, the concentrations at 5, 10, 50, 100 and 250 ng/ml was 223.788 ng/ml, 212.951 ng/ml, 291.585 ng/ml, 288.065 ng/ml, and 178.564 ng/ml respectively ($p \leq 0.001$). However, the extracellular cathepsin B protein concentration upon treatment with the cytokine was significantly increased. At 5 ng/ml TNFα the protein concentration was measured to be 18.128 ng/ml ($p \leq 0.001$), at 10 ng/ml it was 14.295 ng/ml ($p \leq 0.001$), at 50 ng/ml it was 13.375 ng/ml ($p \leq 0.001$), at 100 ng/ml it was 13.980 ng/ml ($p \leq 0.001$), and at 250 ng/ml the protein concentration was 10.539 ng/ml ($p \leq 0.05$).

Treatment of HT 29 cells with IL-1 β also resulted in a significant decrease ($p \leq 0.001$) in intracellular cathepsin B protein. Cathepsin B protein concentration at 5, 10, 50, 100, and 250 ng/ml was noted to be 299.543 ng/ml, 268.673 ng/ml, 279.089 ng/ml, 288.090 ng/ml, and 288.989 ng/ml respectively. Intracellular cathepsin B concentration in control cells was 587.649 ng/ml. Extracellular cathepsin B protein concentration at 5 ng/ml and 10 ng/ml IL-1 β was measured to be 16.902 ng/ml ($p \leq 0.001$) and 28.906 ng/ml ($p \leq 0.001$) respectively. However, at 50, 100, and 250 ng/ml no significant change in protein concentration was noted. Cathepsin B protein concentration in extracellular control cells was noted to be 9.688 ng/ml.

Table 3.8 gives an overview of the intracellular and extracellualar cathepsin B protein concentration upon treatment of HT 29 cells with various concentrations of TNFα and IL-1 β.

	Intracellular Cathepsin B in HT 29 cells	Extracellular Cathepsin B in HT 29 cells		Intracellular Cathepsin B in HT 29 cells	Extracellular Cathepsin B in HT 29 cells
Dilution 1/2	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ½	Average Concentration ng/ml	Average Concentration ng/ml
Control	587.649	9.688	Control	587.649	9.688
TNFα 5ng/ml	223.788	18.128	IL-1β 5ng/ml	299.543	16.902
TNFα 10ng/ml	212.951	14.295	IL-1β 10ng/ml	268.673	28.906
TNFα 50ng/ml	291.585	13.375	IL1β 50ng/ml	279.089	9.962
TNFα 100ng/ml	288.065	13.980	IL-1β 100ng/ml	288.090	9.998
TNFα 250ng/ml	178.564	10.539	IL-1β 250ng/ml	288.989	9.264

Table 3.8: *Intracellular and extracellular cathepsin B protein in HT 29 cells following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

3.5.1.1.3Determination of Cathepsin B Protein Concentration in HUVECs, Following Stimulation with TNF α and IL-1 β

In HUVECs, following treatment with TNFα, there was a significant decrease in intracellular cathepsin B protein concentration. At 5, 10, 50, 100, and 250 ng/ml the protein concentration obtained upon treatment with TNFα was 124.720 ng/ml, 130.658 ng/ml, 124.365 ng/ml, 128.076 ng/ml, and 120.705 ng/ml respectively ($p \leq 0.001$), while the protein concentration in control cells was 164.342 ng/ml. However, extracellular cathepsin B protein was significantly increased ($p \leq 0.001$) upon treatment with the cytokine. There was a dramatic increase in protein concentration, at 5 ng/ml TNFα, the cathepsin protein concentration was measured to be 1309.927 ng/ml, at 10 ng/ml it was 1429.089 ng/ml, at 50 ng/ml it was 1492.004 ng/ml, at 100 ng/ml it was 1289.382 ng/ml and at 250 ng/ml it was 1210.732 ng/ml, this was in comparison to the cathepsin B protein concentration in control endothelial cells (380.739 ng/ml).

	Intracellular Cathepsin B in HT 29 cells	Extracellular Cathepsin B in HT 29 cells		Intracellular Cathepsin B in HT 29 cells	Extracellular Cathepsin B in HT 29 cells
Dilution 1/2	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ½	Average Concentration ng/ml	Average Concentration ng/ml
Control	587.649	9.688	Control	587.649	9.688
TNFα 5ng/ml	223.788	18.128	IL-1β 5ng/ml	299.543	16.902
TNFα 10ng/ml	212.951	14.295	IL-1β 10ng/ml	268.673	28.906
TNFα 50ng/ml	291.585	13.375	IL1β 50ng/ml	279.089	9.962
TNFα 100ng/ml	288.065	13.980	IL-1β 100ng/ml	288.090	9.998
TNFα 250ng/ml	178.564	10.539	IL-1β 250ng/ml	288.989	9.264

Table 3.8: *Intracellular and extracellular cathepsin B protein in HT 29 cells following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

3.5.1.1.3Determination of Cathepsin B Protein Concentration in HUVECs, Following Stimulation with TNF α and IL-1 β

In HUVECs, following treatment with TNFα, there was a significant decrease in intracellular cathepsin B protein concentration. At 5, 10, 50, 100, and 250 ng/ml the protein concentration obtained upon treatment with TNFα was 124.720 ng/ml, 130.658 ng/ml, 124.365 ng/ml, 128.076 ng/ml, and 120.705 ng/ml respectively ($p \leq 0.001$), while the protein concentration in control cells was 164.342 ng/ml. However, extracellular cathepsin B protein was significantly increased ($p \leq 0.001$) upon treatment with the cytokine. There was a dramatic increase in protein concentration, at 5 ng/ml TNFα, the cathepsin protein concentration was measured to be 1309.927 ng/ml, at 10 ng/ml it was 1429.089 ng/ml, at 50 ng/ml it was 1492.004 ng/ml, at 100 ng/ml it was 1289.382 ng/ml and at 250 ng/ml it was 1210.732 ng/ml, this was in comparison to the cathepsin B protein concentration in control endothelial cells (380.739 ng/ml).

Treatment of HUVECs with IL-1 β showed similar results as shown with TNFα, since these also resulted in a significant decrease ($p \leq 0.001$) in intracellular cathepsin B protein concentration and a significant increase ($p \leq 0.001$) in its extracellular concentration. In comparison to intracellular cathepsin B protein concentration, 164.342 ng/ml, in control cells, the concentration of cathepsin B protein following 5 ng /ml treatment with IL-1 β was 119.674 ng/ml. At concentrations 10 ng/ml, 50 ng/ml, 100 ng/ml and 250 ng/ml the intracellular cathepsin B concentration was measured to be 128.793 ng/ml, 120.466 ng/ml, 132.921 ng/ml, and 128.447 ng/ml respectively. Extracellular protein concentration in control cells was 380.739 ng/ml, however following treatment with 5 ng/ml IL-1β the concentration was increased to 1300.743 ng/ml, at 10 ng/ml to 1294.092 ng/ml, at 50 ng/ml to 1827.945 ng/ml, at 100 ng/ml to 1328.002 ng/ml and at 250 ng/ml the concentration was increased to 1828.092 ng/ml. The results can be seen summarised in table 3.9.

	Intracellular Cathepsin B in HUVECs	Extracellular Cathepsin B in HUVECs		Intracellular Cathepsin B in HUVECs	Extracellular Cathepsin B in HUVECs
Dilution ½	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ½	Average Concentration ng/ml	Average Concentration ng/ml
Control	164.342	380.739	Control	164.342	380.739
TNFα 5ng/ml	124.720	1309.927	IL-1β 5ng/ml	119.674	1300.743
TNFα 10ng/ml	130.658	1429.089	IL-1β 10ng/ml	128.793	1294.092
TNFα 50ng/ml	124.365	1492.004	IL1β 50ng/ml	120.466	1827.945
TNFα 100ng/ml	128.076	1289.382	IL-1β 100ng/ml	132.921	1328.002
TNFα 250ng/ml	120.705	1210.732	IL-1β 250ng/ml	128.447	1828.092

Table 3.9: *Intracellular and extracellular cathepsin B protein in HUVECs following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

Liver cells were used as positive control in these experiments. The intracellular cathepsin B protein concentration, determined by Elisa, was measured to be 292.681 ng/ml, while extracellular protein concentration was 399.621 ng/ml.

	Intracellular Cathepsin B in Liver cells	Extracellular Cathepsin B in Liver cells
Dilution ½	Average Concentration ng/ml	Average Concentration ng/ml
Control	292.681	399.621

Table 3.10: *Intracellular and extracellular cathepsin B protein in Liver cells.*

3.5.2 Determination of Dilution of DPP IV Protein for ELISA

As with cathepsin B protease it was necessary to obtain a standard curve to evaluate the dilution factor that was to be used for further identifying the best dilution factor for establishing protein content of DPP IV in samples that were obtained from cell lines following treatment with the cytokines. After Elisa was carried out on various dilutions of samples, the best suited dilution factor was noted to be 1/4. At this dilution factor the concentration of DPP IV in intracellular control cells in HRT 18 cells was 1014.097 ng/ml, in intracellular HT 29 cells it was 668.386 ng/ml, and in intracellular HUVECs control cells 27.461 ng/ml. However, in extracellular HRT 18 control cells the concentration of DPP IV was 61.126 ng/ml, in extracellular HT 29 control cells concentration of DPP IV was 81.843 ng/ml and in HUVECs the concentration was 0.271 ng/ml.

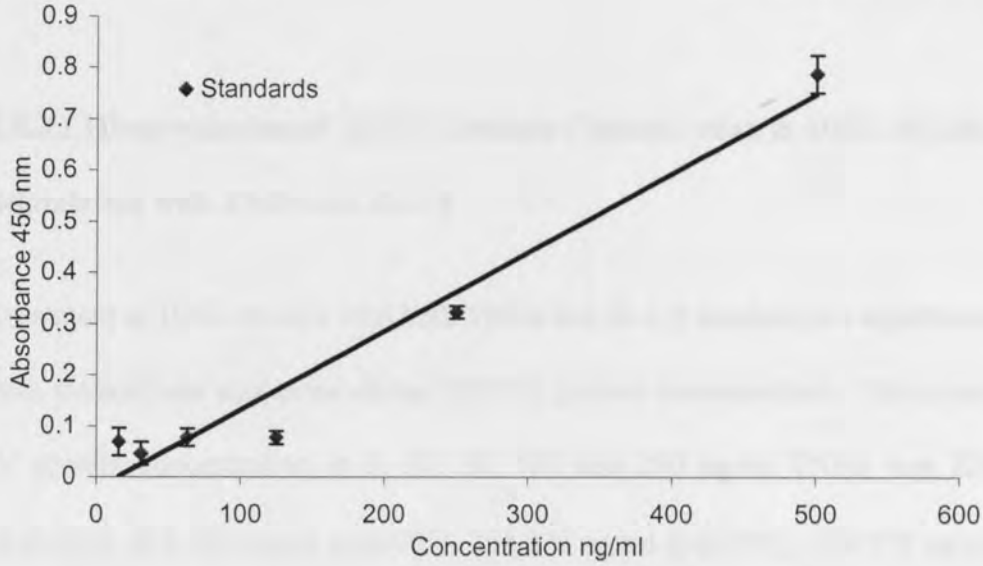


Figure 3.30: *Linear graph representing standard curve for DPP IV to determine the appropriate dilution factor.*

3.5.2.1 ELISA for DPP IV

Once the appropriate dilution factor was obtained for protein sample to be assayed for DPP IV, ELISA was carried out to determine the concentration of DPP IV in the samples derived from cell lines HRT 18, HT 29, HUVECs, and thyroid cells (positive control). The standard curve used to determine the concentration the DPP IV in the cell lines can be seen in figure 3.31.

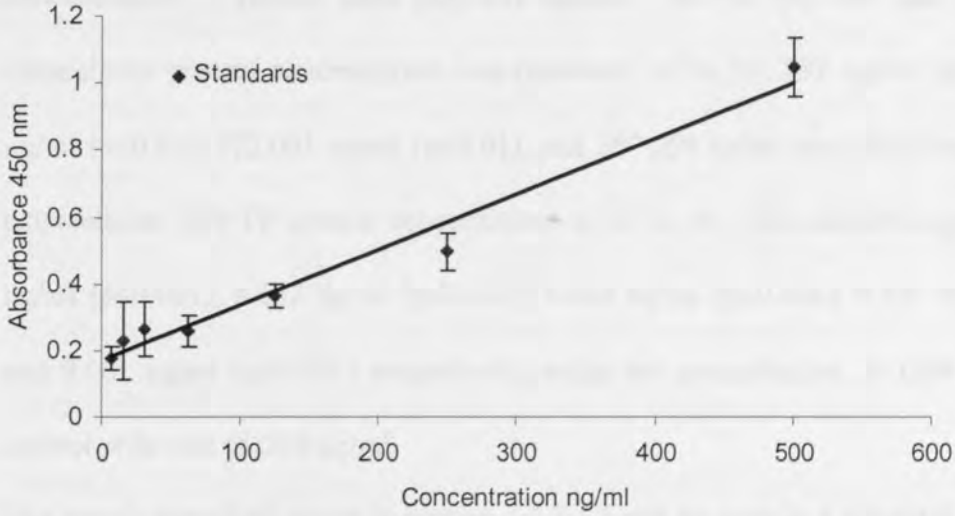


Figure 3.31: *Linear graph representing standard curve for DPP IV to determine the protein concentration in HRT 18, HT 29, HUVECs, and Thyroid cells.*

3.5.2.1.1Determination of DPP IV Protein Concentration in HRT 18 Cells, Following Stimulation with TNFα and IL-1 β

Treatment of HRT 18 cells with both TNFα and IL-1 β resulted in a significant decrease in both intracellular and extracellular DPP IV protein concentration. The intracellular DPP IV protein concentration at 5, 10, 50, 100 and 250 ng/ml TNFα was 326.960 ng/ml ($p \leq 0.001$), 354.026 ng/ml ($p \leq 0.001$), 334.272 ng/ml ($p \leq 0.001$), 324.979 ng/ml ($p \leq 0.001$), and 384.235 ng/ml ($p \leq 0.001$) respectively. Intracellular DPP IV concentration in HRT 18 control cells was measured to be 438.211 ng/ml.

At 5, 10, 50, 100, and 250 ng/ml the extracellular DPP IV protein concentration was 42.057 ng/ml ($p \leq 0.001$), 30.076 ng/ml ($p \leq 0.001$), 40.186 ng/ml ($p \leq 0.001$), 42.079 ng/ml ($p \leq 0.001$), and 9.079 ng/ml ($p \leq 0.001$) respectively, while extracellular protein concentration in control cells was 65.254 ng/ml.

Treatment of HRT 18 cells with 5 ng/ml IL-1 β resulted in a decrease in intracellular DPP IV protein concentration, (380.829 ng/ml, $p \leq 0.01$) compared to an intracellular protein concentration in control cells (438.211 ng/ml). At 10, 50, 100 and 250 ng/ml, the intracellular protein concentration was measured to be 390.832 ng/ml ($p \leq 0.01$), 380.127 ng/ml ($p \leq 0.01$), 322.091 ng/ml ($p \leq 0.01$), and 387.059 ng/ml ($p \leq 0.001$) respectively. The extracellular DPP IV protein concentration at 5, 10, 50, 100, and 250 ng/ml was 10.893 ng/ml ($p \leq 0.001$), 8.032 ng/ml ($p \leq 0.001$), 9.004 ng/ml ($p \leq 0.001$), 9.587 ng/ml ($p \leq 0.001$), and 9.001 ng/ml ($p \leq 0.001$) respectively, while the concentration of DPP IV in HRT 18 control cells was 65.254 ng/ml.

The results described above in section 3.5.2.1.1 can be seen in a tabulated format in table 3.11.

	Intracellular DPP IV in HRT 18 cells	Extracellular DPP IV in HRT 18 cells		Intracellular DPP IV in HRT 18 cells	Extracellular DPP IV in HRT 18 cells
Dilution 1/4	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ¼	Average Concentration ng/ml	Average Concentration ng/ml
Control	438.211	65.254	Control	438.211	65.254
TNFα 5ng/ml	326.960	42.057	IL-1β 5ng/ml	380.829	10.893
TNFα 10ng/ml	354.026	30.076	IL-1β 10ng/ml	390.832	8.032
TNFα 50ng/ml	334.272	40.186	IL1β 50ng/ml	380.127	9.004
TNFα 100ng/ml	324.979	42.079	IL-1β 100ng/ml	322.091	9.587
TNFα 250ng/ml	384.235	9.079	IL-1β 250ng/ml	387.059	9.001

Table 3.11: *Intracellular and extracellular DPP IV protein in HRT 18 following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

3.5.2.1.2Determination of DPP IV Protein Concentration in HT 29 Cells, Following Stimulation with TNF α and IL-1 β

In TNFα treated HT 29 cells a significant decrease in intracellular DPP IV protein concentration and a significant increase in extracellular protein concentration was noted. In comparison to control HT 29 cells, where the intracellular DPP IV concentration was 530.533 ng/ml, treatment with TNFα at 5ng/ml concentration resulted in a decrease in DPP IV protein concentration, 322.908 ng/ml ($p \leq 0.001$), at concentrations 10, 50, 100 and 250 ng/ml DPP IV concentration measured was 301.870 ng/ml ($p \leq 0.001$), 315.318 ng/ml ($p \leq 0.001$), 322.089 ng/ml ($p \leq 0.001$), and 302. 244 ng/ml ($p \leq 0.001$) respectively. Extracellular DPP IV protein concentration following treatment with TNF α was 51.029 ng/ml ($p \leq 0.05$) at 5 ng/ml, 50.632 ng/ml ($p \leq 0.05$) at 10 ng/ml, 49.969 ng/ml ($p \leq 0.05$) at 50

ng/ml, 50.852 ng/ml ($p \leq 0.05$) at 100 ng/ml, and 49.673 ng/ml ($p \leq 0.05$) at 250 ng/ml. The results above are summarised in table 3.12.

When HT 29 cells were subjected to IL-1β, there was a significant decrease in both intracellular and extracellular DPP IV protein concentration, compared to protein concentration in control cells, where intracellular concentration was 530.533 ng/ml and extracellular concentration was 44.466 ng/ml (table 3.12). Intracellular DPP IV protein concentration at 5 ng/ml IL-1 β was 328.723 ng/ml ($p \leq 0.001$), at 10 ng/ml it was 302.847 ng/ml ($p \leq 0.001$), at 50 ng/ml was 311.650 ng/ml ($p \leq 0.001$), at 100 ng/ml was 310.980 ng/ml ($p \leq 0.001$), and at 250 ng/ml was 220.277 ng/ml ($p \leq 0.001$).

At 5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, and 250 ng/ml IL-1 β extracellular DPP IV protein concentration noted was 13.099 ng/ml, 14.055 ng/ml, 14.507 ng/ml, 14.546 ng/ml, and 13.010 ng/ml respectively ($p \leq 0.001$).

	Intracellular DPP IV in HT 29 cells	Extracellular DPP IV in HT 29 cells		Intracellular DPP IV in HT 29 cells	Extracellular DPP IV in HT 29 cells
Dilution 1/4	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ¼	Average Concentration ng/ml	Average Concentration ng/ml
Control	530.533	44.466	Control	530.533	44.466
TNFα 5ng/ml	322.908	51.029	IL-1β 5ng/ml	328.723	13.099
TNFα 10ng/ml	301.870	50.632	IL-1β 10ng/ml	302.847	14.055
TNFα 50ng/ml	315.318	49.969	IL1β 50ng/ml	311.650	14.507
TNFα 100ng/ml	322.089	50.852	IL-1β 100ng/ml	310.980	14.546
TNFα 250ng/ml	302.244	49.673	IL-1β 250ng/ml	220.277	13.010

Table 3.12: *Intracellular and extracellular DPP IV protein in HT 29 following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

3.5.2.1.3Determination of DPP IV Protein Concentration in HUVECs, Following Stimulation with TNF α and IL-1 β

A significant decrease ($p \leq 0.001$) in intracellular and extracellular DPP IV protein concentration was observed in HUVECs following incubation with TNFα and IL-1 β. Incubation of endothelial cells with TNFα at 5 ng/ml resulted in decrease in intracellular DPP IV protein concentration, which was measured to be 10.989 ng/ml. At 10 ng/ml, 100 ng/ml, and 250 ng/ml the protein concentration was 8.002 ng/ml, 10.587 ng/ml, and 22.989 ng/ml respectively, while at 50 ng/ml no protein concentration was detected. In control cells the intracellular DPP IV protein concentration was 59.751 ng/ml. Extracellular DPP IV protein concentration in TNFα treated endothelial cells, at 5 ng/ml, was 32.900 ng/ml, at 10 ng/ml was 24.090 ng/ml, at 50 ng/ml was 24.289 ng/ml, at 100 ng/ml was 24.845 ng/ml and at 250 ng/ml was 24.043 ng/ml, comparison to DPP IV protein concentration in endothelial control cells which was 86.653 ng/ml.

As with TNFα, a decrease in both intracellular and extracellular DPP IV protein concentration was obtained when endothelial cells where subjected to treatment with IL-1 β. In endothelial control cells the intracellular DPP IV protein concentration measured was 59.751 ng/ml, while treatment of endothelial cells with IL-1 β at 5, 10, 50, 100 and 250 ng/ml resulted in intracellular DPP IV protein concentration of 30.986 ng/ml, 20.622 ng/ml, 28.569 ng/ml, 29.529 ng/ml and 20.002 ng/ml respectively. Furthermore, extracellular DPP IV protein concentration following treatment of endothelial with 5, 10, 50, 100 and 250 ng/ml of IL-1 β, was measured to be 39.967 ng/ml, 36.019 ng/ml, 36.518 ng/ml, 35.332 ng/ml and 36.376 ng/ml respectively.

This was in comparison to the extracellular DPP IV concentration in endothelial cells (86.653 ng/ml).

The results obtained, following treatment of HUVECs with various concentrations of TNFα and IL-1 β can be seen in a tabulated format in table 3.13.

	Intracellular DPP IV in HUVECs	Extracellular DPP IV in HUVECs		Intracellular DPP IV in HUVECs	Extracellular DPP IV in HUVECs
Dilution 1/4	Average Concentration ng/ml	Average Concentration ng/ml	Dilution ¼	Average Concentration ng/ml	Average Concentration ng/ml
Control	59.751	86.653	Control	59.751	86.653
TNFα 5ng/ml	10.989	32.900	IL-1β 5ng/ml	30.986	39.967
TNFα 10ng/ml	8.002	24.090	IL-1β 10ng/ml	20.622	36.019
TNFα 50ng/ml	-17.897	24.289	IL1β 50ng/ml	28.569	36.518
TNFα 100ng/ml	10.587	24.845	IL-1β 100ng/ml	29.529	35.332
TNFα 250ng/ml	22.989	24.043	IL-1β 250ng/ml	20.002	36.376

Table 3.13: *Intracellular and extracellular DPP IV protein in HUVECs following treatment with cytokines, TNFα and IL-1 β at 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, and 250ng/ml.*

In thyroid cells (positive control) the intracellular DPP IV protein concentration was 462.288 ng/ml, and extracellular protein concentration was 152.568 ng/ml.

	Intracellular DPP IV in Thyroid cells	Extracellular DPP IV in Thyroid cells
Dilution ¼	Average Concentratio n ng/ml	Average Concentration ng/ml
Control	462.288	152.568

Table 3.14: *Intracellular and extracellular DPP IV protein in Thyroid cells.*

3.6 Inhibitor Assay

Inhibitors Mu-Phe-Hph-FMK, for cathepsin B enzyme, and Gly-Pro-Gly-Gly, for DPP IV enzyme, was used in order to assess their ability/extent to inhibit the enzyme activity. Varying concentrations of the two inhibitors were used in order to assess the concentration optimal for enzyme inhibition.

3.6.1 Cathepsin B Inhibition by Mu-Phe-Hph-FMK

Cell lines HRT 18, HT 29, and HUVECs were incubated with varying concentrations, 10 μM, 40 μM, 80 μM, and 100 μM, of cathepsin B inhibitor Mu-Phe-Hph-FMK. The three cell lines were incubated with the inhibitor for 15 minutes and 30 minutes, to determine the optimal time for the inhibition of cathepsin B enzyme. The inhibitor assay was carried out in triplicate to ensure accuracy.

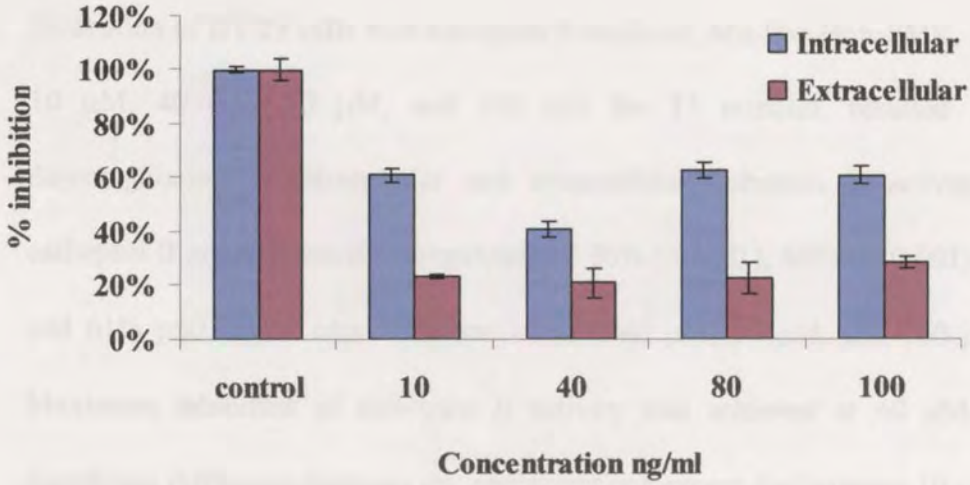
3.6.1.1 Inhibition of Cathepsin B activity by Mu-Phe-Hph-FMK in HRT 18 cells

Once HRT 18 cells grown on 96 well plates were confluent, the cells were treated with 10 μM, 40 μM, 80 μM, and 100 μM Mu-Phe-Hph-FMK inhibitor. Inhibition of cathepsin B was measured with a fluorometer after 15 minutes and 30 minutes of treatment with the inhibitor. A significant downregulation in intracellular and extracellular cathepsin B activity was noted in cells treated with 10 μM, 40 μM, 80 μM, and 100 μM Mu-Phe-Hph-FMK inhibitor for 15 minutes, this can be seen in figure 3.32 (a) . At 10 μM and 100 μM, Mu-Phe-Hph-FMK inhibited intracellular cathepsin B activity by 39% ($p \leq 0.001$ and $p \leq 0.01$ respectively), while at 80 μM 38% ($p \leq 0.01$) inhibition was noted. Maximum inhibition of intracellular cathepsin B activity was noted at 40 μM (59% $p \leq 0.001$).

However, extracellular cathepsin B activity at 10 μM, 40 μM, 80 μM, and 100 μM was downregulated by 77%, 79%, 78%, and 72% respectively. Significance of the results were $p \leq 0.01$ for all concentrations.

Nevertheless, treatment of HRT 18 cells with cathepsin B inhibitor, Mu-Phe-Hph-FMK, for 30 minutes resulted in a significant downregulation in intracellular and extracellular cathepsin B activity (figure 3.32, b). The activity was significantly lower then that achieved with incubation of the cells for 15 minutes with the inhibitor. Intracellular cathepsin B activity was reduced by 63%, 66%, 57%, and 45% at 10 μM, 40 μM, 80 μM, and 100 μM inhibitor concentrations ($p \leq 0.01$ at all concentrations). There was a significant difference between all concentrations except for between 10 μM and 40 μM, and 10 μM and 80 μM. Furthermore, extracellular cathepsin B activity upon treatment with the cathepsin B inhibitor was downregulated by 90% at 10 μM inhibitor concentration and 89% at 40 μM and 80 μM, and 88% at 100 μM ($p \leq 0.001$ at all concentrations). However, no significant difference was observed between the inhibitor concentrations.

(a)



(b)

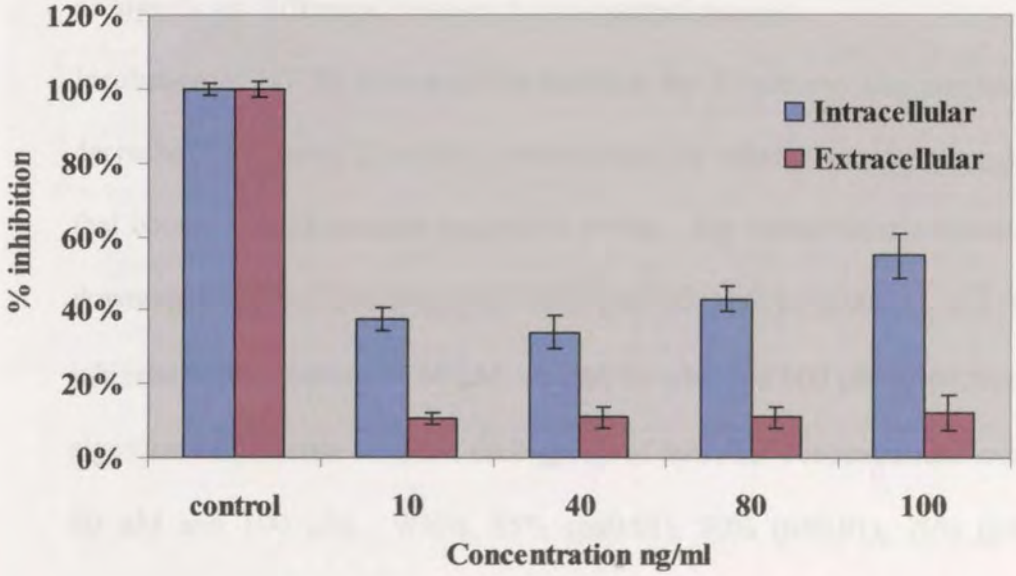


***Figure 3.32**:- % inhibition of intracellular and extracellular cathepsin B activity in HRT 18 cells, following incubation with cathepsin B inhibitor, FMK. (a) 15 minute incubation (b) 30 minute incubation.*

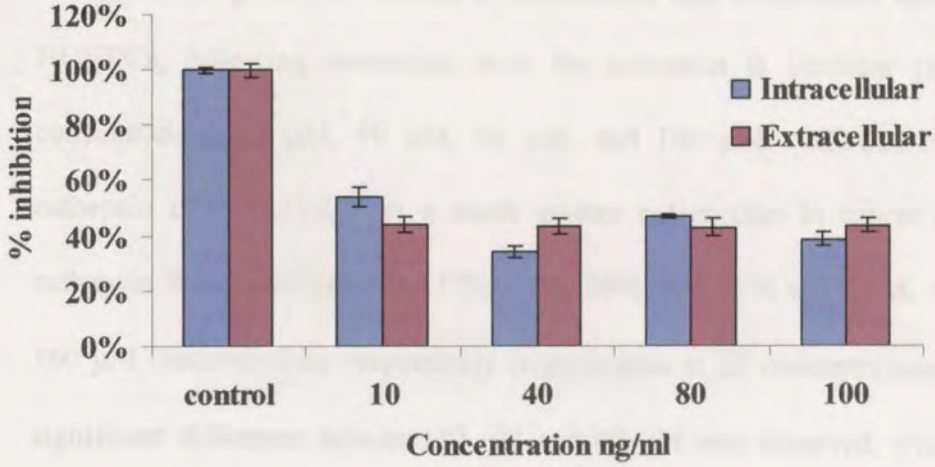
3.6.1.2 Inhibition of Cathepsin B activity by Mu-Phe-Hph-FMK in HT 29 cells

Incubation of HT 29 cells with cathepsin B inhibitor, Mu-Phe-Hph-FMK, at concentrations 10 μM, 40 μM, 80 μM, and 100 μM for 15 minutes, resulted in a significant downregulation in intracellular and extracellular cathepsin B activity. Intracellular cathepsin B activity was downregulated by 46% ($p \leq 0.01$), 66% ($p \leq 0.001$), 53% ($p \leq 0.001$) and 61% $p \leq 0.01$), at concentrations 10 μM, 40 μM, 80 μM, and 100 μM respectively. Maximum inhibition of cathepsin B activity was achieved at 40 μM. There was a significant difference between the concentrations except for between 10 μM and 100 μM. Extracellular cathepsin B activity at 10 μM and 100 μM was downregulated by 56% and at 40 μM and 80 μM, 57 % decrease in cathepsin B activity was noted. However there was no significant difference between the concentrations used.

Incubation of HT 29 cells with the inhibitor for 30 minutes also resulted in a significant decrease in cathepsin B activity, nevertheless the activity was downregualted further than that obtained at 15 minutes incubation period. The intracellular cathepsin B activity was downregulated by 75% ($p \leq 0.01$), 79% ($p \leq 0.001$), 60% ($p \leq 0.01$), and 71% ($p \leq 0.01$) at inhibitor concentrations of 10 μM, 40 μM, 80 μM, and 100 μM respectively. There was a significant difference between each group of inhibitor concentrations except for between 80 μM and 100 μM. While, 85% ($p \leq 0.01$), 80% ($p \leq 0.01$), 76% ($p \leq 0.01$) and 80% ($p \leq 0.001$) decrease in the extracellular enzyme activity was noted at 10 μM, 40 μM, 80 μM, and 100 μM respectively. Significant difference between inhibitor concentrations was only noted between 10 μM and 40 μM, and between 10 μM and 80 μM.

The data above is represented in figure 3.33 (a) and (b).

(a)



(b)

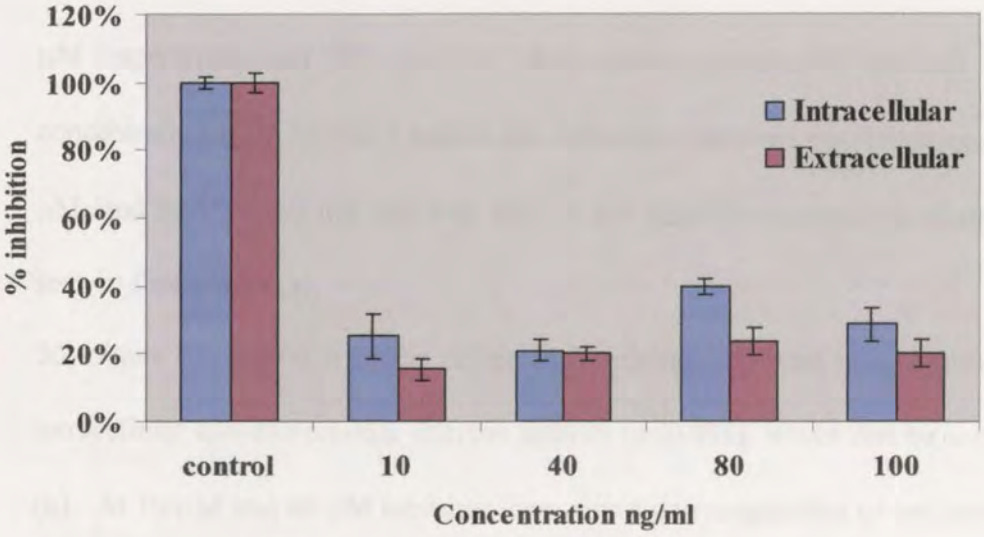


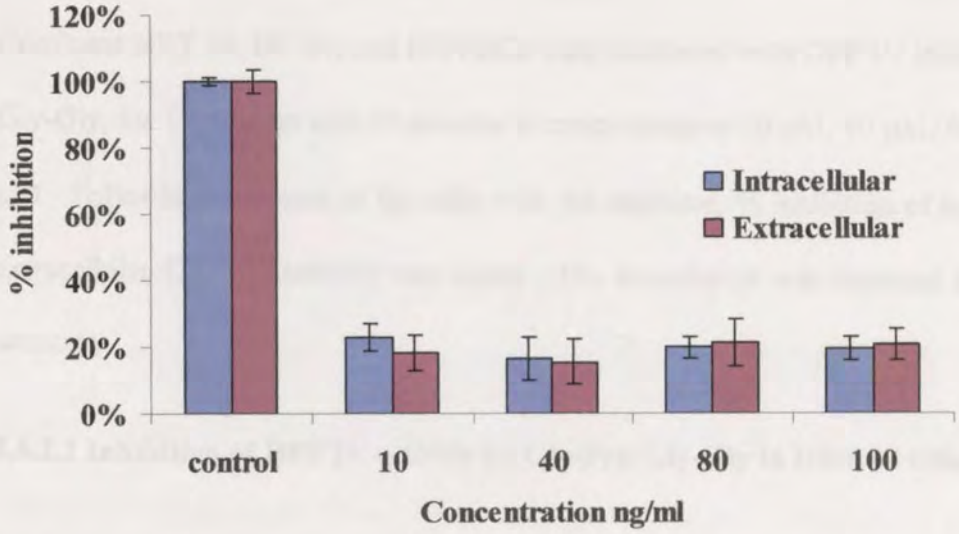
***Figure 3.33**:- % inhibition of intracellular and extracellular cathepsin B activity in HT 29 cells, following incubation with cathepsin B inhibitor, FMK. (a) 15 minute incubation (b) 30 minute incubation.*

3.6.1.3 Inhibition of Cathepsin B activity by Mu-Phe-Hph-FMK in HUVECs

There was a significant decrease in intracellular and extracellular cathepsin B activity in HUVECs, following incubation with the cathepsin B inhibitor (for 15 minutes), at concentrations 10 μM, 40 μM, 80 μM, and 100 μM. Mu-Phe-Hph-FMK, inhibited cathepsin B in HUVECs at a much greater extent than in cancer cells. Intracellular cathepsin B was decreased by 77%, 84%, 80%, and 81% at 10 μM, 40 μM, 80 μM, and 100 μM concentrations respectively (significance at all concentrations was $p \leq 0.001$). A significant difference between 10 μM and 40 μM was observed, whereas no significant difference was seen between the other concentrations. Extracellular cathespin B activity was downregulated by 82% ($p \leq 0.01$) and 85% ($p \leq 0.001$) at concentrations 10 μM and 40 μM respectively, and 79% ($p \leq 0.001$) decrease was noted at 80 μM and 100μM inhibitor concentration. There was a significant difference between concentrations 40 μM and 80 μM, and between 40 μM and 100 μM. A bar graph representation of above data can be seen in figure 3.34 (a).

30 minute incubation with the cathepsin B inhibitor resulted in a significant decrease in intracellular and extracelluar enzyme activity ($p \leq 0.001$), which can be seen in figure 3.34 (b). At 10 μM and 40 μM inhibitor there was a downregulation of cathepsin B activity by 93%, at 80 μM enzyme activated was reduced by 92%, and at 100 μM by 91%. There was a significant difference between inhibitor concentration of 10 μM and 100 μM, 40 μM and 100 μM, and between 80 μM and 100 μM. Extracellular cathepsin B activity in HUVECs at 10 μM and 40 μM was downregulated by 93%. Whereas, % decrease in the enzyme activity at 80 μM and 100 μM inhibitor concentration was 89% and 86%. There was a significant difference between the different inhibitor concentrations except for between 10 μM and 40 μM.

(a)



(b)

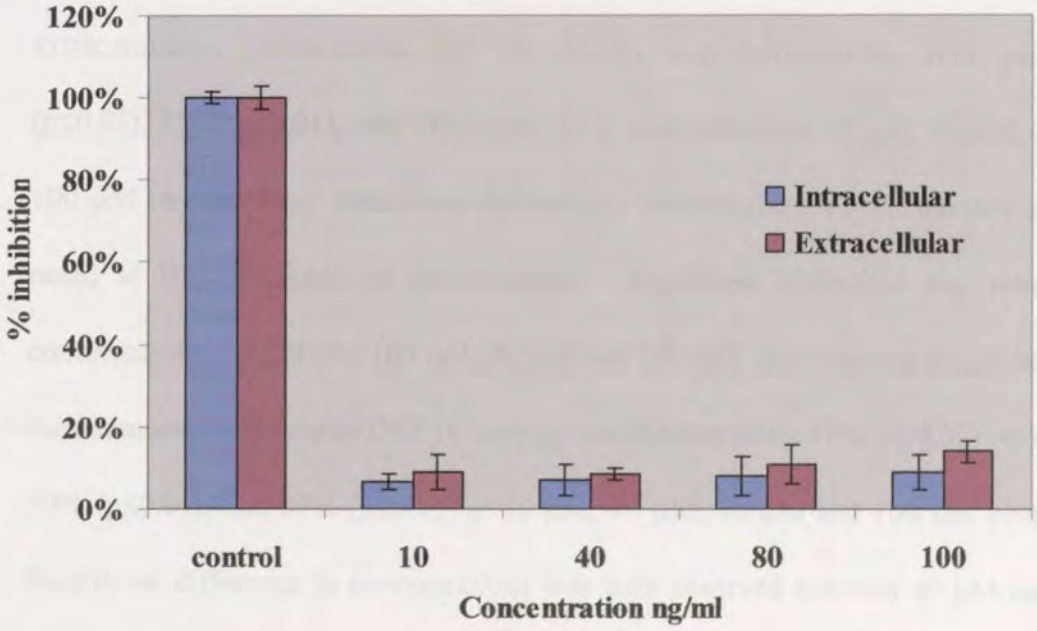


***Figure 3.34**:- % inhibition of intracellular and extracellular cathepsin B activity in HUVECs, following incubation with cathepsin B inhibitor, FMK. (a) 15 minute incubation (b) 30 minute incubation.*

3.6.2 DPP IV Inhibition by Gly-Pro-Gly-Gly

Confluent HRT 18, HT 29, and HUVECs were incubated with DPP IV inhibitor, Gly-Pro-Gly-Gly, for 15 minutes and 30 minutes at concentrations 10 μM, 40 μM, 80 μM, and 100 μM. Following treatment of the cells with the inhibitor, % inhibition of intracellular and extracellular DPP IV activity was noted. The experiment was repeated three times for accuracy.

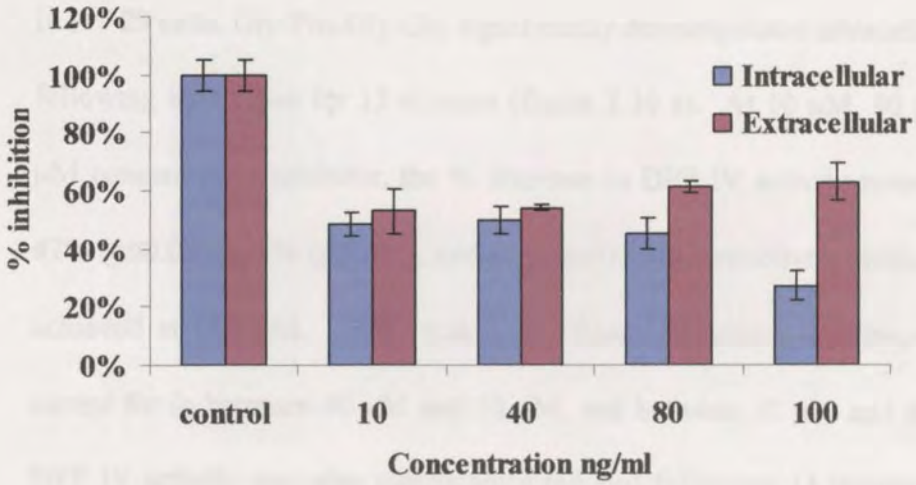
3.6.2.1 Inhibition of DPP IV activity by Gly-Pro-Gly-Gly in HRT 18 cells

Incubation of HRT 18 cells for 15 minutes with DPP IV inhibitor, Gly-Pro-Gly-Gly, resulted in a significant downregulation in enzymes activity both intracellularly and extracellularly. Intracellular DPP IV activity was decreased by 51% ($p \leq 0.05$), 50% ($p \leq 0.05$), 55% ($p \leq 0.01$), and 73% ($p \leq 0.01$) at concentrations 10 μM, 40 μM, 80 μM, and 100 μM respectively. Maximum decrease in intracellular DPP IV enzyme activity was noted at 100 μM inhibitor concentration. Significant difference was noted between concentrations 10 μM and 100 μM, 40 μM and 100 μM, and between 80 μM and 100 μM. Furthermore, extracellular DPP IV activity was decreased by 47% ($p \leq 0.05$), 46% ($p \leq 0.05$), 39% ($p \leq 0.01$), and 37% ($p \leq 0.05$) at 10 μM, 40 μM, 80 μM and 100 μM concentrations. Significant difference in concentrations was only observed between 10 μM and 100 μM, and 40 μM and 80 μM. The above data can be seen represented as a bar graph in figure 3.35 (a).

30 minute incubation of HRT 18 cells resulted in a significant decrease in intracellular and extracellular DPP IV activity, as can be seen in figure 3.35 (b). This decrease in activity was greater then that achieved by incubating the cells for a period of 15 minutes with the inhibitor.

There was 80%, 77%, 81%, and 83% inhibition of intracellular DPP IV activity in HRT 18 cells following incubation with 10 μM, 40 μM, 80 μM and 100 μM concentration of Gly-Pro-Gly-Gly respectively (significance at all concentrations was $p \leq 0.01$). Significant difference between inhibitor concentrations was observed between 10 μM and 40 μM, 10 μM and 100 μM, and 40 μM and 100 μM. Extracellular DPP IV activity following treatment with the inhibitor was downregulated by 69% ($p \leq 0.01$), 60% ($p \leq 0.01$), 57% ($p \leq 0.001$), and 52% ($p \leq 0.01$) at concentrations 10 μM, 40 μM, 80 μM, and 100 μM respectively. Significant difference between concentrations was observed between 10 μM and 40 μM, and in between 10 μM and 100 μM.

(a)



(b)

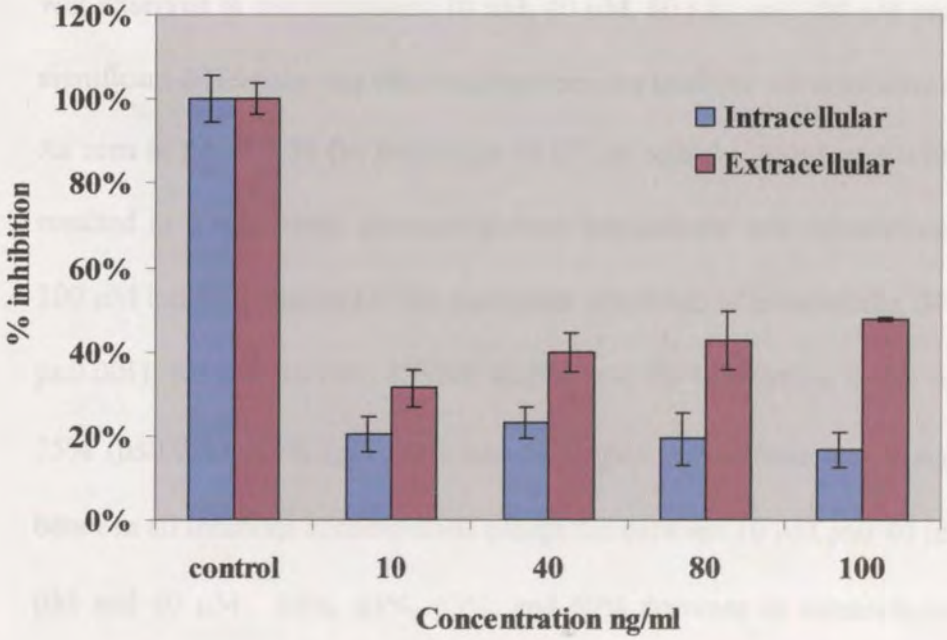


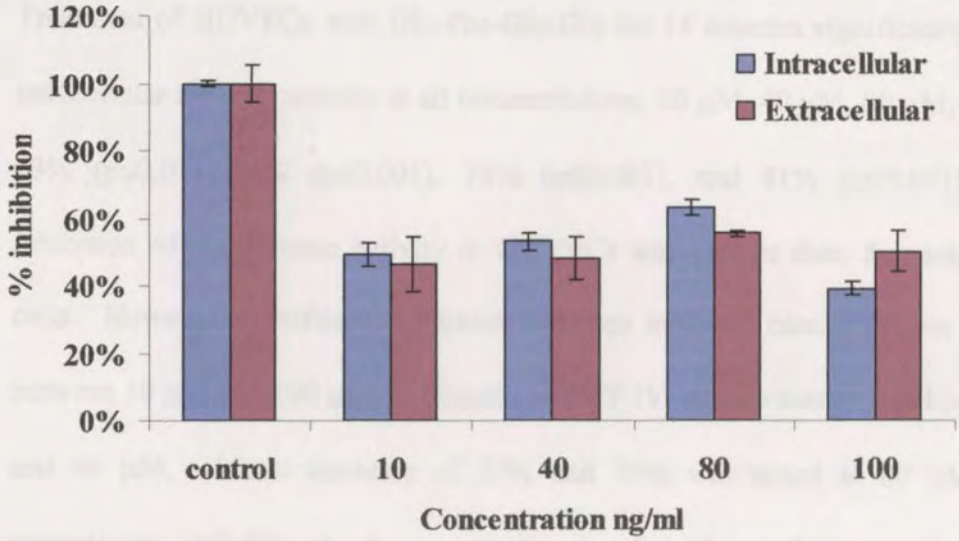
***Figure 3.35**:- % inhibition of intracellular and extracellular DPP IV activity in HRT 18 cells, following incubation with DPP IV inhibitor, Gly-Pro-Gly-Gly. (a) 15 minute incubation (b) 30 minute incubation.*

3.6.2.2 Inhibition of DPP IV activity by Gly-Pro-Gly-Gly in HT 29 cells

In HT 29 cells, Gly-Pro-Gly-Gly significantly downregulated intracellular DPP IV activity, following incubation for 15 minutes (figure 3.36 a). At 10 μM, 40 μM, 80 μM, and 100 μM concentration inhibitor, the % decrease in DPP IV activity noted was 51% ($p \leq 0.01$), 47% ($p \leq 0.001$), 37% ($p \leq 0.01$), and 61% ($p \leq 0.001$) respectively, with maximum inhibition achieved at 100 μM. There was a significant difference in between all concentrations except for in between 40 μM and 80 μM, and between 10 μM and 40 μM. Extracellular DPP IV activity was also significantly reduced following 15 minute incubation with the inhibitor. A decrease of 54% ($p \leq 0.05$), 52% ($p \leq 0.05$), 44% ($p \leq 0.01$), and 50% ($p \leq 0.01$) was observed at concentrations 10 μM, 40 μM, 80 μM, and 100 μM respectively, while no significant difference was observed between the inhibitor concentrations.

As seen in figure 3.36 (b) incubation of HT 29 cells for 30 minutes with Gly-Pro-Gly-Gly, resulted in a significant decrease in both intracellular and extracellular DPP IV activity. 100 μM inhibitor resulted in the maximum inhibition of intracellular DPP IV activity (76% $p \leq 0.001$), while at 10 μM, 40 μM, and 80 μM the % decrease in the enzyme activity was 55% ($p \leq 0.001$), 65% ($p \leq 0.001$) and 54% ($p \leq 0.01$). There was a significant difference between all inhibitor concentration except for between 10 μM and 40 μM, and between 10 μM and 80 μM. 58%, 65%, 63%, and 69% decrease in extracellular DPP IV enzyme activity was noted at inhibitor concentrations of 10 μM, 40 μM, 80 μM, and 100 μM respectively (($p \leq 0.01$ at all concentrations). There was a significant difference between 10 μM and 100 μM, and between 40 μM and 100 μM.

(a)



(b)

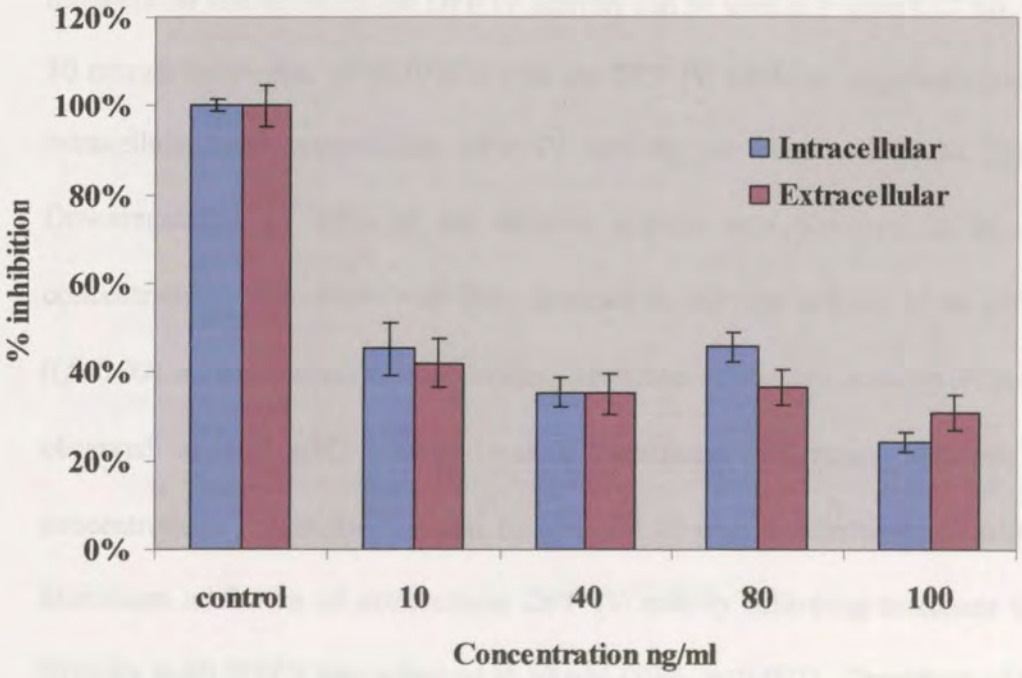


***Figure 3.36**:- % inhibition of intracellular and extracellular DPP IV activity in HT 29 cells following incubation with DPP IV inhibitor, Gly-Pro-Gly-Gly. (a) 15 minute incubation (b) 30 minute incubation.*

3.6.2.3 Inhibition of DPP IV activity by Gly-Pro-Gly-Gly in HUVECs

Treatment of HUVECs with Gly-Pro-Gly-Gly for 15 minutes significantly downregulated intracellular DPP IV activity at all concentrations, 10 μM, 40 μM, 80 μM, and 100 μM, by 73% ($p \leq 0.01$), 79% ($p \leq 0.001$), 78% ($p \leq 0.001$), and 81% ($p \leq 0.001$) respectively. Inhibition of the enzyme activity in HUVECs was greater than that achieved in cancer cells. However, significant difference between inhibitor concentrations was only noted between 10 μM and 100 μM. Extracellular DPP IV activity was reduced by 72% at 10 μM and 40 μM, while a decrease of 77% and 79% was noted at 80 μM and 100 μM respectively ($p \leq 0.001$ at all concentrations). Significant difference between inhibitor concentrations was only noted between 40 μM and 100 μM. The decrease in the intracellular and extracellular DPP IV activity can be seen in Figure 3.37 (a)

30 minute incubation of HUVECs with the DPP IV inhibitor significantly downregualted intracellular and extracellular DPP IV activity, as can be seen in figure 3.37 (b). Downregulation of 89% of the enzyme activity was observed at 10 μM inhibitor concentration, while there was 88% decrease in enzyme activity at 40 μM and 80 μM (($p \leq 0.001$ at all concentraions). Greatest inhibition of enzyme activity (92%decrease) was observed at 100 μM. There was a significant difference between all inhibitor concentrations except for between 10 μM and 40 μM, and between 40 μM and 80 μM. Maximum inhibition of extracellular DPP IV activity following treatment with Gly-Pro-Gly-Gly in HUVECs was achieved at 10 μM (89%, $p \leq 0.001$). Treatment of the cells with 40 μM inhibitor resulted in 86% ($p \leq 0.01$) decrease in enzyme activity and treatment with 80 μM and 100 μM resulted in 85%, ($p \leq 0.01$ and $p \leq 0.001$ respectiviely) decrease in the enzyme activity. Significant difference was observed between all concentration except for between 40 μM and 100 μM, and between 80 μM and 100 μM.

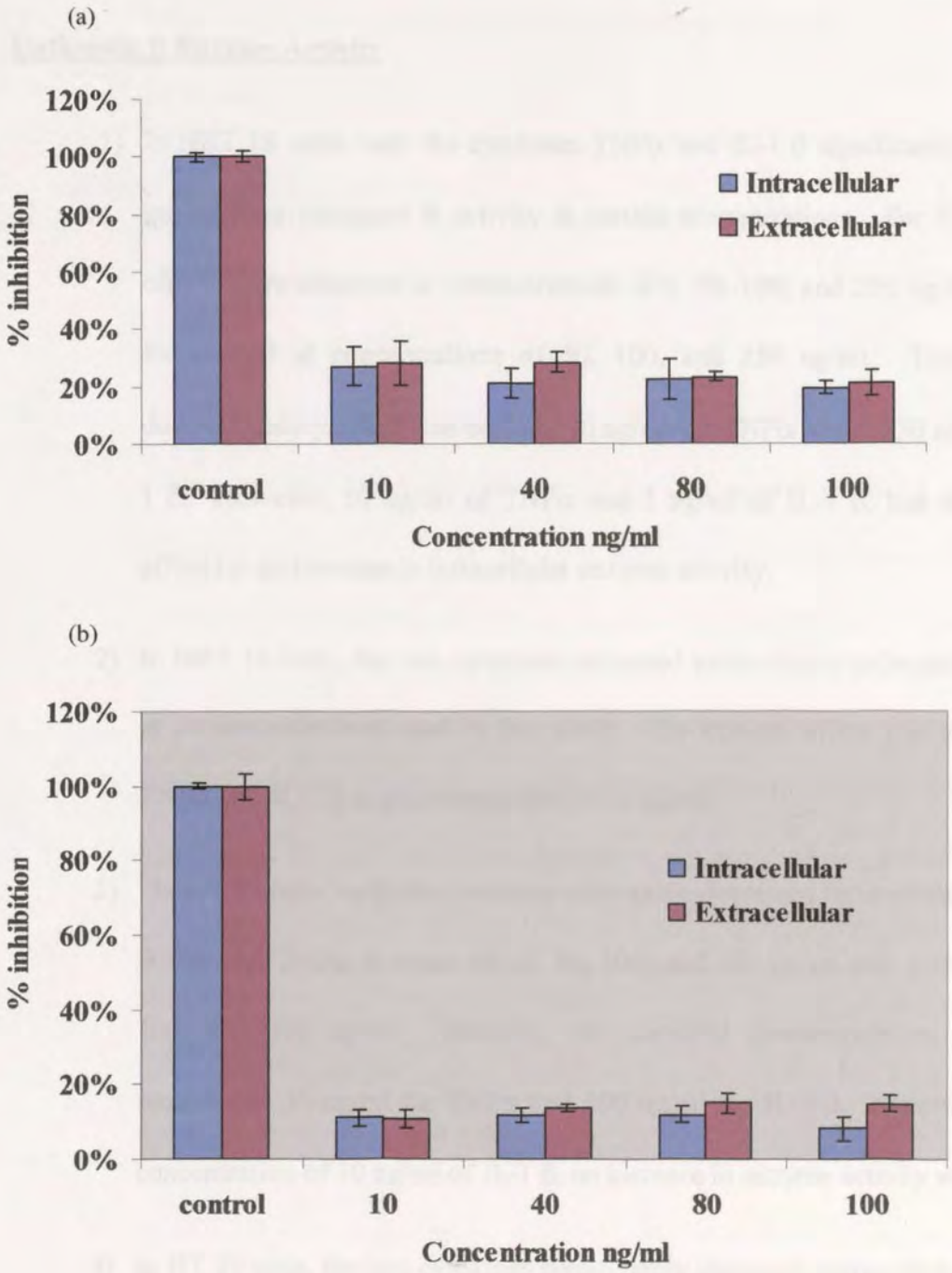


***Figure 3.37**:- % inhibition of intracellular and extracellular DPP IV activity in HUVECs, following incubation with DPP IV inhibitor, Gly-Pro-Gly-Gly. (a) 15 minute incubation (b) 30 minute incubation.*

Summary of the Results:-

Cathepsin B Enzyme Activity

1) In HRT 18 cells, both the cytokines TNFα and IL-1 β significantly decreased intracellular cathepsin B activity at certain concentrations. For TNFα, these effects were observed at concentrations of 5, 50, 100, and 250 ng/ml; whereas for IL-1 β at concentrations of 50, 100, and 250 ng/ml. The maximum downregulatory effect was noted at 50 ng/ml for TNFα and at 250 ng/ml for IL-1 β. However, 10 ng/ml of TNFα and 5 ng/ml of IL-1 β, had the opposite effect i.e. an increase in intracellular enzyme activity.

2) In HRT 18 cells, the two cytokines increased extracellular cathepsin B activity at all concentrations used in this study. The optimal effect was achieved by TNFα and IL-1 β at a concentration of 10 ng/ml.

3) In HT 29 cells, both the cytokines once again decreased intracellular cathepsin B activity; TNFα at doses of 10, 50, 100, and 250 ng/ml and IL-1β at 5, 50, 100, and 250 ng/ml. However, the maximal downregulatory effect was recorded at 50 ng/ml for TNFα and 100 ng/ml for IL-1 β. Nevertheless, at a concentration of 10 ng/ml of IL-1 β, an increase in enzyme activity was noted.

4) In HT 29 cells, the two cytokines significantly increased extracellular cathepsin B activity at all concentrations. Optimal effect was achieved by TNFα at a concentration of 5 ng/ml and by IL-1β at 10 ng/ml.

5) In HUVECs, the two cytokines significantly increased both intracellular and extracellular cathepsin B activity. Maximum increase in both the intracellular and extracellular activity was achieved by 5 ng/ml concentration of TNFα,

while IL-1 β caused maximum effect on intracellular activity at 100 ng/ml and on extracellular activity at 5 ng/ml.

Cathepsin B Protein Content

6) In HRT 18 cells, intracellular cathepsin B protein was highest when the cells were treated with TNFα at a concentration of 10 ng/ml, while other doses also increased cathepsin B protein but to a lesser extent. IL-1β showed a varied effect, treatment of the cells with 5 and 10 ng/ml increased intracellular cathepsin B protein, while 50, 100 and 250 ng/ml decreased intracellular cathepsin B protein. In these doses maximum inhibition was achieved by IL-1 β at a concentration of 250 ng/ml.

7) In HRT 18 cells, extracelluar cathepsin B protein was increased at 5 and 250 ng/ml of TNFα, with maximum increase at 5 ng/ml. No significant change in protein was measured at 10, 50 and 100 ng/ml concentration of TNFα. However, when the cells were treated with IL-1 β extracellular protein was decreased progressively at concentrations of 50 ng/ml and 250 ng/ml and increased linearly at 5 and 10 ng/ml.

8) In HT 29 cells, there was a decrease in intracellular cathepsin B protein following treatment with TNFα and IL-1 β at all concentrations, with maximum effect achieved by TNFα at 250 ng/ml and by IL-1 β at 10 ng/ml.

9) In HT 29 cells, extracellular cathepsin B protein was increased by TNFα at all concentrations, producing maximum effect at 5 ng/ml. IL-1 β also increased extracellular cathepsin B protein, but only at concentrations 5 ng/ml and 10 ng/ml with maximum effect achieved at 10 ng/ml.

10) In HUVECs, TNFα and IL-1 β both downregulated intracellular cathepsin B protein and upregulated extracellular cathepsin B protein. Maximum intracellular effect was achieved by TNFα at 250 ng/ml and at 5 ng/ml by IL-1 β, while maximum extracellular effect was achieved by TNFα at 50 ng/ml and by IL-1 β at 250 ng/ml.

Certain concentrations of TNFα and IL-1 β demonstrated a correlation between cathepsin B enzyme activity and cathepsin B protein content. For example, in HRT cell, there was a significant increase in cathepsin B enzyme activity and its protein level extracellularly when treated with TNFα, at concentrations of 5, 100 and 250 ng/ml. When treated with IL-1 β, a significant decrease in both intracellular cathepsin B activity and its protein content was noted at doses 50, 100 and 250 ng/ml. However, IL-1 β at 5 ng/ml and 10 ng/ml resulted in an increase in the enzymes extracellular protein level, there was also an increase in the enzyme activity at these concentrations.

In HT 29 cells, TNFα significantly decreased cathepsin B activity and its protein level intracellularly, and increased both enzyme activity and protein levels extracellularly at all concentrations. IL-1 β also showed the same effect i.e. it significantly decreased cathepsin B enzyme activity and its protein intracellularly, however the increase in the enzyme activity and its protein was only noted at concentrations 5 and 10 ng/ml.

In HUVECs, both the cytokines, TNFα and IL-1β, increased both cathepsin B activity and its protein level extracellularly. This was achieved at all the concentrations.

DPP IV Activity

11) In HRT 18 cells, TNFα decreased intracellular DPP IV activity at all concentrations (5, 10, 50, 100, and 250 ng/ml), while IL-1β downregulated enzyme activity only at 5, 10 and 50 ng/ml (no significant change in activity was observed at 100 and 250 ng/ml . The maximum effect that was achieved by TNFα was at a concentration of 250 ng/ml, while that achieved by IL-1β was at 10 ng/ml.

12) In HRT 18 cells, TNFα increased extracellular activity of DPP IV at all concentrations, maximum effect was achieved at 5 ng/ml. Whereas, IL-1 β produced maximum upregulatory effect at 10 ng/ml, to a lesser extent 5 and 50 ng/ml also showed upregulatory effect, while no significant change in activity was noted at concentrations 100 and 250 ng/ml.

13) In HT 29 cells, TNFα downregulated intracellular DPP IV activity at all concentrations. Optimal effect was produced at the concentration of 100 ng/ml. On the other hand IL-1 β decreased the enzyme activity at only 250 ng/ml.

14) In HT 29 cells, TNFα increased extracellular activity of DPP IV at all concentrations, however, maximum effect was achieved at 250 ng/ml. Whereas, IL-1 β showed no significant change in DPP IV extracellular activity.

15) In HUVECs, TNFα at all concentrations and IL-1β at 250 ng/ml increased intracellular of DPP IV enzyme activity. TNFα produced maximum effect at a concentration of 10 ng/ml. However, no significant change in the extracellular enzyme activity was observed by TNFα. A significant increase in extracellular

enzyme activity was observed when the cells were treated with 5 and 100 ng/ml of IL-1 β.

DPP IV Protein Content

16) In HRT cells, both the cytokines downregulated the intracellular DPP IV protein following treatment at all concentrations. TNFα and IL-1 β both showed maximum inhibition of intracellular activity at 100 ng/ml.

17) In HRT cells, both the cytokines downregulated the extracellular DPP IV protein following treatment at all concentrations. TNFα showed maximum inhibition of extracellular DPP IV protein at 250 ng/ml, while IL-1 β produced maximum inhibition at 10 ng/ml.

18) In HT 29 cells, TNFα downregulated intracellular DPP IV protein at all concentrations. However, maximal inhibition was achieved at a concentration of 10 ng/ml. IL-1 β also downregulated intracellular DPP IV protein, showing maximum effect at 250 ng/ml.

19) In HT 29 cells, TNFα upregulated extracellular protein at all concentrations. Maximal effect was achieved at a concentration of 5 ng/ml. IL-1 β on the other hand downregulated extracellular DPP IV protein at all concentrations, showing maximum effect at 250 ng/ml.

20) In HUVECs, both the cytokines downregulated intracellular and extracellular DPP IV protein at all concentrations. Maximum inhibition of intracellular protein was achieved by TNFα at a concentration of 50 ng/ml, while maximum inhibition of the extracellular protein was achieved at 250 ng/ml. IL-1 β had an

optimal effect on the intracellular DPP IV protein at 250 ng/ml and on extracellular protein at 100 ng/ml.

At certain doses TNFα and IL-1 β demonstrated a correlation with DPP IV activity and its protein. For example, in HRT 18 cells, TNFα at all concentrations and IL-1β at 5, 10 and 50 ng/ml resulted in a significant decrease in DPP IV enzyme activity and its protein level intracellularly.

In HT 29 cells, TNFα resulted in a significant decrease in the intracellular DPP IV enzyme activity and its protein level along with increase in its enzyme activity and protein level extracellularly at all concentrations. While, IL-1 β treatment of HT 29 cells, resulted in DPP IV enzyme activity and its protein level being proportional only at 250 ng/ml i.e. both the enzyme activity and its protein level were decreased at this concentration.

In HUVECs, IL-1 β showed proportionality between extracellular DPP IV enzyme activity and its protein level at 5 and 100 ng/ml concentrations.

In addition

Viability studies showed that:-

21) Both the cytokines, TNFα and IL-1β, resulted in change in cell viability in all three cell lines, HRT 18, HT 29 cells and HUVECs.

- In HRT 18 cells, at 30 hours (incubation period for optimal cathepsin B activity) TNFα resulted in no significant change in the cell number at all concentrations, and at 12 hours (incubation period for optimal DPP IV activity) it caused an increase in cell number only at a dose of 250 ng/ml, suggesting a protective mechanism possibly due to the involvement of NF-κB pathway and synthesis of

cytoprotective proteins. At 30 hours, IL-1 β resulted in no significant change in cell number of HRT 18 cells at all concentrations, suggesting once again a protective mechanism of cells such as increase in Bcl_2 expression. However, at 12 hours IL-1 β resulted in decrease HRT 18 cell viability at concentrations of 50, 100 and 250 ng/ml, with greatest effect at 250 ng/ml, this could be due to the induction of NO by IL-1 β.

- In HT 29 cells, at 18 hours (incubation period for optimal cathepsin B activity) TNFα showed no significant effect on the cell viability at all concnetrations, however at 42 hours (incubation period for optimal DPP IV B activity) a decrease in cell viability was observed at 10, 50 and 250ng/ml (maximum effect at 10 and 50 ng/ml), suggesting the involvement of TNFR1 or TRAIL in the induction of cell death.

  IL-1β at 18 hours incubation decreased the cell viability of the HT 29 cells at concentrations 10, 50 and 250 ng/ml, the maximum decrease in cell number was achieved at the concentration of 10 ng/ml. A decrease in cell number was also noted following incubation of HT 29 cells with IL-1 β for 42 hours at concentrations 10 and 50 ng/ml, where 50 ng/ml showed a greater effect.

- Both the cytokines, TNFα and IL-1 β, significantly decreased cell viability of HUVECs, at 12 hours (incubation period for optimal cathepsin B activity) and 48 hours (incubation period for optimal

DPP IV activity). At 12 hours incubation with TNFα, decrease in cell viability was observed at concentrations 10, 50 and 250 ng/ml (250 ng/ml showed the maximum effect). However, at 48 hours incubation with all the concentrations decreased the cell viability, maximum decrease was noted at a dose of 5 ng/ml.

Following 12 hour incubation with IL-1 β decrease in cell number was noted at concentrations 10, 50, 100 and 250 ng/ml, maximum decline in cell number was achieved by 250 ng/ml. At 48 hour incubation with IL-1 β decrease in cell viability was achieved by doses 5, 10, 50 and 100 ng/ml, with maximum effect at a concentration of 50 ng/ml.

Inhibitor Studies showed that:-

22) From the two time points, 15 minutes and 30 minutes incubation period, maximum inhibition of both the enzymes, cathepsin B and DPP IV, by Mu-Phe-Hph-FMK and Gly-Pro-Gly-Gly respectively, was achieved at 30 minutes incubation in all the three cell lines.

23) Inhibitor Mu-Phe-Hph-FMK produced maximum inhibition of intracellular cathepsin B activity, following 30 minutes incubation, in HRT 18 cells at 40 μM. Greatest inhibition of extracellular enzyme activity was produced at 10 μM. Other doses, 80 μM and 100 μM also inhibited the enzyme, however the maximal inhibition was noted at above concentrations.

24) In HT 29 cells, maximum inhibition of intracellular and extracellular cathepsin B activity was achieved at 40 μM and 10 μM concentrations respectively, following 30 minute incubation with the inhibitor. Inhibitor concentrations of

80 μM and 100 μM also resulted in the inhibition of the enzyme but to a lesser extent.

25) In HUVECs, maximum inhibition of intracellular and extracellular cathepsin B activity was achieved by Mu-Phe-Hph-FMK at concentrations 10 μM and 40 μM respectively (incubation period 30 minutes).

26) Inhibitor Gly-Pro-Gly-Gly produced maximum inhibition of intracellular DPP IV activity in HRT 18 cells at 100 μM concentration following 30 minutes incubation. Maximum inhibition of the enzyme extracellularly was achieved at 10 μM. Other doses 40 μM and 80 μM also caused an inhibition of the enzyme, but to a lesser extent.

27) In HT 29 cells, maximum inhibition of both intracellular and extracellular DPP IV activity was achieved by Gly-Pro-Gly-Gly at 100 μM concentration following 30 minutes incubation.

28) In HUVECs, maximum inhibition of intracellular DPP IV activity was produced at 100 μM concentration, and inhibition of extracellular enzyme activity at 10 μM concentration (incubation period 30 minutes).

It is well established fact that cell culture provides an effective means of investigating various biochemical, morphological and physiological aspects of different cell types including endothelial cells and cancer cells. Endothelial cells, the lining cells of all blood vessels, are involved in many physiological and pathophysiological processes, including homeostasis, vasoregulation, inflammation, angiogenesis, and the extravasation of fluid macromolecules, hormones, and leucocytes (Nachman & Jaffe 2004). The recognition that endothelial cells are involved in all these processes went in parallel with the ability of investigators to study these cells in culture (Hinsbergh & Draijer 1996).

A convenient source of endothelial cells has been the human umbilical vein, which was used in this study. A Human umbilical cord usually contains three large blood vessels, of which the vein is the largest. Two arteries are also present which are usually closed by contraction of the vessels. Endothelial cells derived from human umbilical veins were first successfully cultured *in-vitro* in 1973 (Jaffe *et al.*, 1973). Weibel-Palade bodies (Rod-shaped/elongated storage granules for vWF specific to endothelial cells) and the von Willebrand factor antigen were used as morphological and immunocytochemical markers to identify the cells as being endothelial in nature, which can be seen in figure 3.1 (a) & (b), and figure 3.5 (a) & (b) respectively. In addition, CD 31 also known as platelet-endothelium cell adhesion molecule (PECAM-1) was also used as a immunocytochemical marker to identify endothelial cells, which can be seen in figure 3.2 (a), (b) and 3.3.

In the present study, growth of endothelial cells was slow in comparison to the cancer cells (HRT 18 and HT 29). This difference in the rate of growth between the cells is due to the fact that normal cells (HUVECs) go through a cell cycle at a controlled/regulated rate, whereas cancer cells have a disrupted cell cycle producing doubling of the cells in an uncontrolled manner. Therefore, the cancer cells divide too fast and pile up on top of one another. In the present study all the five cell types showed to have different cell doubling times. The cell doubling time for HRT 18 was calculated to be 34 hrs, while the other cell types had longer cell doubling time. Cell doubling time for HT 29 was 53 hrs, for HUVECs was 39 hrs, for liver cells was 56 hrs and for thyroid cells was 62 hrs. Both the cancer cell types HRT 18 and HT 29 grew at a faster rate, reaching 3,560,000 cells/96 well plate and 2,809,000 cells/96 well plate on day 6 respectively. However, HUVECs grew at much slower rate than the cancer cells, reaching only 1,489,000 cells/96 well plate on day 6 (figure 3.0). This is because primary cells such as the endothelial cells that were isolated from human umbilical vein are capable of one or two divisions in culture, and given the right conditions can survive for some time. Having reached the limit the cell continues to live and be metabolically active but not divide (a state termed senescence) and may eventually die. However, cancer cells can exceed this limit and appear to be immortal in that they can simply continue dividing (Shay *et al.*, 2001).

Normal cells when grown in culture need to be in a reasonably close proximity to each other and cannot grow when seeded at low density. This probably relates to the need for factors produced by neighbouring cells for survival and growth. This was noted in the present study whilst growing the endothelial cells isolated from human umbilical cords. Cancer cells lose this requirement and can survive and grow at low densities (Pause *et al.*, 1998). When normal cells grow in culture they continue dividing (assuming the presence of growth factors and nutrients) until the surface of the dish is covered by a single layer of

cells just touching each other they touch each other, when cell division stops. This probably occurs as a consequence of signals from one cell affecting its neighbour leading to a switching off of division. This is called contact inhibition (cell contact leads to inhibition of cell division), which can be seen in figure 3.1 (b) where HUVECs grown in culture formed a monolayer. However, on the other hand cancer cells continue dividing at high densities and do not receive or send the signal to stop dividing, this was noted in the present study when the cancer cells where viewed under the microscope. Cancer cells may be immortal; that is, proliferate indefinitely in culture (Shay *et al.*, 2001). The two cancer cell types, HRT 18 and HT 29, did not maintain contact inhibition *in-vitro*, i.e. as they spread out across the culture flask, when two adjacent cells touch, this does not signal them to stop growing in contrast to normal cells. Loss of contact inhibition is a classic sign of cancer cells (Ouellette *et al.*, 2000). As a result when viewed under a light microscope the cancer cells not only appeared to form monolayer in culture but also were piled up on top of one another.

Figure 3.0 shows growth curve patterns of cell lines HRT 18, HT 29, liver cells, thyroid cells and HUVECs, and also show the lag, exponential or log, and stationary phases of the growth cycle (Phases are described in detail under section 1.11.1). In all the five cell lines, HRT 18, HT 29, liver cells, thyroid cells and HUVECs, the lag phase lasted from day 0 to day 1, as the cells were grown in good cell conditions. At day 2 there was a considerable increase in cell number with time in all cell lines that were studied, indicating that the cells were in their log phase.

Contact inhibition of HUVECs results in decrease in its proliferative activity resulting in the occurrence of quiescent cells (Fuse *et al.*, 2000). However, tumour growth has been found mostly to be erratic, with alternating growth phases and periods of no growth/temporary dormancy or rapid growth (Speer *et al.*, 1984). When viewed under a light microscope the tumour cells, HRT 18 and HT 29 cells, appeared to be of different sizes and were crowded together in a disorderly fashion, whilst endothelial cells displayed a cobblestone morphology (figure 3.1 a, and b) at confluence, which is regarded as a characteristic feature of endothelial cells (Ratcliffe *et al.*, 1999).

To identify the endothelial nature of the cells obtained in culture, positive and negative criteria were used. A number of determinants can be used to identify endothelial cells such as, vWF, CD 31, V, E cadherin, PAL-E antigen, EN-4 antigen, Ulex europaeus lectin-1, Dil-acetylated LDL uptake, and Angiotensin converting enzyme (Hinsbergh & Draijer 1996). However, in this study the determinants that were used to positively identify endothelial cells were vWF and CD 31, whereas in negative samples the primary antibody was omitted and hence no staining in the cells was noted as can be seen in figure 3.4 for CD31 and 3.7 for vWF in the present study.

von Willebrand Factor (Factor VIII Related Antigen) and CD 31 are the most widely used endothelial cell markers for studying angiogenesis/neovascularisation (Jackson 2003, Ruggeri 2000). However, their localization in the cell and function differ from each other. von Willebrand Factor is synthesised by endothelial cells, causing adhesion of platelets to injured vessel walls and it functions as a carrier and stabiliser for coagulation of Factor VIII. von Willebrand Factor antibody reacts specifically with the endothelial cells of blood vessels and is a useful marker for the identification of endothelial lineage in tumours.

CD 31 is a type I integral membrane glycoprotein and a member of the immunoglobulin super family of cell surface receptors. It is strongly expressed by all endothelial cells and

weakly by several types of leucocytes (Parums *et al.*, 1990). According to several studies, staining of blood vessels with CD31 antibodies has shown to be suitable for the identification of angiogenesis in several types of malignancies such as lung (Giatromanolaki, Koukaris, Chetty 1996), breast (Horak *et al.*, 1992) and colorectal cancer (Takahashi 1996). Monoclonal antibody to CD31 is very specific for the staining of vascular endothelial cells in normal and cancerous tissue.

After treating the HRT 18 cells with TNFα and IL-1β, a downregulation of intracellular cathepsin B activity, with an increase in the enzymes extracellular activity was noted. This was in contrast to the control, where no treatment was applied to the HRT 18 cells. Such findings suggested that both the cytokines induced the release of cathepsin B from the HRT 18 cells resulting in an increased expression of the enzyme extracellularly, implying their role as important modulators of proteolytic activity. As a decrease in intracellular activity was observed it could be said that the cytokines may have released most of the intracellular protease or may have inactivated the enzymes present inside the cells. Studies conducted by Halaby & co-workers (2002) have shown an increase in cathepsin B activity and staining intensity in MCF7 cells in response to treatment with TNFα (1 ng/mL). In contrast to this the present study demonstrated decrease in intracellular cathepsin B activity in HRT 18 cells following treatment with TNFα at concentrations 5, 50, 100, and 250 ng/ml (figure 3.16 a). Decrease in intracellular cathepsin B activity was also noted in TNFα (5, 10, 50, 100, and 250 ng/ml) treated HT 29 cells (figure 3.17 a).

IL-1β showed a greater effect on the intracellular cathepsin B activity whereas TNFα was shown to have a greater effect on the extracellular activity.

Results for HT 29 cells were similar to those obtained for HRT 18 cells. There was again a significant downregulation of cathepsin B intracellular activity, and upregulation of the

enzyme's extracellular activity following treatment with TNFα (figure 3.17 a). There was 100% increase in extracellular cathepsin B activity by both the cytokines. This could be due to a number of reasons such as the treatment may have resulted in the release of a mature form of cathepsin B, or else a non active form that was already present could have been activated by the treatment, resulting in increased basal enzyme activity. Proteases in normal cells are regulated at every step of their biosynthesis. In tumour cells, misregulation at any one or more step may result in increase activity of the enzyme (Yan, Sameni, Sloane 1998). Altered trafficking of cathepsin B, resulting in its localisation in the plasma membrane and the release of abnormal amounts of precursors of cathepsin B has also been demonstrated by Heidtmann *et al* (1997).

Other studies have demonstrated increased cathepsin B levels in human tumours, and have suggested a role in invasion and metastasis (Berquin & Sloane 1996) (Bervar *et al.*, 2003). Although lysosomal localization of the enzyme suggests it functions primarily as a component of the protein degradation system, several data have pointed to alternative localization of cathepsin B, including the nucleus, the cytoplasm, and the plasma membrane. Additionally, there is evidence of the presence of the active enzyme in the extracellular matrix (Reddy, Zhang, Weiss 1995) suggesting escape of the active enzyme from lysosome or an extracellular mechanism for proenzyme activation. Extracellular cathepsin B has been implicated in inflammatory airway disease (Burnett *et al.*, 1995) and in bone and joint disorders (Buttle 1994).

In addition, altered forms of cathepsin B have been found in human lung tumours (Krepela *et al.*, 1995). Lysosomal enzymes are processed and transported to lysosomes via receptor dependent pathways such as mannose 6-phosphate pathway. Defects in the transport systems have been identified in fibroblasts from patients with lysosomal storage disease and in some murine tumour cell lines. In both cases, the defect resulted in the release of

lysosomal enzymes (Sloane *et al.*, 1987). Therefore, it is plausible that cytokines TNFα and IL-1β could have interfered with the trafficking of the protease resulting in the release of a latent form of the enzyme, which was then activated by other protease enzymes present outside the cells, such as uPA. TNFα and IL-1 β may have directly activated cathepsin B or indirectly by upregulating other proteases. Nevertheless, in HT 29 cells in the present study TNFα seemed to be a more potent modulator of cathepsin B at intracellular level, while both showed an equal effect on the enzyme extracellularly.

In cervical smooth muscle cells TNFα has been indicated to induce cathepsin B enzyme (Watari *et al.*, 1999). TNFα and IL-1 β both have been shown to induce matrix metalloproteinase (MMP-9) expression in normal human fibroblasts, osteosarcoma and fibrosarcoma cell lines (Hozumi *et al.*, 2001).

Studies by Morgan & co-workers (1998) have shown that IL-1β could modulate MMP-9 expression in human trophoblast continuous cell lines, where expression of MMP–9 in these cells is crucial for invasion. Malignant tumour cells frequently overexpress MMP-9 and much research has been focussed in understanding its role in cancer cells (Morgan, Kniss and McDonell 1998).

Cathepsin B activity has also been detected intracellularly and extracellularly in both human osteoblast-like cells and human osteosarcoma cells, IL-1β has shown to significantly increase cathepsin B activity in both the cell types intracellularly and extracellularly (Aisa 1996). In addition, TNFα at high concentrations has been known to exert cytotoxic effects on tumour cells, virally infected cells and endothelial cells. It also has the ability to induce MMP, in an autocrine and paracrine fashion (Pope *et al.*, 2000) and also upregulates uPA (Ribatti & Vacca 1999). Both TNFα and IL-1 have been reported to activate the transcription of genes for MMP and tPA at low concentrations.

Activated proteases are able to then digest extracellular matrix thereby facilitating migration of tumour cells. Thus induction of protease production by these cytokines could contribute to angiogenesis.

Nevertheless, it has been noted that high concentrations of TNFα suppresses tPA production and induces the secretion of plasminogen activator inhibitor type 1 (PAI-1) resulting in inhibition of angiogenesis.

In endothelial cells both the cytokines significantly upregulated intracellular and extracellular cathepsin B activity (figure 3.18 a and b). This was in contrast to cancer cells, where the cytokines actually downregulated intracellular cathepsin B activity, this can be seen in figure 3.16 (a) (b) and 3.17 (a) (b). Huet & co-workers (1993) have demonstrated that TNFα and IL-1 stimulate the secretion of a latent cysteine proteinase activity from synovial fibroblast-like cells. Another group Lemaire *et al* (1997) has shown, using the enzymatic substrates Z-Arg-Arg-AMC, that treatment of synovial fibroblast-like cells with TNFα resulted in a marked increase in cathepsin B secretion.

The increase in the enzymes intracellular activity indicated that the cytokines increase the synthesis of cathepsin B in endothelial cells, or activate the dormant form to a mature form. In addition, alterations in activity of cathepsin B enzyme may reflect changes in its synthesis, activation and processing, intracellular trafficking and delivery and in the inhibition (Sloane *et al.*, 1990). Addition of both the cytokines changed the enzyme's activity showing that the factors may exert their function at any stage of the enzyme regulation.

Cell surface and secreted cathepsin B has been implicated in the invasive and metastatic phenotype of numerous types of cancer. Studies by Hulkower and coworkers (2000) have demonstrated extracellular cathepsin B activity in cell lines such as U87 glioma cells, HT-1080 fibrosarcoma, HCT-116 colon, PC-3 prostate using the Z-Arg-Arg-AMC substrate.

This is in agreement with the results obtained in the present study, where extracellular cathepsin B activity was noted in HRT 18 and HT 29 cells. In addition, the present study also showed a significant increase in the enzymes extracellular activity in HRT 18 and HT 29 cells when treated with cytokines TNFα and IL-1β (figure 3.16 (a) (b) and 3.17 (a) (b) respectively).

The activities of DPP IV and cathepsin B has been previously investigated by Sedo & coworkers (1991) in primary human lung tumours and matched lung parenchyma, using continuous-rate fluorometric assays of the enzymes, and a significant correlation between the enzyme activity and tumour was observed (Sedo, Krepela, Kasafirek 1991).

TNFα is a multifunctional peptide with respect to its angiogenic capability of promoting the expression, synthesis, and secretion of uPA, a serine protease, by endothelial cells, and by enhancing collagenase synthesis by endothelial cells *in-vitro* (Baillie, Winslet, Bradley 1995).

Previously not much work has been done on DPP IV activity relating it to tumour progression. In the present study TNFα and IL-1β had similar effects on the activity of DPP IV as it had on the activity of cathepsin B, in both cancer cell lines. There was a significant downregulation of intracellular activity and upregualtion of extracellular activity. Once again these results suggest that the treatments could be responsible for the increase in extracellular activity by either activating a latent form of the enzyme into a mature form, and additionally may also have induced its release from the cells. This increase in DPP IV activity may result in the metabolism of some peptides that change the tissue environment to one favourable for cancer growth (Wilson *et al.*, 2000). Decrease in its intracellular activity could be attributed to the fact that the treatments resulted in the denaturation of the enzyme or simply destroyed its active site either directly or indirectly.

TNFα showed greater effects than IL-1 β on DPP IV activity in the cell lines. Other studies have shown that the invasiveness of tumour cells and expression of proteolytic enzymes, including u-PA, were enhanced by the treatment with TNFα *in-vitro* (Wu *et al.*, 1999).

In endothelial cells in the present study TNFα increased the expression of the enzyme both intracellularly and extracellularly. IL-1β on the other hand showed a downregulation of extracellular activity, with an increase in intracellular activity, indicating either that the synthesis of the protein was increased within the cell or the cytokine treatment had resulted in the activation of the latent form of the enzyme. Decrease in extracellular activity of the enzyme suggested that the enzyme already present outside the cell (extracellular) was denatured by the treatment or the cytokine had inhibited the release/induction of the enzyme. Cytokine TNFα has been shown to modulate DPP IV activity (Nemoto *et al.*, 1999). In HUVECs, an increase in DPP IV activity was observed when the cells were treated with TNFα (Silva *et al.*, 2003).

Although the therapeutic effects of TNFα and IL-1β continue to be explored in the clinic, the results from the present work as well as several other studies, including Suganuma *et al* (1999) implicate their role in promoting cancer. Since an upregulation of both the enzymes is evident extracellularly the results from the present study support such findings. TNFα and IL-1β act as mediators by increasing extracellular levels of enzymes that are thought to play a critical role in tumour progression by causing the degradation of extracellular matrix and the basement membrane.

In addition to elucidating the effect of various concentrations of TNFα and IL-1 β on the intracellular and extracellular activity of cathepsin B and DPP IV in the cell lines, HRT 18, HT 29, and HUVECs, the present study also investigated the effect of these cytokines on the viability of the cell lines. Since addition of the cytokines influenced the activity/expression of the two proteases it was necessary to investigate whether the change in the protease activity was due to cell death, or other causes.

Cytokines such as TNFα and IL-1 β have been implicated to play an important role in cancer progression and also in cancer regression (Ben-Baruch 2003). Studies involving cytokines have been shown to have controversial results, with respect to its effect on cells, for instance the pro and anti apoptotic features of TNFα and IL-1β on different cell types. Therefore it is of great importance to study the pleoitropic nature of these cytokines.

Cathepsin B, which is commonly over-expressed in human primary tumours, was shown by Foghsgaard *et al* (2001) to have two opposing effects in malignancy. Firstly, the enzyme was shown to cause apoptosis in malignant cells and, while on the other hand it facilitated invasion of the tumour. Therefore, MTT test was carried out to assess whether the increase in extracellular cathepsin B activity was due to cell death (apoptosis) caused by cathepsin B enzyme, whose activity was influenced by addition of TNFα and IL-1 β. Similarly, DPP IV enzyme has been associated with the progression of various tumours, including that of the thyroid, and hence the addition of cytokines which influenced the role of these enzymes in the three cell lines needed further elucidation of the precise action or mechanism by which these cytokines enhance or inhibit tumour growth.

Cathepsin B is synthesised as a proenzyme and transported into lysosomes, where it is processed and activated either by lysosomal proteases or by autoactivation (Mach *et al.*, 1992). This compartmentation of cathepsin B within acidic vesicles prevents it from

inducing cell injury. However, during treatment of the cancer cells, HRT 18 and HT 29, and HUVECs with TNFα and IL-1 β increased cathepsin B was detected extracellularly (figure 3.16 (a) (b), 3.17 (a) (b) and 3.18 (a)(b) respectively).

TNFα, a multifunctional cytokine, has been known to possess cytostaic/cytotoxic activity, including apoptosis (Malagarie-Cazenave *et al.*, 2002) and, tumour promoting activity (Ashkenazi & Dixit 1999). TNFα and IL-1 β both have been found to cause tumour regression and increase survival time in a variety of cancer patients (Nakamoto *et al.*, 2000) (Elkordy *et al.*, 1997). The direct anti-tumour properties of the two proinflammatory cytokines are generally considered to reside in their ability to inhibit tumour growth or cause cell death (Stoelcker *et al.*, 2000). Reports have shown data suggesting a new model by which both TNFα and IL-1 β impair cell cycle progression (Shen *et al.*, 2002). Hence, the dual role of TNF α and IL-1 β required further assessment of these cytokines in the cell lines HRT 18, HT 29, and HUVECs

Tumour cell apoptosis is mediated by a variety of intracellular and extracellular signals. Cytokines from the TNF family are the primary extracellular mediators of apoptosis. These cytokines are TNF, FasL, and TRAIL, all of which trigger apoptosis by binding their respective receptors. TNF, and FasL induce apoptosis of normal as well as tumour cells. TRAIL preferentially induces apoptosis in a wide variety of tumour cells while remaining non-toxic to most normal cells as a result of which it is being intensely investigated as a promising anticancer agent (Vigneswaran *et al.*, 2005).

TNFα has been shown to inhibit proliferation and induce apoptosis of cultured MCF-7 cells (Shen *et al.*, 2002). One of the mechanisms by which TNF induces apoptosis is through upregulating the cathepsin B activity. Studies have shown that cathepsin B possesses a caspase-processing activity and is upregulated during apoptosis in human

breast carcinoma cells, MCF-7 (Mathiasen *et al.*, 2001) suggesting that lysosomal enzymes play a role in regulating caspases, proteins that are downstream effectors of apoptosis, and are also implicated in mammary tumour regression. Apoptosis was induced in MCF-7 cells by treating the cells with TNFα (1 ng/mL) for 24 h at 37°C. However, in the present study no significant change in cell viability was observed when HRT 18 and HT 29 cells were treated with TNFα (5, 10, 50, 100, and 250 ng/ml) for 30 minutes and 18 hours respectively (time optimal for maximal cathepsin B activity). This could be due to the fact that a higher concentration was used, which had a different effect on the cells since cytokines have been shown to have pleiotropic affects. A study conducted by Liu & co-workers (2003) has shown that ligation of death receptors such as TNF-R1 triggers apoptosis and that NF-κB counteracts this process by activating the transcription of anti-apoptotic genes. The mechanism by which NF-κB protects cells against TNFα induced apoptosis is by inhibition of the lysosomal pathway of apoptosis. NF-κB can protect cells from death after TNF-R1 stimulation, by extinguishing cathepsin B activity in the cytosol. This activity of NF-κB is mediated, at least in part, by the upregulation of *Serine protease inhibitor 2A* (*Spi2A*), a potent inhibitor of cathepsin B (Liu *et al.*, 2003). This could also be one of the reasons why in the present study no significant change in cell viability was noted when HRT 18 and HT 29 cells were treated with TNFα. As in the present study biochemical studies confirmed decrease in cathepsin B activity. In addition, cathepsin B deficiency in hepatocytes has been shown previously to increase their resistance to TNF induced apoptosis (Vigneswaran *et al.*, 2005).

Animal models have shown that NK cells play an essential role in preventing the colonization of tumour cells and subsequent growth of metastases. Furthermore, tumour cells that are resistant to NK cell-mediated apoptosis are able to rapidly form metastases *in-vivo* (Vigneswaran *et al.*, 2005).

Activated NK cells constitutively express TRAIL, while cancer cells express the corresponding TRAIL-receptors. Interaction of the ligand and the receptor mediate the apoptosis of tumour cells. TRAIL, which exerts potent tumoricidal activity, is the key molecule mediating NK-cell protection against tumour metastasis.

MTT assays carried out by Vigneswaran et al., (2005) showed that TRAIL induced apoptosis in both primary and metastatic oral cancer cells. However, no such conclusions can be drawn from present study, where there was no significant change in the cell viability of HRT 18 and HT 29 following treatment with TNFα for 30 hrs and 18 hrs respectively (optimal time for cathepsin B activity). This could be due to the fact that there was significant downregulation of cathepsin B activity, the mediator of TRAIL induced apoptosis, in these cells. Cathepsin B has been shown to mediate apoptosis by increasing the mitochondrial permeability via cleavage of Bid and thereby acting upstream of the activation of executor caspase-3. Also, cathepsin B acts as an executor protease in caspase-independent apoptosis of tumour cells mediated by TNF. Cathepsin B is the primary participant in the execution of apoptosis in cancer cells as well as in normal T-lymphocytes in the absence of caspase activation (Vigneswaran et al., 2005).

A combination of IL-1, IFN, and TNF has been shown to induce nitric oxide synthase mRNA expression and nitric oxide generation in the human colon carcinoma cell line HT 29, leading to apoptosis of these cells . In the present study, treatment of HT 29 for 18 hours with IL-1β alone resulted in a decrease in cell viability (figure 3.24 b), possibly due to the production of NO, an inducer of apoptosis, as suggested by Wright & co-workers (1999).

In addition, HuT 78, a T-cell line derived from a Sezary lymphoma, is resistant to the

cytotoxic effects of TNF, suggesting that TNF may be a growth factor for this cell line (O'Connell *et al.,* 1995). The identification of two TNF receptors on cells suggested initially that individual receptors might mediate the individual functions of killing and proliferation. Due to the occurrence of TNFα receptors on nearly all cells, TNFα demonstrates a wide variety of biological action. It has cytolytic and cytostatic effects on tumour cells (Maury & Teppo 1987). TNF exerts its biological functions by interactions with two members of the TNF receptor superfamily, namely TNF-R1 and TNF-R2. However, both receptors have since been found to mediate both proliferation and cytotoxicity (Probert *et al.*, 1993). Therefore, both may trigger proliferation in HuT 78 (O'Connell *et al.,* 1995).

TNF was first identified as an oncolytic agent that promotes the hemorrhanic necrosis and regression of some malignancies by inducing an inflammatory response in tumour capillary beds (Kunkel *et al.*, 1989). TNF also acts directly on transformed cells, eliciting a cytotoxic response from some and inhibiting the proliferation of others; however, many transformed cells are resistant to the cytotoxic and cytostatic activities of TNF (Machester, Heston, Donner 1993). TNF can induce the inhibition of cell proliferation and apoptosis in some cells (Yarden & Kimchi 1986). High concentrations of TNFα has been shown by Machester & co-workers (1993) to have cytotoxic and antiproliferative effects on cervical squamous cell carcinoma cell line, ME180, while low dose was shown to induce growth, again this was not consistent with the results from the present study where high TNFα concentration showed no significant change in cell viability in HRT 18 (30 hrs) and HT 29 (18 hrs). One possible explanation why there was not a significant downregulation in cell number could be due to the part played by the NF-kB. TNFα has been shown to activate the transcription factor NF-kB, and it has been demonstrated that NF-kappa B plays a protective role in the apoptosis induced by TNFα (Beg & Baltimore 1996). It has been

shown that NF-κB is required for cell survival in TNFα treated fibroblasts, fibrosarcoma cells, Jurkat cells, porcine endothelial cells, transforming growth factor β1 treated B cells, Ras-transformed NIH3T3 cells, and a protease inhibitor-treated B cells (Zen *et al.,* 1999). In addition the present study showed no significant decrease in cell viability by TNFα in HRT 18 (30 hrs) and HT 29 cells (18 hrs) it can be possible that maybe in these cell lines NF-κB was involved as a protective mechanism. Nevertheless, there was a significant increase in HRT 18 cells at 12 hour incubation period with TNFα (figure 3.23 b).

TNFα has been shown by Gómez & co-workers (1996) to mediate cell death in L929 cells and within 36 hrs TNF promoted a 7-fold increase in the reduction of MTT in TNF-sensitive cells. While, in the present study 30 hr incubation (incubation time for optimal cathepsin B activity) of HRT 18 cells with TNFα and IL-1 β had no significant change in the cell viability. Furthermore, in HT 29 cells 42 hr incubation with both the cytokines there was a decrease in cell viability, this can be seen in figure 3.25 b.

Vascular endothelial cells are primary targets of cytokine induced cell death leading to tissue injury. The activity assay carried out in the present study suggested the release of cathepsin B and DPP IV in the extracellular media, nevertheless studies conducted by Li & Pober (2005) have reported that TNFα in the presence of IFN-γ activates cathepsin B, and triggers its release from lysosomes, as well as a caspase death pathway in HUVECs. It has been shown that vascular-like structure formation requires apoptotic cell death through activation of a caspase-dependent mechanism, one of the critical steps leading to apoptosis, and the release of lysosomal enzymes such as cathespin B, into the cytosol activates the apoptotic machinery. (Halaby *et al.,* 2002).

A diverse group of signals induce apoptosis, but it is known that TNFα is one of the prime signals that induce apoptosis in a host of cells (Guicciardi *et al.,* 2000).

Stimulation of TNF-R1, by TNFα, triggers apoptosis not only by activating caspases but also by causing the release of cathepsins B from the lysosome into the cytoplasm where they induce apoptosis (Liu *et al.*, 2003). The loss of lysosome integrity and the release of cathepsins and other digestive enzymes is a critical event in the induction not only of apoptosis but also of coagulative necrosis. This explains the decrease in the viability of HUVECs, when treated with TNFα in the present study. Since the present study also elucidated increase in cathepsin B activity in HUVECs therefore it is possible that the decrease in cell number can be due to the fact that the cells had an increased expression of intracellular cathepsin B activity, which mediated the apoptosis of the cells. In addition it has been shown that TNFα promotes apoptosis of endothelial cells (Geng *et al.*, 1996).

TNFα has been shown to inhibit endothelial cell proliferation *in-vitro* (Liebovich *et al.*, 1987). However, the precise mechanisms of its antiproliferative properties are unknown. Thus, some effects of TNFα on cellular proliferation appear to be entirely mediated through intracellular mechanisms that alter the cell's ability to progress through the proliferative cycle. Other effects, however, are indirect in that they result from changes in extracellular matrix or autocrine production of other cytokines by growth factors e.g. TGF-ß. Furthermore, it is also possible that the cytokines arrests the cell cycle in G-1 phase (Frater-Schroder *et al* ., 1987).

TNFα inhibits endothelial cell proliferation and promotes the migration of cultured microvascular endothelial cells, although not of large-vessel endothelial cells (Liebovich *et al.*, 1987). TNFα is angiogenic in a variety of *in-vivo* assays and promotes capillary tube formation *in-vitro* but inhibit endothelial cell proliferation *in-vitro* (Folkman & Ingber 1992).

Both TNF and IL 1 appear to activate in cells cytolytic mechanisms as well as antagonising mechanisms which can protect cells from cytolysis (Holtmann, Hahn, Wallach 1988).

IL-1 β has shown to induce programmed cell death in isolated rat ECL (Enterochromaffin-like cells, which are histamine-containing endocrine cells in the gastric mucosa) cells via activation of NF-kappaB (Mahr *et al.*, 2000). IL-1beta (100 pg/mL) increased the rate of programmed cell death 2-3 fold in ECL cells after 24 hours of incubation (total of 12%-14%). IL-1β has also been shown to be toxic to cultured human islet β cells and to induce apoptosis in these cells. The cytotoxic effect of IL-1β is potentiated by other cytokines such as TNF and interferon γ (Papaccio *et al.*, 2002). IL-1 induces apoptosis of pancreatic cells and chondrocytes (Geng *et al.*, 1996).

TNFα causes cytolysis or cytostasis of certain transformed cells, (Granger, Orr, Yamamoto 1985) although it has little effect on many cultured normal human cells (Helson *et al.*, 1975). Sato *et al.*, (1986) on the other hand have shown TNFα to be directly toxic to vascular endothelial cells. This was in consistent with the results obtained in the present study. In HUVECs, 48 hr treatment with TNFα resulted in an almost 50 % reduction in cell viability (figure 3.27 b). The decrease in cell viability in HUVECs confirmed by the MTT test and increase in cathepsin B activity confirmed by activity assay suggested that the cathepsin B could play an important role in decreasing the viability of the cells, by inducing apoptosis. Similarly, studies by Guicciardi & co-workers (2000) have implicated cathepsin B in TNFα induced apoptosis in hepatocytes.

Furthermore, treatment with IL-1 β for 48 hrs also resulted in reduction of endothelial cell viability, however this reduction in cell viability was not as high as that achieved by TNFα. It is well known that treatment of HUVECs with TNFα also results in induction of IL-1 β (Dinarello *et al.*, 1986), resulting in reduction in cell viability even further. The

results obtained for HUVECs were in contrast to that obtained for transformed cells, where the cell viability was not reduced as much.

Studies conducted by Yasuhara & co-workers (2005) have shown IL-1β to cause apoptotic death of chondrocytes *in-vitro* via overproduction of NO. Nevertheless, IL-1β and NO are also known to function as anti-apoptotic mediators in various types of cells, including chondrocytes. For example studies conducted by Kuhn, Hashimoto, and Lotz, (2000) have suggested a protective role of IL-1β. They showed IL-1β to modulate the Fas death cascade in chondrocytes by mechanisms that involve tyrosine phosphorylation events and NF-kB-dependent gene activation. Thus, it remains controversial whether IL-1β or NO contributes to chondrocyte death. Therefore the decrease in cell viability in the present study may be due to NO production, however, further experimentation is required for the elucidation of the exact mechanism.

As can be seen in figure 3.26 b, 12 hour incubation of HUVECs with TNFα and IL-1 β also resulted in decrease in cell viability of HUVECs, however the decrease was not as much as that achieved by incubation for 48 hrs.

Other actions of TNFα include growth of human fibroblasts, and other cell lines (Sugarman *et al.*, 1985) activation of polymorphonuclear neutrophils (Shalaby *et al.*, 1985) and osteoclasts (Bertolini *et al.*, 1986) and induction of prostaglandin E2 and collagenase production (Dinarello *et al.*, 1986), TNFα is currently being evaluated in treatment of certain cancers and AIDS-Related Complex. TNFα and IL-1β cause tumour regression and increase median survival time in a variety of cancer patients. Studies by Shen & co-workers (2002) tested the novel idea that the two cytokines inhibit breast cancer proliferation by impairing mitotic and antiapoptotic activities that are mediated by growth factor receptors.

In the current studies, ELISA was carried out in order to detect cathepsin B and DPP IV protein in control and cytokine treated HRT 18, HT 29 cells, and HUVECs.

In regards to TNFα treated transformed cells (HRT 18) there was an increase in protein content and a decrease in enzymes activity intracellularly at all concentrations, however there was a correlation between extracellular protein content of cathepsin B and its enzyme activity. A correlation was demonstrated between intracellular cathepsin B protein and enzyme activity treatment with IL-1 β at all concentrations but extracellular cathepsin B protein was not in correlation with the enzymes activity at concentrations of 50 ng/ml, 100 ng/ml and 250 ng/ml.

Nevertheless, in TNFα and IL-1 β treated HT 29 cells there was a correlation between both intracellular and extracellular protein content of cathepsin B and its enzyme activity, i.e. there was a downregulation in intracellular protein content and its activity, and an upregulation in extracellular protein and its activity.

Protein concentrations of cathepsins B has been previously measured by Lah & co-workers (2000) in invasive breast tumour cytosols. These potential biological prognostic indicators were compared with other histopathological parameters, such as tumour size, lymph node involvement, tumour-node-metastasis stage, histological grade, DNA analysis, and steroid receptors. Cathepsin B protein level was found to be higher in larger tumours. As prognostic markers, cathepsin B concentration was significantly linked with increased risk of recurrence.

Foghsgaard & co-workers (2001) showed that in WEHI-S fibrosarcoma cells, TNFα induced an increase in cytosolic cathepsin B activity followed by cellular death with apoptotic features. Treatment of the cells with TNFα resulted in neither a significant increase in the protein levels of pro-cathepsin B or mature cathepsin B. However,

treatment of the cells resulted in cellular redistribution of the protein. Cathepsin B was shown to disappear from perinuclear granules (co localizing with lysosomal markers) and was distributed diffusely throughout the cell. This was not consistent with the findings from the present study for HRT 18 cells, where treatment with of the cells with TNFα resulted in downregulation of cytosolic cathepsin B activity and a significant increase in its protein levels.

In disease conditions, increases in the expression of cathepsin B occur at both the gene and protein levels. At the gene level, the altered expression results from gene amplification, elevated transcription, use of alternative promoters and alternative splicing. These molecular changes lead to increased cathepsin B protein levels and in turn redistribution, secretion and increased activity (Yan & Sloane., 2003).

In TNFα and IL-1 β treated HRT 18 cells there was a correlation between intracellular protein content and enzyme activity of DPP IV. However, in terms of extracellular DPP IV protein content and enzyme activity there was no correlation between the two. In TNFα treated HT 29 cells there was a correlation between intracellular and extracellular DPP IV protein content and enzyme activity.

In HUVECs there was a no correlation between intracellular protein content and enzyme activity of cathepsin B following treatment with TNFα and IL-1β, i.e. downregulation of intracellular cathepisn B protein and an upregulation in its enzyme activity was observed. However, there was a correlation between extracellular protein content and enzyme activity following treatment with both TNFα and IL-1 β, since there was a significant increase in both cathepsin B protein and its enzyme activity. At protein level, using ELISA, it has been shown that the cytokine IL-6, does not induce increased amounts of

cathepsin B in human endothelial cell lines (in contrast to IL-1β), while it induced increase amounts of cathepsin L (Gerber *et al.*, 2000). Huet & co-workers (1993) demonstrated that TNFα and IL-1β stimulated the secretion of a cathepsin B activity and protein from synovial fibroblast-like cells. Similar pattern was observed in the present study where treatment of HUVECs with TNFα and IL-1β resulted in secretion of cathepsin B activity and protein.

Work of Fujiwara *et al* (1994) indicated that TNFα and IL-1 β stimulate DPPIV protein and activity in human luteinising granulosa cells *in-vitro*. They concluded that these cytokines were involved in the differentiation of granulosa cells during corpus luteum formation.

Upregulation of DPP IV expression on human gingival fibroblasts was examined in response to various stimulants including IL-1 β, TNFα, gamma interferon; lipopolysaccharide from *Porphyromonas gingivalis*, *Prevotella intermedia*, *Escherichia coli*, and *Prevotella* glycoprotein, which augmented DPP IV expression on gingival fibroblasts (Nemoto *et al.*, 1999).

The present study found no correlation between DPP IV protein and enzyme activity in endothelial cells, when treated with TNFα and IL-1β. Kehlen *et al.* (1998) demonstrated that both TGF-β1 and TNFα down-regulated aminopeptidase A and DPP IV mRNA as well as protein expression in renal tubular epithelial cells and renal cell carcinoma cells in culture. Similar findings were obtained in present study, where a downregulation of DPP IV protein expression was observed in HUVECs and cancer cells (HRT 18 and HT 29).

Inhibitors Mu-Phe-Hph-FMK, for cathepsin B enzyme, and Gly-Pro-Gly-Gly, for DPP IV enzyme, were used in order to assess their ability/extent to inhibit the enzyme activity. Varying concentrations of the two inhibitors were used in order to assess the concentration optimal for enzyme inhibition.

The inhibition of cathepsin B has been postulated to be directly responsible for the abolition of the invasion process in several tumour cells lines (Sinha *et al.*, 2001).

Treatment for cancer, traditionally dominated by cytotoxic and hormonal therapies, is currently being revolutionized by the emergence of innovative, targeted therapies with the potential to offer improved efficacy and reduced toxicity.

In the present studies optimal inhibition of cathepsin B enzyme was achieved by 40 μM Mu-Phe-Hph-FMK at incubation period of 30 minutes, in all the three cell lines HRT 18, HT 29, and HUVECs. Inhibition of cathepsin B in HUVECs, figure 3.34 b, was more prominent than that in the two cancer cells (figure 3.32 b, for HRT 18 cells and figure 3.33 b, for HT 29 cells).

FMK is a trapping group responsible for irreversible inhibition, but is non-cytotoxic. Inhibition occurs when the the FMK group covalently bonds to the –SH of an adjacent cysteine residue on the target protein.

Several inhibitors, as discussed under section 1.4 are used in inhibition assays. However, Mu-Phe-Hph-FMK (inhibitor of cathepsin B) is not been investigated to so much extent. Since not many studies have been conducted using the inhibitor Mu-Phe-Hph-FMK for cathepsin B, therefore the study conducted was to explore the possible usefulness of this inhibitor.

Studies carried out by Lee & co-workers (2002) assessed the inhibition of cathepsin B by Mu-Phe-HPh-FMK in promastigotes cells, at 100 μM for 60 mins. There was no inhibition of cathepsin B observed in cells treated with Mu-Phe-HPh-FMK. However the present study did note an inhibition of enzyme activity in cell lines HRT 18, HT 29, and HUVECs (incubated with the inhibitor for 30 minutes). Maximum inhibition was observed at 40 μM (intracellular) and 10 μM (extracellular). The discripency in the two studies could be due to the different cell lines producing different effect and also the different incubation period.

DPPIV has been implicated in several diseases, therefore it is important to develop selective inhibitors for human DPPIV that are able to control the biological function of DPP IV.

Inhibitors of the regulatory protease DPP IV are currently under development in preclinical and clinical studies (several pharmaceutical companies, now in Phase III) as potential drugs for the treatment of type 2 diabetes (Mest & Mentlein, 2005).

anti-Substituted β-methylphenylalanine derived amides have been shown to be potent DPP-IV inhibitors (Xu *et al.*, 2005). 4-(2-Aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF-HCL) is a irreversible inhibitor of serine proteases, AEBSF is much less toxic than PMSF and DFP.

DPP IV has been implicated in immune disorders, HIV-1 infection and tumour progression and hence its inhibitors are of immense potential value. Lung cancer is one type of cancer that is particularly difficult to treat (Foltz *et al.*, 2004). Therefore, assessment of many new inhibitors of DPP IV is viewed as a major step towards improved options in the treatment of cancers.

N-Peptidyl-O-acylhydroxylamines and boronic acid analogues of proline and alanine are two known DPP IV inhibitors. The major drawbacks for their therapeutic use are for the hydroxylamines, the toxicity and for the boronic acid derivatives, the chemical liability. A low toxicity, acceptable stability and a high specificity are essential criteria for the design of inhibitors that are suitable, not only for experimental, but also for therapeutic use. (Borloo & De Meester 1994).

The present study assessed the inhibition capability of Gly-Pro-Gly-Gly in cell lines, HRT 18, HT 29, and HUVECs. The inhibitor inhibited DPP IV at optimal when incubated for 30 minutes at 100 μM. Studies conducted by Bauvois (1990) have shown murine thymocytes to be inhibited by phenylmethane sulfonyl fluoride, diprotin A, Gly-Pro-Ala and Gly-Pro-Gly-Gly.

The purpose of the present study was to shed some light on the usefulness of cytokines TNFα and IL-1 β on the treatment of cancer. It is a well known fact that both the cytokines play an important role in tumour progression, and also in tumour regression, therefore the present study investigated the possible role of these cytokines in tumour regression. In addition, since the role of proteases, including cathepsin B and DPP IV, have been implicated in cancer progression the study undertaken hoped that the treatment of cancer cells with cytokines would modulate cathepsin B and DPP IV activity and hence would regress tumour growth.

Results from the present study showed HRT 18 cancer cells and HT 29 cells to respond differently to the treatment of the cells with cytokines (TNFα and IL-1 β), as appose to the response that was shown by the endothelial cells isolated from human umbilical vein. The cancer cells HRT 18 and HT 29 cells both showed no significant change in cell number when treated with TNFα for 30 hours and 18 hours respectively (time points that gave optimal cathepsin B activity in controls), whereas a significant decrease in cell number was noted in normal endothelial cells, suggesting that the cancer cells had a mechanism of self protect, whereas normal cells were unable to do so.

Cancer cells acquire a mechanism that protects them from apoptosis. Cathepsin B has been shown by several studies to be involved in, or to participate in the induction of apoptosis. Furthermore, since the present study also detected decrease in intracellular catrhepsin B activity in the cancer cells, and increase the enzyme activity in endothelial cells, it is possible that the cancer cells showed no significant change in cell viability by mechanism that decreased intracellular cathepsin B activity, while on the other hand normal endothelial cells were unable to adopt to this and have an increase in its intracellular cathepsin activity, which may have played a role in the death of the endothelial cells.

The information that cancer cells use different mechanism then normal cells in order for then to survive gives us an opportunity to explore mechanisms involved in both the cell types.

The revealations made from the present study shed some important insight into the involvement of cytokines and proteases in either progression or regression of cancer. The study conducted determined the role of the cytokines in the progression of cancer at higher concentrations.

Therefore, it can be overall concluded that the cytokines TNFα and IL-1β play a vital role in modulating cathepsin B and DPP IV expression. In small concentrations, the cytokines upregulate the enzymatic activity. However, higher doses have the opposite effect i.e. downregulation of the enzyme activity. Thus, by manipulating the dose of these cytokines, the enzyme activity can be increased or decreased. In addition, the viability studies also revealed that the cytokines also have a varied effect on different cell types depending on the dose used.

Therefore, full understanding of the molecular mechanism(s) of TNFα and IL-1 β will provide the basis for a pharmacological approach intended to inhibit or potentiate selected biological actions of these cytokines.

Moreover, cathepsin B and DPP IV play a vital role in the angiogenic process by destroying the basement membrane and making way for new blood vessels. Therefore, it can be postulated that TNFα and IL-1 β have the potential of being used as modulators of angiogenesis. This potential applied role of both the cytokines combined with the potent inhibitory effects of Mu-Phe-Hph-FMK (cathepsin B) and Gly-Pro-Gly-Gly (DPP IV) offer distinct opportunity to significantly influence the future of cancer treatment.

The focus of the present study was to understand the role and influence of cytokines, TNFα and IL-1 β, on the two proteases cathepsin B and DPP IV, and also to understand the role of the cytokines and proteases in tumour angiogenesis and metastasis.

A major emphasis of research on cancer progression has focussed around model systems to characterise each of the sequential events making up the metastatic cascade. The *in-vitro* experiments by which cancer progression have been assessed has generally been compared with results of an *in-vivo* metastasis model in which tumour cells are injected in mice to determine the number of metastasis.

Considerable insight in both the molecular and cellular biology of angiogenesis has been obtained by *in-vitro* studies using endothelial cells (Cockerill, Gamble, Vadas 1995). In addition, steps in angiogenic cascade such as endothelial proliferation, migration and differentiation can be analysed *in-vitro* (Fan & Polverini 1997).

Although a strong role for cathepsin B in growth and metastasis of many types of tumour has been established by invasion and metastasis studies *in-vitro*, there is insufficient data demonstrating the efficacy of cathepsin B inhibitors as anti-metastatic agents in *in-vivo* models (Hulkover *et al.*, 2000). Nevertheless, study regarding involvement of DPP IV and its inhibitors either *in-vitro* or *in-vivo* is greatly lacking. Indeed a compound that may cause a significant effect *in-vitro* may not elicit the same response *in vivo* as such, it is crucial that the compounds to be tested should be studied *in-vivo*.

Similarly, a single cytokine may elicit a reaction under a certain condition, however, this same reaction could be reversed under other conditions. For example, the type, duration and as well as the extent of cellular activities induced by a particular cytokine can be influenced by the

micro-environment of the cell. For this reason, it would valuable to investigate the influence of cytokines TNFα and IL-1 β, on *in-vivo* models.

Chick chorioallantoic membrane (CAM), rabbit cornea assay, sponge implant models, matrigel plugs and conventional tumour models are all classical *in-vivo* models that can be used to evaluate potency of both angiogenic and anti angiogenic compounds (Ribatti & Vacca 1999).

Future studies will focus on the CAM model, an established *in-vivo* angiogenesis assay (Maas *et al.*, 1999). It was introduced by Folkman and his colleagues to assay the angiogenic activity of various tumours, normal tissues, and cells (Splawinski *et al.*, 1988).

This particular *in-vivo* assay mimics the angiogenic process in the developing chick using the accessible chorioallantoic membrane with its developing network of blood vessels. Since the early chick embryo lacks a mature immune system, xenografts from mammalian species are able to establish and grow (Folkman 1975). Usually the CAM appears approximately 7 days following fertilisation. At this stage, materials to be analysed can be implanted into the CAM. Figure 5.1 shows an image of CAM after implantation of an angiogenic inhibitor, which eventually leads to inhibition of vessel growth within the CAM (figure 6.0 b).

(a) (b)

(http://glycotech.com/angio/angio4.jpg)

Figure 6.0: *Image (a) refers to control and image (b) shows inhibition of vascular development by an angiogenic inhibitor.*

The aim of the future study would be to assess the angiogenic properties of proteases and cytokines, and to quantitate the vascular response.

Certain enzyme inhibitors such as pepstatins, organophosphates and coumarins block the action of proteases and are able to cut off the blood vessel formation in tumours (Sinha *et al.*, 1995). Hence, inhibitor studies would also be carried out. Effect of various cathepsin B and DPP IV inhibitors, such as Leupeptin, L-trans-epoxysuccinyl-leucyl amido (4-guanidino) butane (E64), ZPhe-Arg-Arg-CHN2 and thiazolidines will examine the ability of enzyme inhibitors to inhibit angiogenesis. These results would also allow us to elucidate the possible mechanism(s) by which such inhibitors would inhibit vessel formation.

The many specific activities of individual cytokines have been the basis for current concepts of therapeutical intervention, in particular tumour therapy. A better understanding of the proteases and inhibitors involved in tumour progression may also allow for therapeutic intervention at the earlier stages of tumour progression.

There is accumulating evidence that tumour growth and metastases are angiogenesis-dependent, and there is also evidence that proteases are involved in metastasis. Therefore, the experiments will shed some light on the mechanism(s) by which these enzymes promote angiogenesis.

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http://www.anticancer.com/HDRA_reader.jpg

http://www.dojindo.com/newimages/MTT-RS.jpg

http://www.uq.edu.au/vdu/ELISA.htm

http://glycotech.com/angio/angio4.jpg

Appendix 1

Materials

Amersham Pharmacia Biotech, Buckingh.U.K.	TNFα (human recombinant), IL-1β (human recombinant).
BDH Laboratory Supplies, Poole, UK.	DPX mountant.
Chemicon International, Harrow, U.K.	sCD26 Elisa kit.
Dako Limited, Bucks. U.K.	Mouse anti-human CD 31, biotinylated goat anti-mouse IgG, rabbit anti-human vWF, biotinylated goat anti-rabbit IgG.
ECACC, Salisbury, U.K.	HRT 18 and HT 29 cell lines, Human liver cells, human thyroid follicular epithelial cells.
Exclusive group plc, U.K.	Isoton II
Fisher Scientific, Leicestershire, UK.	Cryogenic vials, Tips.
IDS, Tyne & Wear, U.K.	Human cathepsin B elisa kit.
Life Technologies, Paisley, U.K.	HBSS, MEM, RPMI, PBS, DMEM, Iscoves, FCS, NEAA, chamber slides, tissue culture flasks, universals, pipettes, petri dish.
Merck Biosciences Ltd, Nottingham, U.K.	Diprotin, CA-074.
Roche Diagnostic, Lewes, East Sussex, UK.	Collagenase A
Serotec, Kidlington, Oxford, UK.	Rabbit anti-human vWF, biotinylated goat anti-rabbit IgG.

Sigma Co Ltd., Poole, Dorset.	Tri-reagent, isopropanol, fibronectin, Gly-Pro 7amido-4Methyl Coumarin hydrobromide, Nα-CBZ-Arg-Arg 7amido-4Methyl Coumarin hydrochloride, heparin, ECGS, penicillin-streptomycin, Depc, BSA, SDS, Anti sheep IgG peroxidase conjugate, Anti mouse IgG peroxidase conjugate, DAB, DMF, DTT, MES, β mercaptaethanol, Folin-ciocalteau phenol reagent, EDTA, MTT reagent, Mu-Phe-Hph-FMK, Gly-Pro-Gly-Gly.
Three S Healthcare, Southgate, London, UK.	Avon kwill filling tube.
Vector Laboratories, U.K.	Vectastain ABC kit.

Umbilical cords were obtained from St. George's hospital in Tooting.

Appendix 2

Cell culture growth media

HRT 18 cells - RPMI 1640 media was supplemented with 10% FCS, 2mM glutamine, 1% streptomycin and penicillin and 1% fungizone.

HT 29 cells - MEM media was supplemented with 10% FCS, 1% NEAA, 2mM glutamine, 1% streptomycin and penicillin, 1% fungizone.

HUVECs - Iscove's media was supplemented with 20% FCS, 2% heparin, 3mg/ml ECGS, 1% streptomycin and penicillin and 1% fungizone.

Liver cells - EMEM media was supplemented with 10% FCS, 1% NEAA, 2mm glutamine, 1% streptomycin and penicillin and 1% fungizone.

Thyroid cells - RPMI 1640 media was supplemented with 10% FCS, 2mM glutamine, 1% streptomycin and penicillin and 1% fungizone.

Appendix 3

Enzyme Assay

Cathepsin B

Substrate: Z-Arg-Arg-AMC
Made into 20mM stock by dissoving 5 mg in 402 μl DMF, then 1mM working solutions diluted with ddw.
Buffer: 0.1 M MES pH 5.5 with 0.1% v/v Triton X-100
DTT: 100mM solution by dissolving 18 mg/ml ddw.

Assay
Total volume per well (96 well plate) = 200 μl

Sample	50 μl (media) extracellular	0 μl (ie cells) intracellular
Buffer (0.1 M pH 5.5)	135 μl	185 μl
DTT (100 mM)	5 μl	5 μl
Substrate (1 mM)	10 μl	10 μl

DPPIV

Substrate: Gly-Pro-AMC
Made into 10mM stock by dissolving 5 mg in 1218 μl DMF, then 1 mM working solutions.
Buffer: 0.1 M Tris-HCL pH 8.0 with 0.1% v/v Triton X-100

Assay
Total volume per well (96 well plate) = 200 μl

Sample	50 μl (media) extracellular	0 μl (ie cells) intracellular
Buffer 0.1 M pH 8.0)	140 μl	190 μl
Substrate (1 mM)	10 μl	10 μl

Appendix 4

Immunocytochemistry

Endothelial cell marker

	CD 31	vWF
Primary Antibody	Mouse anti-human CD 31	Rabbit anti-human vWF
Dilution	1:10	1:100
Secondary Antibody	Biotinylated goat anti-mouse IgG	Biotinylated goat anti-rabbit IgG
Dilution	Already prepared (neat)	1:200

Appendix 5

ELISA Dilutions

	Intracellular Cathepsin B protein in HRT 18 cells		Extracellular Cathepsin B protein in HRT 18 cells	
Dilution Control	Average Absorbance nm	Average Concentration ng/ml	Average Absorbance nm	Average Concentration ng/ml
1 in 1	2.098	178.41	0.045	0.291
1 in 2	2.491	237.696	0.002	0.002
1 in 4	2.020	167.465	-0.016	-1.000
1 in 8	0.553	19.221	-0.009	-1.000
1 in 16	0.335	8.318	-0.023	-1.000
1 in 32	0.180	2.946	-0.024	-1.000
1 in 64	0.074	0.667	-0.030	-1.000

	Intracellular Cathepsin B protein in HT 29 cells		Extracellular Cathepsin B protein in HT 29 cells	
Dilution Control	Average Absorbance nm	Average Concentration ng/ml	Average Absorbance nm	Average Concentration ng/ml
1 in 1	2.772	284.177	0.089	0.908
1 in 2	2.517	241.856	0.035	0.191
1 in 4	1.101	60.745	0.008	0.016
1 in 8	0.522	17.455	-0.010	-1.000
1 in 16	0.317	7.585	-0.018	-1.000
1 in 32	0.181	2.974	-0.014	-1.000
1 in 64	0.081	0.776	-0.029	-1.000

	Intracellular Cathepsin B protein in Endothelial cells		Extracellular Cathepsin B protein in Endothelial cells	
Dilution Control	Average Absorbance nm	Average Concentration ng/ml	Average Absorbance nm	Average Concentration ng/ml
1 in 1	2.311	209.697	0.095	1.013
1 in 2	2.062	173.324	0.037	0.209
1 in 4	1.349	85.297	0.058	0.444
1 in 8	0.565	19.923	0.028	0.131
1 in 16	0.243	4.865	0.032	0.164
1 in 32	0.127	1.645	0.043	0.269
1 in 64	0.050	0.346	-0.022	-1.000

	Intracellular DPP IV protein in HRT 18 cells		Extracellular DPP IV protein in HRT 18 cells	
Dilution	Average Absorbance nm	Average Concentration ng/ml	Average Absorbance nm	Average Concentration ng/ml
1 in 1	2.477	1618.768	-0.018	3.508
1 in 2	1.836	1203.785	-0.06	-23.683
1 in 4	1.543	1014.097	0.071	61.126
1 in 8	0.234	166.652	0.11	86.375
1 in 16	0.096	77.311	-0.067	-28.215
1 in 32	0.028	33.288	-0.08	-36.631
1 in 64	0.061	54.652	-0.061	-24.331

	Intracellular DPP IV protein in HT 29 cells		Extracellular DPP IV protein in HT 29 cells	
Dilution	Average Absorbance nm	Average Concentration ng/ml	Average Absorbance nm	Average Concentration ng/ml
1 in 1	2.037	1333.912	-0.004	12.571
1 in 2	1.688	1107.970	0.008	20.340
1 in 4	1.009	668.386	0.103	81.843
1 in 8	0.237	168.594	-0.042	-12.030
1 in 16	0.075	63.716	0.015	24.872
1 in 32	0.086	70.837	0.117	90.906
1 in 64	0.068	59.184	-0.054	-19.799

	Intracellular DPP IV protein in Endothelial cells		Extracellular DPP IV protein in Endothelial cells	
Dilution	Average Absorbance nm	Average Concentration ng/ml	Average Absorbance nm	Average Concentration ng/ml
1 in 1	0.149	111.623	-0.013	6.745
1 in 2	-0.007	10.629	-0.065	-26.920
1 in 4	0.019	27.461	-0.023	0.271
1 in 8	0.019	27.461	0.207	149.172
1 in 16	0.085	70.19	0.032	35.877
1 in 32	-0.052	-18.504	0.015	24.872
1 in 64	0.216	154.999	-0.066	-27.568