ISOLATION, PURIFICATION AND PARTIAL CHARACTERISATION OF CANCER PROCOAGULANT FROM PLACENTAL AMNION-CHORION MEMBRANES AND ITS ROLE IN ANGIOGENESIS, INFLAMMATION AND METASTASIS

By

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Promoter: Prof CL Frost

DEDICATION

In loving memory of Germaine Scheepers and Simthembile Pambuka, my friends and fellow scientists. You will always be remembered.

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DECLARATION

I, Jason Krause (203007174), hereby declare that the thesis for the degree *Philosophiae Doctor* is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification, .

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ABSTRACT

Cancer procoagulant (EC 3.4.22.26) is an enzyme that is derived from tumour and foetal tissue, but not normal tissue. It is a direct activator of factor X and has been isolated from amnion-chorion membranes as well as from extracts and cells from human melanoma. The presence of cancer procoagulant has been associated with the malignant phenotype, as well as having a particularly high activity in metastatic cells. Cancer procoagulant activity is elevated in the serum of early stage breast cancer patients and decreased to normal in the advanced stages of the disease.

In this study, cancer procoagulant was successfully isolated from amnion-chorion membranes and purified to homogeneity. The molecular weight of cancer procoagulant was determined using SDS-PAGE and was found to be 68 kDa. Cancer procoagulant was delipidated and it was shown that its activity was increased by the presence of lipids in a dose-dependent manner. Recovery of cancer procoagulant after delipidation is poor, consequently, a larger mass of sample is required to obtain sufficient amounts of delipidated material for N-terminal amino acid analysis. The optimum pH of cancer procoagulant was determined to be pH 8 and its optimal temperature was found to be 50°C. Novel synthetic substrates were designed to assay for cancer procoagulant activity. Currently, 2 potential candidates have been identified, namely, PQVR-AMC and AVSQSKP-AMC. Cancer procoagulant-induced expression of cytokines is differently modulated in the less aggressive MCF-7 cell line as compared to the metastatic and more aggressive MDA-MB-231 cell line. There are marked similarities in the inflammatory response produced by cancer procoagulant in hTERT-HDLEC and MDA-MB-231 cells, which are both associated with migratory capacity. Furthermore, cancer procoagulant-induced PDGF-β expression in hTERT-HDLEC and MDA-MB-231 cells could point to involvement of cancer procoagulant in wound healing and metastatic spread, respectively. Cancer procoagulant induced the motility of MDA-MB-231, MCF-7 and hTERT- cells *in vitro* in a time- and dose-dependent manner.

Together, these results suggest that cancer procoagulant plays a role in the migration of breast cancer cells as well as the migration of endothelial cells.

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List of Abbreviations

17b-HSD	17-b hydroxysteroid dehydrogenase
AMC	7-amino-4-methylcoumarin
Ang-1	Angiopoetin 1
ATRA	All-trans retinoic acid
AVSQSKP-AMC	Alanine-Valine-Serine-Glutamine-Serine-Lysine-Proline-AMC
AVYQPQP-AMC	Alanine-Valine-Tyrosine-Glutamine-Proline-Glutamine-Proline-AMC
bFGF	Basic fibroblast growth factor
bFGF-2	Basic fibroblast growth factor 2
Boc	Butyloxycarbonyl
Boc-AVR-AMC	Boc-Alanine-Valine-Arginine-AMC
Boc-AVSQSKP-AMC	Boc-Alanine-Valine-Serine-Glutamine-Serine-Lysine-Proline-AMC
Boc-AVYQPQP-AMC	Boc-Alanine-Valine-Tyrosine-Glutamine-Proline-Glutamine-Proline-AMC
Boc-PQVR-AMC	Boc-Proline-Glutamine-Valine-Arginine-AMC
Boc-QVR-AMC	Boc-Glutamine-Valine-Arginine-AMC
Boc-RGD-AMC	Boc-Arginine-Glycine-Aspartate-AMC
CAC	Colitis-associated-cancer
CathB	Cathepsin B
CathD	Cathepsin D
CBB	Coomassie Brilliant Blue
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
c-MET	Met proto-oncogene (hepatocyte growth factor receptor)
CMFDA	5-cloromethylfluorescein diacetate
COX-2	Cyclooxygenase-2
CP	Cancer procoagulant
CSC	Cancer stem cell
CXCR4	C-X-C chemokine receptor type 4
DIC	Disseminated intravascular coagulation
DLD-1	Human colon adenocarcinoma cells
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethylsulfoxide
E_0	Total enzyme concentration
E1	Oestrone
E2	Oestradiol
ECM	Extracellular matrix
ECs	Endothelial cells
EGM-2MV	Microvascular endothelial cell growth medium
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-Mesenchymal Transition
EP	E-series prostaglandin receptors
Eph	Ephrins
ER	Oestrogen receptor
FBS	Foetal bovine serum

FGF	Fibroblast growth factor
Flk-1	Foetal liver kinase 1
FXa	Activated factor ten
gp	Glycoprotein
HCT-116	Human colorectal carcinoma 116 cells
HDLEC	Human dermal lymphatic cells
HGF	Hepatocyte growth factor
HGF/SF	Hepatocyte growth factor/scatter factor
HIF-1	Hypoxia-inducible factor 1
HT-29	Human colon adenocarcinoma grade II cell line
hTERT	Human telomerase reverse transcriptase
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IGF	Insulin growth factor
IL	Interleukin
IL-1	Interleukin 1
IL-1R	Interleukin-1 receptor
IL-1-β	Interleukin 1 beta
IL-6	Interleukin 6
JAK	Janus kinase
KDR/Flk-1	Kinase insert domain receptor/foetal liver kinase 1
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protease
MAPK	Mitogen activated protein kinase
MCF-7	Michigan Cancer Foundation 7
MDA-MB-231	Mammary gland adenocarcinoma cells
MET	Mesenchymal-to-epithelial transition
MM	Multiple myeloma
MMP-2, -8	Metalloproteinase-2, -8
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MVD	Microvessel density
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated β cells
NK	Natural killer
P53	Tumour protein 53
PA	Plasminogen activator
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor type-1
PAR-1, -2, -3, -4	Protease-activated receptor -1, -2, -3, -4
PARs	Protease-activated receptors
PDGF-BB	Platelet-derived growth factor beta homodimer
PDGFR-β	Platelet-derived growth factor beta receptor
PDGF-β	Platelet-derived growth factor β
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2

PGF	Placental growth factor
PQVR-AMC	Proline-Glutamine-Valine-Arginine-AMC
PTEN	Phophatase and tensin homologue
RAS	Rat sarcoma
RFU	Relative fluorescence units
RGD-AMC	Arginine-Glycine-Aspartate-AMC
RVV	Russel's Viper Venom
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	Scatter factor
sFlt-1	Soluble fms-like tyrosine kinase-1
siRNA	Small interfering ribonucleic acid
STAT	Signal transducer and activator of transcription
SW-480	Human colon adenocarcinoma cell line
TAMs	Tumour-associated macrophages
TAP	Tick anti-coagulant peptide
TF	Tissue factor
TGF	Transforming growth factor
TGF-β	Transforming growth factor β
Th1	T helper cells
TIMPs	Tissue inhibitors of matrix metalloproteases
TN-C	Tenascin-C (human)
TNFR(s)	Tumour necrosis factor alpha receptor(s)
TNF-α	Tumour necrosis factor alpha
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF-A, -B-, C-, D, -E	Vascular endothelial growth factor A, B, C, D, E
VEGFR-1, -2, -3	Vascular endothelial growth factor receptor 1, 2, 3

1. LITERATURE SURVEY

1.1 Metastasis

Metastasis can be defined as the spread of malignant cells from a primary tumour to distant sites (Al-Mehdi *et al*., 2000). It poses the biggest problem to cancer treatment and is the main cause of death of cancer patients (Duffy *et al*. 2008; Wittekind and Neid, 2005; Chaffer and Weinberg, 2011; Riihimaki *et al*., 2013). It occurs in a series of discrete steps (Figure 1.1), which have been modelled into a "metastatic cascade". These steps include: Epithelial–Mesenchymal Transition (EMT), invasion, anoikis (detachment-induced cell death), angiogenesis, transport through vessels and outgrowth of secondary tumours. A "pre-metastatic niche" (presented in Figure 1.1 as step 0) is formed before metastasis manifests itself. This "pre-metastatic niche" is induced by a distant primary tumour and is mediated by bone marrow-derived cells. Following the establishment of the "pre-metastatic niche", cells in the primary tumour undergo Epithelial–Mesenchymal Transition (EMT) and acquire invasive properties (step 1, Figure 1.1) (Bonnomet *et al*., 2010). Cells undergoing EMT also acquire cancer stem cell (CSC)-like characteristics. These characteristics include self-renewal capacity, dormancy, active DNA repair, re-differentiation, drug resistance, and resistance to apoptosis. This causes resistance to chemotherapeutic agents (Monteiro and Fodde, 2010; Chaffer and Weinberg, 2011). In step 2, the basement membrane is degraded and remodelling of the Extracellular Matrix (ECM) takes place. This is accomplished by proteinases which facilitate tumour cell invasion (Tryggvason *et al*., 1987; Duffy *et al*., 2008). Step 3 involves invasion of the surrounding tissue by either single tumour cells (3a) or collectively (3b). The fourth step involves intravasation of tumour cells into the newly formed vessels within or in the vicinity of the tumour. Tumour cells are transported in step 5 through the vasculature and arrested in a capillary bed where they extravasate (step 6, Figure 1.1) and can remain dormant for years (Bonnomet *et al*., 2010). In step 8, some disseminated cells form a secondary tumour or macrometastasis. This requires, in addition to ECM remodelling, angiogenesis (step 9, Figure 1.1). Cells that are outside their normal microenvironment go through anoikis, which is apoptosis that is induced by cell detachment (Howe *et al*., 2012). Anoikis could impede the formation of

metastasis at several steps of the cascade, as indicated by the stars in the Figure 1.1. Not all steps of the metastatic cascade necessarily occur in a linear way as outlined above. For instance, pre-malignant tumours can already be vascularized while the timing of induction of the pre-metastatic niche remains mysterious.

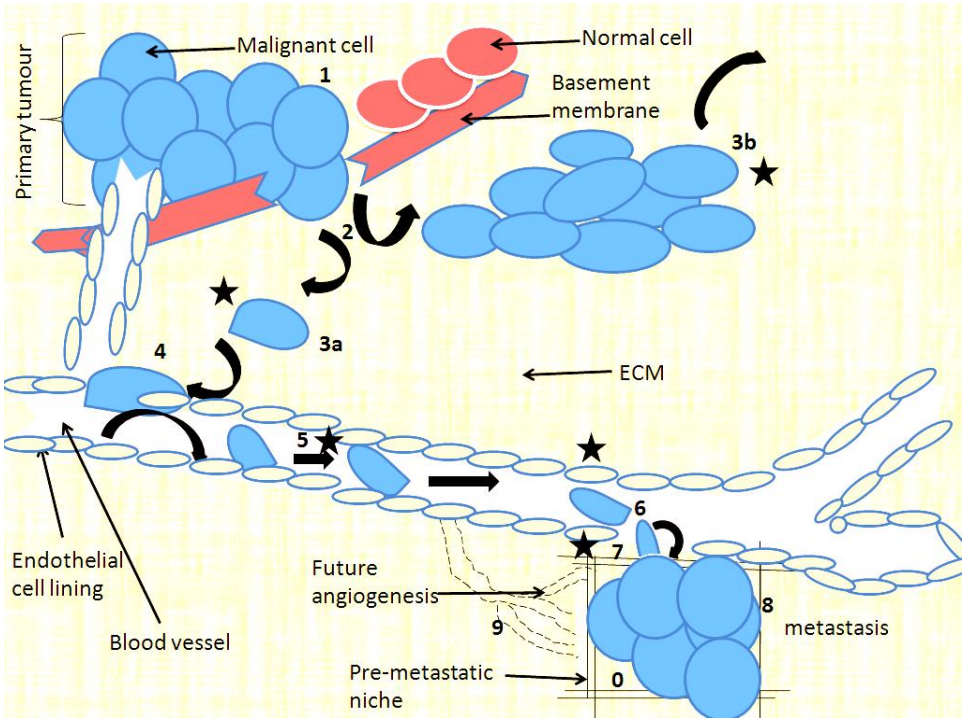


Figure 1.1 The metastatic cascade. The steps of this cascade is outlined in the text (Adapted from Geiger and Peeper, 2009). 0, pre-metastatic niche; 1, EMT; 2, ECM remodelling; 3a, invasion (single cells); 3b, invasion (collective); 4, intravasation; 5, tumour cell arrest; 6 and 7, extravasation; 8, macrometastasis/secondary tumour formation; 9, angiogenesis.

Despite the prevalence of secondary tumours in cancer patients, metastasis is an extremely inefficient process (Wong *et al*., 2001). To successfully colonize a distant site, a cancer cell must complete all of the steps of the cascade. Failure to complete any one step results in failure to colonize and proliferate in the distant organ (Wittekind and Neid, 2005). As a result, tumours can shed millions of cells into the bloodstream daily, yet very few clinically relevant metastases are formed (Wittekind and Neid, 2005). In spite of this metastatic inefficiency, the total extent of metastatic spread is huge and, as mentioned before, represents the primary cause of cancer morbidity and mortality (Wittekind and Neid, 2005; Chaffer and Weinberg, 2011). A comprehensive understanding of the biological and pathological intricacies underpinning the process of metastasis is still lacking. This is due, in part, to the complexity of the metastatic cascade, which encompasses not only the biology of the

tumour cell but also the rest of the organism in which it resides. Many models have been developed to attempt to provide a working hypothesis upon which to base further research (Hunter *et al*., 2008).

Previously, it was believed that the development of metastases is a late event following the arrival of the primary tumour at a certain critical mass. This model is referred to as the linear-progression model (Foulds, 1958). Recent evidence shows that tumour cells disseminate at a relatively early stage leading to parallel growth of metastasis along with primary tumour growth. This model is known as the parallel-progression model (Klein, 2009). Metastatic spread has also been viewed as a unidirectional process with dissemination of cancer cells occurring from primary tumours only in order to form secondary metastases. Contrary to this classical belief, recent experimental evidence suggests that cancer progression is a multi-directional process in which tumour cells are capable of self-seeding the primary tumour (*Kim et al*., 2009; Comen et al., 2011). Many of the models that have been developed thus far are, however, somewhat inconsistent with current biological observation and that none sufficiently explain all of the complexities associated with the metastatic process. In spite of this, valuable insights have been gleaned from many of them, which have helped to further comprehend the underlying mechanisms of tumour dissemination and colonization.

1.1.1 Metastasis and coagulation

The coagulation cascade and thrombosis play a crucial role in metastatic spread of tumour cells (Rickles and Falanga, 2009). Thrombosis is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system (Jain *et al*., 2010). Virchow's triad describes the three broad categories of factors that are thought to contribute to thrombosis: hypercoagulability, haemodynamic changes (stasis, turbulence) and endothelial injury or dysfunction (Kyrle and Eichinger, 2005). It is named after German physician Rudolf Virchow (1821-1902) (Kyrle and Eichinger, 2009; Koster *et al*., 1995). The triad proposes that thrombosis is the result of alterations in blood flow, vascular endothelial injury, or alterations in the constitution of the blood. Virchow's triad is a useful concept for clinicians and

pathologists in understanding the contributors to venous, and perhaps arterial, thrombosis (Kyrle and Eichinger, 2009; Bagot and Arya, 2008). While the triad is now frequently applied to describe thrombosis arising within the arterial circulation, many continue to restrict it to that occurring within the venous vasculature (Bagot and Arya, 2008). Thrombus formation can result in hypoxia. Hypoxia leads to the production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), which in turn initiates the angiogenesis pathway. Within the circulatory system, platelets and fibrin guard tumour cells from elimination by the immune system through the formation of a fibrin mesh, thereby promoting the arrest of tumour cells at the endothelium (Ho-Tin-Noe *et al*., 2009). This favours the formation of secondary tumours. Furthermore, platelets play a protective role in the maintaining the tumour vascular integrity (Ho-Tin-Noe *et al*., 2009; Gay and Felding-Habermann, 2011). Tissue factor (TF) produced by tumour cells can lead to the activation of the coagulation cascade. It is capable of promoting metastasis by coagulation-independent mechanisms, such as causing increased VEGF production to promote angiogenesis, thereby maintaining tumour cell growth (Zhang *et al*., 1994; Folkman, 1996; Fernandez and Rickles, 2002).

Cancer cells depend partially on the activation of the coagulation cascade to become metastatic (Rickles and Falanga, 2009). Activated factor X (FXa) was shown to significantly decrease the migration of various cancer cells of different origins (breast, lung and colon cancer cells) (Senden *et al*., 1998). It has also been proven that FXa mediated inhibition of cancer cell migration was specific, since it was inhibited by tick anti-coagulant peptide (TAP, a specific FXa inhibitor) but not by Hirudin (a specific thrombin inhibitor). Additionally, the FXa effect was dose dependent, with a maximal inhibitory effect reached at 0.75U/ml FXa (corresponding to 130.5nM) (Borensztajn *et al*., 2009). The authors determined that FXa acted via protease-activated receptor (PAR)-1-dependent signaling, and PAR-1 desensitization, as well as knocking-down PAR-1 expression, abolished the FXa effects. They also established that Giα is not involved in FXa mediated inhibition of cell migration because its effects were not reverted by pertussis toxin. These results suggest that, apart from its role in blood coagulation, FXa plays a vital role in cancer cell migration (Borensztajn *et al*., 2009).

The discovery of a family of seven trans-membrane domain receptors known as the protease-activated receptors (PARs) has provided a possible link between the activation of the coagulation cascade and cancer progression (Borensztajn *et al*., 2009). Activation of PARs is a unique process. Most receptors require ligand binding for activation, but PARs require proteolytic cleavage instead (Asokananthan *et al*., 2002). A new tethered ligand is released through the removal of the N-terminal extracellular region. This region interacts with the body of the receptor, inducing transmembrane signalling to G proteins and in this manner regulates many cellular functions including reorganization of the cytoskeleton, cell migration along with modulation of cell survival (Shi *et al*., 2004). There are currently four known PARs (PAR-1 to -4). Thrombin activates PAR-1, -3 and -4, whereas PAR-2 is activated by FVIIa (Shi *et al*., 2004; Asokananthan *et al*., 2002; Wu *et al*., 2010). There is evidence that mediation of intracellular signalling by FXa occurs via activation of either PAR-1 and/or PAR-2 (Borensztajn *et al*., 2009). It appears that when FXa complexes with TF and FVIIa, it activates PAR-2, whereas soluble FXa activates both PAR-1 and PAR-2. It appears that the cell type and the range of receptors on individual cells are of major importance for determining whether FXa signalling is dependent on PAR-1 or PAR-2 activation (Borensztajn *et al*., 2008; Borensztajn *et al*., 2009). In particular, the activation of PAR is also tightly regulated by heterologous receptor crosstalk. For example, during angiogenesis, a negative regulatory control on PAR-2 signalling is exerted by TF. This is released by phosphorylation of a cytoplasmic domain TF (Belting *et al*., 2004; Ahamed and Ruf, 2004). Furthermore, various alternative receptors such as those of the sphingosine 1-phosphate receptor family may also modify FXa-dependent activation of PAR (Niessen *et al*., 2008). Many vital physiological and pathological processes are influenced by cell motility. For example, migration of fibroblasts and epithelial cells is required for wound healing, whereas cancer cell metastasis is also highly dependent on cell migration (Borensztajn *et al*., 2009). For cell motility to occur, two processes are important, namely: chemokinesis and chemotaxis (Shi *et al*., 2004). Chemokinesis is defined as the induction of random, non-directional motility in response to a ligand without any orientational cues. Chemotaxis on the other hand, represents a directional response of cells toward a ligand gradient (Anand-Apte and Zetter, 1997). A chemokinetic effect can occur without a chemotactic effect; however, no agonist has been

described which exclusively influences chemotaxis without having an influence on chemokinesis (Entschladen *et al*., 2005; Geiger and Peeper, 2009).

Cancer cell migration is inhibited by FXa in a specific, dose-dependent manner (Borensztajn *et al*., 2009). Specifically, FXa inhibits chemokinesis of all cancer cell lines tested, whereas it inhibited chemotaxis of the indolent MCF-7, A549, DLD-1 and HCT-116 cells, but not the more aggressive HT-29, SW-480 and MDA-MB-231 cells. The inhibition of chemokinesis was not as a result of modification of cell adhesion properties (Borensztajn *et al*., 2009). With regard to the mechanism of FXa-mediated inhibition of cell migration, it was shown that siRNA treatment of MCF-7 and MDA-MB-231 cells specifically knocking-down PAR-1 expression resulted in the observation that the inhibitory effects of FXa were mediated via PAR-1 activation (a notion confirmed by receptor desensitization) (Borensztajn *et al*., 2009; Falanga *et al*., 2009).

1.1.2 Molecular mechanism of metastasis and coagulation

The unravelling of the molecular mechanism of coagulation-mediated metastasis has been attempted by researchers since its discovery. Of particular interest is the MET oncogene. The MET proto-oncogene product (hepatocyte growth factor receptor) (c-MET) is a receptor tyrosine kinase. Its ligand is hepatocyte growth factor (HGF). When c-MET is bound to HGF activation of a wide range of different cellular signalling pathways, including those involved in proliferation, motility, migration and invasion takes place. Besides its importance in the control of tissue homeostasis under normal physiological conditions, c-MET has also been found to be abnormally activated in human cancers through mutation, amplification or protein over-expression (Comoglio *et al*., 2008). The c-MET proto-oncogene is located on chromosome 7q21-31. The protein product of this gene is the c-MET tyrosine kinase (Boon *et al*., 2002; Epstein *et al*. 1996; Gambarotta *et al*. 1996; Boccaccio *et al*. 1994). It is a cell surface receptor that is expressed in epithelial cells of many organs, such as the pancreas, liver, prostate, kidney, muscle and bone marrow, during both embryogenesis and adulthood (Peruzzi and Bottaro, 2006; Birchmeier *et al*., 2003; Comoglio and Trusolino, 2002; Comoglio *et al*., 2008). The MET oncogene encodes the tyrosine kinase receptor for hepatocyte growth factor/scatter factor

(HGF/SF), and drives a physiological cellular programme known as ‘invasive growth’, which underlies tissue morphogenesis and repair. Abnormal execution of this programme in time and localization has been associated with neoplastic transformation, invasion and metastasis (Boccaccio *et al*., 2005). Activation of the MET oncogene has been reported in a wide array of human tumours including lung (Bean *et al*., 2007), gastric (Bertotti *et al*., 2010) and skin tumours (Syed *et al*., 2011), either as a consequence of germline or sporadic mutations, or, more commonly, of overexpression, probably caused by hypoxia-induced transcription (Boccaccio and Comoglio, 2009). Figure 1.2 provides a comprehensive summary highlighting the main role players in coagulation-mediated metastasis and illustrates some of the crosstalk between a generalised cancer cell and endothelial cell. These include the activated oncogenes (MET and RAS), hypoxia-inducible factor-1 (HIF-1), tumour suppressor genes (PTEN and P53), tissue factor (TF), cyclo-oxygenase 2 (COX-2) and plasmingoen-activator inhibitor 1 (PAI-1) and will be discussed in this section.

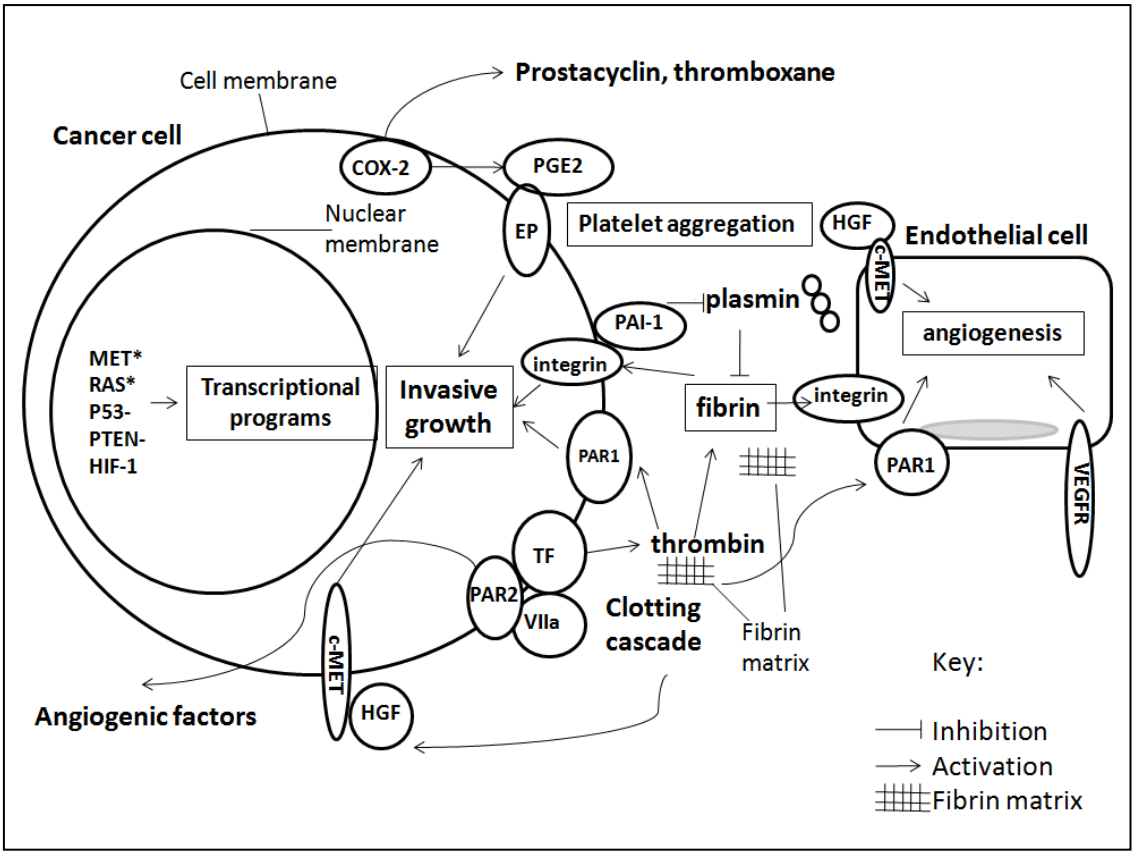


Figure 1.2 Haemostasis genes promote tumour progression. Activated oncogenes (MET*, RAS*), hypoxia-inducible factor-1 (HIF-1) and the loss of tumour suppressor genes (PTEN, P53) leads to the induction of transcriptional upregulation of tissue factor (TF), cyclo-oxygenase 2 (COX-2), and plasminogen-activator inhibitor 1 (PAI-1), resulting in promotion of haemostasis activation and fibrin deposition. This, in turn, leads to formation of a provisional matrix that favours angiogenesis and supports integrin-mediated cell adhesion and migration. Hepatocyte growth factor (HGF) is activated by coagulation proteases. This in turn leads to the activation of the receptor encoded by the MET proto-oncogene (c-MET), which is expressed by endothelial and cancer cells. The coagulation cascade generates TF and thrombin which activates cell surface receptors called protease-activated receptors (PAR-1 and 2). Platelet aggregation and prostaglandin E_2 (PGE_2) are modulated by COX-2-catalysed synthesis of prostacyclin and thromboxane. PGE_2 binds cell surface E-series prostaglandin receptors (EP). PAI-1 inhibits plasmin and fibrin degradation and also promotes integrin recycling. MET, TF, PARs, EP, vascular endothelial growth factor receptor (VEGFR), and integrins collaborate in regulating cancer cell invasive growth and angiogenesis (Adapted from Boccaccio and Comoglio, 2009).

MET overexpression can mostly be attributed to a decline in the oxygen concentration in the growing tumour mass, in view of the fact that MET is directly targeted by hypoxia inducible factor (HIF-1) (Pennacchietti *et al*., 2003). Boccaccio *et al*. (2005) recently found that MET, in addition to being co-regulated with coagulation genes in hypoxic cells, also regulates their expression (Boccaccio *et al*., 2005). It is, therefore, an oncogene capable of modulating tumour cell procoagulant activity, with local and systemic effects (Boccaccio and Camoglio, 2009).

A second factor contributing to the molecular basis of coagulation-mediated metastasis is fibrin. Fibrin aids unrestricted tumour growth through promotion of angiogenesis and represents a reservoir of growth and angiogenic factors, such as HGF, VEGF, and fibroblast growth factor, which bind fibrin directly or indirectly through heparin (Mosesson, 2005). By engaging $\alpha_v\beta_3$ integrin (expressed specifically during the angiogenic phase) and vasculoendothelial cadherin, fibrin also provides direct anchorage to endothelial cells (Mosesson, 2005; Bach *et al*., 1998). Due to the association of blood vessels with tumours usually displaying structural alterations and increased permeability, fibrin deposition is enhanced (Fernandez *et al*., 2004). A provisional matrix is formed by fibrin which supports anchorage, proliferation, morphogenesis of endothelia, inflammatory cell infiltration and cancer cell invasive growth (Palumbo *et al*., 2000; Palumbo *et al*., 2002). The fibrin matrix could, therefore, protect small migrating cancer colonies, as well as provide a niche that preserves cell viability and proliferative potential and prevent attack by immune cells (Palumbo *et al*., 2008; Boccaccio and Camoglio, 2009).

Thirdly, tissue factor binds and activates coagulation factor VII, initiating the coagulation cascade and fibrin polymerisation. After binding of factor VII, signals are transducted by the TF cytoplasmic domain. These signals are responsible for the modulation of the biological activities of cancer cells. The interaction of TF with protease-activated receptors (PARs) is a critical TF signalling feature, since it is likely to strengthen the coordination between the activation of blood clotting and cellular responses induced by TF (Boccaccio and Camoglio, 2009). It appears that Factor VIIa (bound to TF) has a preference for activating PAR-2, which is mainly expressed by cancer cells, whereas thrombin preferentially activates PAR-1, -3 and -4, which are predominantly found on endothelial cells and platelets (Coughlin, 2000). PAR1 has also been found to be expressed by cancer cells (Coughlin, 2000; Ruf and Mueller, 2006; Rak *et al*., 2009). TF-PAR-2 signalling possibly modulate other cell surface molecules, such as growth factor receptors and integrins. Consequently, transcriptional programs controlling survival, motility, and angiogenesis are up-regulated (Versteeg and Ruf, 2006; Schaffner and Ruf, 2008). PAR-1 is expressed in endothelia and induces pro-angiogenic programs. It also modulates tissue permeability and induces exposure of sub-endothelial adhesive sites to intravasating cells (Figure 1.2) (Boccaccio and Comoglio, 2009).

Another factor that contributes to coagulation-mediated metastasis is PAI-1. PAI-1 is a secreted protein that inhibits plasminogen activation, thereby preventing that degradation of fibrin and clot removal by plasmin (Boccaccio *et al*., 2005). MET oncogene activation can lead to the transcriptional up-regulation of PAI-1 in tumour cells. This would result in the promotion of the development of the pro-angiogenic and pro-adhesive provisional matrix of fibrin being formed around the tumour. PAI-1 promotes endocytosis of cell surface multi-molecular complexes such as uPA (which is bound and inhibited by PAI-1), uPA receptor, and integrins (Czekay *et al.,* 2003; Stefansson and Lawrence, 2003; Shi *et al*., 2004; Lee *et al*., 2010). This process possibly provides a dynamic interaction between cancer cells and the extracellular matrix, promoting cell migration (Boccaccio and Comoglio, 2009).

COX-2 is another coagulation gene that is strongly upregulated by the MET oncogene (Figure 1.2) (Boccaccio and Comoglio, 2009). It catalyses the synthesis of intermediate prostanoids, prostaglandins G_2 and H_2 (PGG_2 and PGH_2). These molecules converted into prostaglandins and thromboxane by tissue specific synthases, resulting in its secretion. Upon secretion, prostaglandins and thromboxane modulates platelet, endothelial, and cancer cell functions. Even though COX-2 mostly supports the production of the platelet aggregation inhibitor, prostacyclin in endothelial cells, ultimately, COX-2 activation in cancer cells is for the most part erratic, and can include the production of thromboxane, a potent inducer of platelet aggregation, which has been shown to promote metastasis (Nierodzik and Karpatkin, 2006). COX-2 products have been implied in angiogenesis stimulation (Oshima *et al*., 1996; Daniel *et al*., 1999; Boccaccio and Comoglio, 2009; Zhao *et al*., 2012).

1.1.3 Proteases in metastasis

One of the most important biological reactions is proteolysis. Proteolysis is accomplished by a class of enzymes known as proteases (Rakashanda *et al*., 2012). A critical factor that contributes to the success of metastasis is the involvement of proteases (Yang *et al*., 2009). Tumour invasion and metastasis represents a major threat to breast cancer patients. During cancer invasion and metastasis several natural tissue barriers such as basement membranes and interstitial connective

tissue have to be degraded (Tryggvason *et al*., 1987; Zhang *et al*., 2004; Chaffer and Weinberg, 2011). Basement membranes separate organ parenchyma from the underlying stroma and are the first extracellular barriers to be overcome by invading cancer cells (Duffy *et al*., 2008). Type IV collagen and laminin are major constituents of basement membranes and are of the most important proteins in basement membranes (Wilhelm *et al*., 1989; Duffy *et al*., 2008). Other components include proteoglycans, elastin, reticulin and fibronectin (Timpl, 1989). The organised breakdown of the extracellular matrix (ECM) components by proteinases occurs at several stages within the metastatic cascade and represents an integral part of the metastatic process. The matrix metalloproteinases (MMPs) and the serine proteinases, plasminogen activators (PAs) are the proteinases primarily involved in ECM degradation. These will be discussed in this section along with cathepsins B and D.

1.1.3.1 Plasminogen activator

The serine protease, plasminogen activator (PA) is responsible for the conversion of inactive plasminogen to the active plasmin (Duffy, 1990; Testa and Quigley, 1990; Carter *et al*., 2010). Plasmin is a protease with broad substrate specificity and catalyses the hydrolysis of various proteins such as fibrin, fibronectin and laminin. It can also activate inactive proteases such as the procollagenases, generating active collagenases (Folgueras *et al*., 2004). There are two forms in which PA exists, namely tissue-type PA (t-PA) and urokinasetype PA (u-PA). These two forms of PA are the products of two separate genes. The gene encoding t-PA is located on chromosome 8 that of u-PA is found on chromosome 10. t-PA and u-PA have different biological functions. The former is responsible for the dissolution of blood clots while the latter is mainly involved in normal and pathological tissue destructive events, such as those that occur during the spread of cancer. Both t-PA and u-PA are secreted as single chain structures containing one disulphide bond (Duffy, 1990; Testa and Quigley, 1990). Activation of u-PA results in a 54 kDa protein composed of two chains covalently linked by a disulphide bond. The light chain (A chain) is derived from the amino terminal end of the inactive protein and has a molecular weight of 24 kDa. It is comprised of various domains with amino acid sequences homologous to sequences found in other proteins. One such domain, known as the

kringle region, has homology with a similar structure in plasminogen, fibronectin and prothrombin, while another is homologous to the receptor binding region of endothelial growth factor (EGF). The heavy chain (B chain) is slightly larger (molecular weight = 30 kDa) and contains the catalytic site for u-PA. Active site sequences of u-PA are similar to corresponding regions in other serine proteases such as t-PA, trypsin, chymotrypsin, plasmin and thrombin. When the 54 kDa form of u-PA is cleaved, it results in a catalytically active 33 kDa form that consists of the B chain in addition to 21 amino acids of the A chain. *In vivo* studies of the catalytic actions of u-PA suggest that u-PA exerts its effects by binding to its receptor (Stoppelli *et al*., 1985; Vassali *et al*., 1985; Pepper, 2001). Receptor binding appears to concentrate the protease allowing for degradation of the surrounding matrix (Ossowski, 1988). Plasmin exhibits specificity for substrates with Ile in the P1` position, either Leu or Phe in the P2` position and Lys in the P3` position (Kiss *et al*., 1985).

1.1.3.2 Cathepsin B

Cathepsin B (CathB) is a cysteine protease usually located in lysosomes. CathB is expressed as an inactive precursor with a molecular weight of 37 kDa. It is activated by either a cathepsin D-like enzyme (Nishimura *et al*., 1988) or by a metalloprotease (Nishimura *et al*., 1988; Hara *et al*., 1988). In its activated form it has a molecular weight of 23-28 kDa. CathB can have a single or double chain form. It can also exist as a mixture of both single and double chain forms (Sloane *et al*., 1990). Active CathB activates certain collagenases (Eeckhout and Vaes, 1977), as well as the precursor form of u-PA (Kobayashi *et al*., 1991). The optimum pH of CathB isolated from normal tissues is acidic (pH 4.5 to 5.5). Its activity at neutral or alkaline pH is low (Sloane *et al*., 1990). Conversely, CathB isolated from tumour tissue has a higher activity at neutral and slightly alkaline pH (Sloane *et al*., 1990), which may enable it to play a role in cancer spread. A higher proportion of CathB is found on the cell membranes of malignant tissues than on that of normal tissue (Sloane *et al*., 1986; Keren and LeGrue, 1988), which may be due to post-translational glycosylation defects during processing of CathB (Pagano *et al*., 1989). CathB prefers cleaving on the C-terminal side of Arg residues (Gordon and Mourad, 1991). It also demonstrates specificity for Lys, Leu and Pro in the P3 position.

1.1.3.3. Cathepsin D

Cathepsin D (CathD) is a lysosomal aspartic protease with an acidic pH optimum in the range pH 3 to 5 (Gildberg, 2006; Krause *et al*., 2010). Westley and Rochefort (1980) showed that CathD production was induced in oestrogen receptor (ER)-positive breast cancer lines by oestradiol but was produced constitutively in ER-negative cell lines. A later study showed that CathD is a mitogen for oestrogen-deprived breast cancer cell lines (Vignon *et al*., 1986). Briozzo *et al*. (1988) showed that CathD catalyses the degradation of the extracellular matrix. CathD from breast cancer cells can exist in several molecular weight forms (Benes *et al*., 2008). The precursor is a 52 kDa protein and is transported to lysosomes to be processed to an intermediate 48 kDa protein which is then converted into mature forms with molecular weights of 34 and 14 kDa (Rochefort *et al*., 1990). This conversion appears to occur at a lower rate in cancer cells than in normal cells (Murphy *et al*., 1989), resulting in a greater accumulation proportion of the 52 kDa and 48 kDa forms in cancer cells than in non-malignant cells (Capony *et al*., 1989). The presence of hydrophobic residues in the P1 position of CathD substrates is a preferential factor for cleavage (Sun *et al*., 2013).

1.1.3.4 Metalloproteases

There are at least 20 human proteins in the MMP system, each sharing amino acid sequences and homologies. MMPs are divided into five subclasses based on their substrate specificity, namely: collagenases, gelatinases, stromelysins, membrane-type MMPs (MTMMPs), and other MMPs. Together, these MMPs have the ability to break down all ECM components and are, therefore, tightly regulated at several levels including activation and transcription. The presence of specific tissue inhibitors of metalloproteinases (TIMPs) also provides an extra level of regulation (Radisky and Radisky, 2010). The metalloproteases require both calcium and zinc ions as cofactors and are secreted as inactive zymogens. Initially, the metalloproteases were divided into three main groups (Murphy *et al*., 1989) namely interstitial collagenases, type IV collagenases and the stromelysins. Types I, II and III collagens are substrates for interstitial collagenases. A quarter of the cleavage products resulting from collagen hydrolysis are unstable and are further degraded by

the same collagenases or a different protease. The first bond cleaved can either be a Gly-Leu or Gly-Ile bond. The main form of collagen in basement membranes is type IV collagen and is hydrolysed by type IV collagenase. Due to its ability to degrade gelatine, these proteases are sometimes referred to as gelatinases. Collagenase IV can exist in three different forms, namely a 72 kDa form (Liotta *et al*., 1981), a 92 kDa form (Wilhelm *et al*., 1989) and a 100 kDa form (Kato *et al*., 1990). The 72 kDa form contains an exclusive central domain with a sequence of 171 amino acids which resemble the collagen binding domains of fibronectin. The structure of the 92 kDa form is similar to that of the 72 kDa enzyme, but contains an extra sequence of amino acids flanked by its central catalytic domain and its C-terminal end. There are four different forms of stromelysins that are known. These include types 1, 2, 3 and Pump 1 (McDonnell and Matrisian, 1990; Basset *et al*., 1990). The substrate specificity of stromelysins is broad, and they are capable of catalysing the degradation of several components of the extracellular matrix. Stromelysin 1 (MMP-3) catalyses the breakdown of proteoglycans, laminin, fibronectin, gelatin and the non-elical globular portions of type IV collagen (McDonnell and Matrisian, 1990; Reunanen *et al*., 2002).

1.2 Angiogenesis

An important requirement for sustained tumour growth and successful metastasis of cancer cells is the process of angiogenesis. Angiogenesis is the formation of new blood vessels from pre-existing blood vessels (Folkman *et al*., 1966; Folkman, 1990; Dvorak, 2005). The process of angiogenesis is under tight regulation by production of pro- and anti-angiogenic factors. These factors are listed in Table 1.1.

Table 1.1 Promoters and inhibitors of angiogenesis (Makrilia *et al*., 2009).

Inhibitors	Promoters
Angiostatin	Angiogenin transforming growth factors
Basic fibroblast growth factor receptor	Angiopoietin
Endostatin	Angiotropin
Interferon-alpha	Fibroblast growth factor
Interleukin 1,6, 12	Granulocyte-colony stimulating factor
Placental proliferin-related protein	Hepatocyte growth factor
Platelet factor 4	Interleukin 8
Prolactin	Matrix metalloproteinases
Thrombospondin	Placental growth factor
Tissue inhibitors of metalloproteinases (TIMPs)	Platelet-derived growth factor
Transforming growth factor-beta	Proliferin
	Tumor necrosis factor
	VEGF

1.2.1 An overview of angiogenesis

The formation of vasculature has been described by two distinct processes. The first process is termed vasculogenesis, which is the formation of primitive blood vessels from mesoderm by differentiation of angioblasts during embryonic development (Risau and Flamme, 1995). In the second process, following the formation of the primary vascular plexus, expansion of the circulatory network depends on sprouting from pre-existing vessels in a process termed angiogenesis (Folkman, 1995b; Hanahan and Folkman, 1996; Risau, 1997; Yancopoulos *et al*., 2000). The formation of blood vessels in later stages of embryonic development as well as the formation of blood vessels required in adults due to tissue demands is mainly products of angiogenesis (Folkman and Shing, 1992). New blood vessels form naturally during events such as embryogenesis, the female reproductive cycle and wound healing (Yancopoulos *et al*., 2000; Carmeliet *et al*., 1996; Tonnesen *et al*., 2000). Angiogenesis also occurs in many pathological states, such as ischemic diseases, chronic inflammatory reactions, and cancer (Folkman, 1995a; Carmeliet and Jane, 2000; Wong *et al*., 2009). The observation that tumour growth and metastasis requires the formation of new blood vessels was first shown by Folkman *et al*. (1966). The exchange of waste and nutrients is possible via diffusion, provided the tumour cells are located within 100µm of blood vessels (Folkman, 1990; Rijken *et al*., 2000). Tumours growing beyond this threshold require the formation of a new blood supply, which results in the appearance of an angiogenic phenotype (Folkman, 1990; Folkman, 1995b). Folkman *et al*. (1971) proposed that tumour growth and metastasis were dependent on angiogenesis. They hypothesised that inhibition of

angiogenesis could potentially be a strategy to inhibit or arrest tumour growth. It was also shown that pre-malignant tissue cells were capable of acquiring angiogenic capacity as part of the transformation to become fully malignant (Gullino, 1978; Wong *et al*., 2009). Genetic studies have confirmed that the acquisition of an angiogenic phenotype is one of the hallmarks of cancer (Dameron *et al*., 1994; Rak *et al*., 1995; Hanahan and Folkman, 1996; Volpert *et al*., 1997; Maxwell *et al*., 1999; Rak *et al*., 2000; Chan *et al*., 2013).

1.2.2 The mechanism of angiogenesis

Angiogenesis involves a succession of synchronised actions that is initiated through production of various pro-angiogenic factors such as VEGF. Following its expression, VEGF binds to its receptor on endothelial cells (Figure 1.3), resulting in an increase in vascular permeability. This in turn results in extravasation of plasma proteins and dissociation of pericyte coverage (Roberts and Palade, 1997; Dvorak, 2005). Endothelial cells then migrate and proliferate in preparation for the new vasculature (Ausprunk and Folkman, 1977; Dvorak, 2005). The vascular basement membrane and extracellular matrix (ECM) are degraded at the same time, allowing for the sprouting of new vessels. Cathepsin B, matrix metalloproteinases (MMP) and other enzymes as well as the expression of matrix proteins such as fibronectin, laminin, tenascin-C and vitronectin are involved in the breakdown of the ECM (Mikkelsen *et al*., 1995; Zagzag *et al*., 1996; Gladson, 1999; Shepherd and Sridhar, 2003; Ljubimova *et al*., 2006). The new blood vessel is formed by alignment of endothelial cells, tubular morphogenesis and formation of a lumen. The connection of individual sprouts leads to the formation of vascular loops, which in turn initiates blood flow through the vessel loops. Pericytes and/or smooth muscle cells are then recruited for assembly along the endothelial cells outside the new vessel, leading to the maturation of the vessel wall. The final step in the angiogenic process involves the formation of a new basement membrane (Folkman, 1982; Folkman, 1985; Folkman, 1986; Wong *et al*., 2009).

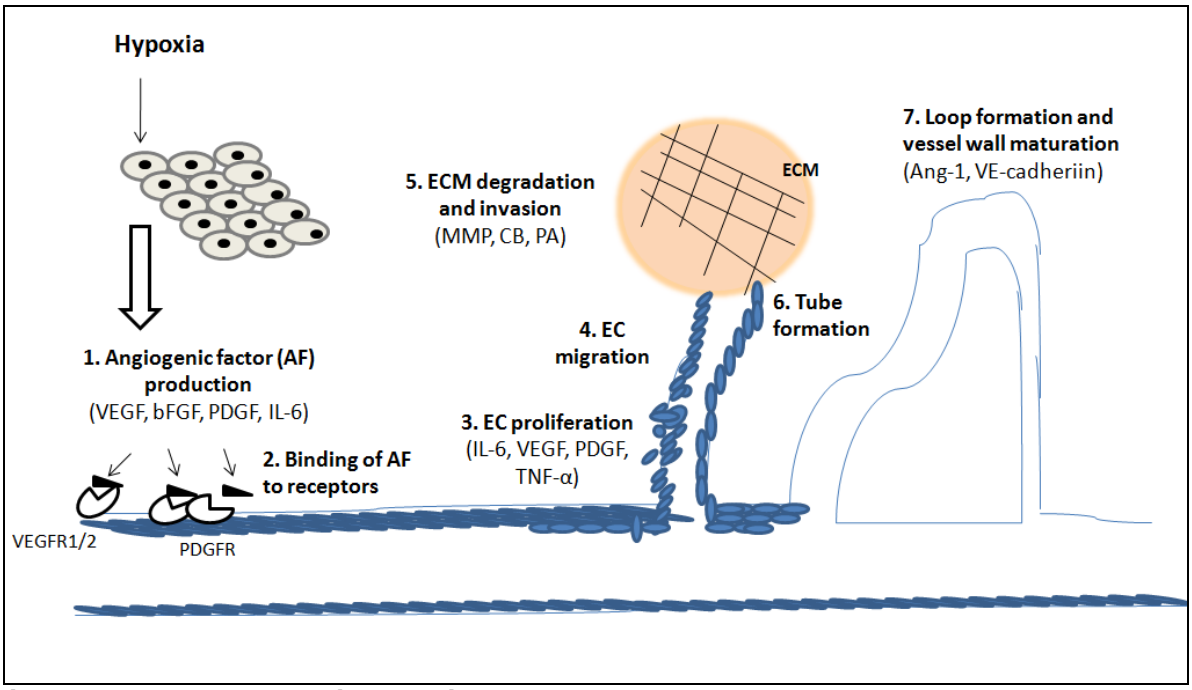


Figure. 1.3 Tumour angiogenesis. Hypoxia triggers (1) tumour cells to secrete angiogenic factors such as vascular endothelial growth factor (VEGF) to initiate angiogenesis (2). VEGF endothelial cell (EC) receptor binding induces endothelial cell proliferation (3) and migration (4), followed by degradation and invasion of the extracellular matrix (ECM) (5). Endothelial cells undergo tube formation (6) and eventually forms a loop followed by vessel wall maturation (7). (Adapted from Wong *et al*., 2009).

1.2.3 Vascular endothelial growth factor (VEGF)

The VEGF family is comprised of VEGF-A, -B, -C, -D, -E (Table 1.2) and placenta growth factor (PGF) (Ferrara *et al*., 2003). Vascular endothelial growth factor-A (VEGF-A or VEGF) is the most documented and most extensively characterized member of the VEGF family (Makrilia *et al*., 2009). It is a tumour-secreted factor that has crucial functions in both normal and tumour-associated angiogenesis (Schweighofer *et al*., 2009). In order for VEGF-A to exert its biological effect it binds to its receptors on the cell surface. These receptors are VEGF receptor-1 [VEGFR-1 (flt-1)], VEGF receptor-2 [VEGFR-2 (KDR/flk-1)] and VEGFR-3. VEGFR-1 and -2 are found on the vascular endothelium and are up-regulated during angiogenesis, while VEGFR-3 is expressed on lymphatics. It appears that the role of VEGFR-1 is complex and studies suggest that it may negatively regulate angiogenesis (Fong *et al*., 1999; Kearney *et al*., 2002), although it has also been shown to contribute to vascular sprouting and metastasis (Kearney *et al*., 2004; Hiratsuka *et al*., 2002; Makrilia *et al*., 2009). Interaction between VEGF-A and VEGFR-2 has an important function in angiogenesis, namely the organised signalling of proliferation and

migration of endothelial cells as well as recruitment of endothelial cell progenitor cells (Gerber *et al*., 1998). It has recently been shown that VEGF-B plays a role in vascular survival rather than being an angiogenic factor (Zhang *et al*., 2009). VEGF-C is a ligand of VEGFR-3 which shares sequence similarity with VEGFR-1 and VEGFR-2 (Joukov *et al*., 1996; Lee *et al*., 1996). VEGFR-3 expression is predominantly found in lymphatic endothelium (Kaipainen *et al*., 1995; Kukk *et al*., 1996; Oh *et al*., 1997). It appears that the chief function of VEGF-C is to regulate the growth of lymphatic vessels. In a study by Jeltsch *et al*. (1997) it was found that transgenic mice over-expressing VEGF-C in kertainocytes develop dilated lymphatic vessels in the skin. It has also been shown that VEGF-C promotes the spread of cancer cells through lymphatic channels (Tsurusaki *et al*., 1999; Yonemura *et al*., 1999). VEGF-D was first isolated from mouse skin fibroblasts. It was found to show sequence homology to VEGF-C (Achen *et al*., 1998; Yamada *et al*., 1997) and is, therefore, thought to have similar biological functions as VEGF-C. VEGF-E is a dimer of 20 kDa. It lacks a basic domain and shows no affinity for heparin, but similar levels of mitotic activity on primary endothelial cells when compared to VEGF-A. VEGF-E binds to KDR/Flk-1 (VEGFR-2) but not to Flt-1 (VEGFR-1) and does not induce autophosphorylation of Flt-1 (Ogawa *et al*., 1998). PGF stimulates cell proliferation and differentiation by binding to its receptors, Flt-1. It shows sequence homology to VEGF-A and has similar biological effects, such as stimulation of endothelial cells (Ratajczak *et al*., 1998).

Table 1.2. Vascular endothelial growth factor family members (Makrilia *et al*., 2009).

Type of VEGF	Mechanism of action	References
VEGF-A	Physiologic and tumour angiogenesis	(Dvorak, 2005)
VEGF-B	Vasculogenesis and activation of invasive enzymes on endothelial cells	(Olofsson, 1996; Grimmond, 1996)
VEGF-C	Lymphangiogenesis and tumour angiogenesis	(Joukov, 1996)
VEGF-D	Angiogenesis	(Yamada, 1997)
VEGF-E	Endothelial cell mitosis and angiogenesis	(Meyer, 1999)
PGF	Vascular permeability	(Maglione, 1991)

1.2.4 The role of cytokines in angiogenesis

Another major contributor to the initiation and progress of angiogenesis are cytokines. Cytokines are low molecular-weight glycoproteins, which are rapidly synthesised and are secreted by various healthy and diseased cells (primarily mononuclear phagocytes and activated T lymphocytes) following stimulation. They are usually pleiotropic, meaning they act on many different adjacent target cells and their effects are often additive, synergistic, or antagonistic. Cytokines regulate survival, growth, differentiation, and the effector functions of cells in multi-cellular organisms (Heinrich *et al*., 1998; Nicolini *et al*., 2006). Unsurprisingly, cytokines have a considerable effect on tumour growth *in vivo* (Yao *et al*., 2007; Marotta *et al*., 2011). They are also produced by cancer cells and may act as tumour growth-promoting or inhibiting factors. They are capable of activating or modulating specific or non-specific anti-tumour responses as a result of their effect on the growth and function of immuno-competent cells. Additionally, since cytokines are mediators of the effector response from innate and acquired cellular immunities, they could, possibly, be involved in the mechanism of evading of the immunosurveillance system by tumour cells (Sooriakumaran and Kaba, 2005; Chin and Wang, 2014). Specific cytokines and their roles in metastasis and angiogenesis will be discussed in detail in Chapter 4.

1.2.5 Membrane-bound factors essential for angiogenesis

Several membrane-bound proteins play important roles in angiogenesis. These include integrins, ephrins, and cadherins and are responsible for numerous functions associated with the assembly of blood vessels. Of particular interest are $\alpha_v\beta_3$-integrin, ephrin-2B, and VE-cadherin, which affect the process of angiogenesis. Integrins are glycosylated, heterodimeric transmembrane adhesion receptors that are involved in cell to cell and cell to ECM interactions. Integrin $\alpha_v\beta_3$ mediates angiogenesis through the binding of ECM components and matrix metalloproteinase-2 (Hodivala-Dilke *et al*., 1999; Brooks *et al*., 1996). Eph receptors and ephrin ligands play a role in the development of blood vessels. For example, Ephrin-B2 co-localizes with its receptor eph-4B at arterial and venous interfaces after the establishment of the primary capillary plexus by vasculogenesis, but before it is remodelled by angiogenesis (Wang *et al*., 1998). VE-cadherin may be involved in the regulation of

molecules passing across the endothelium (Esser *et al*., 1998; Kevil *et al*., 1998) as well as in the mediation of contact inhibition of EC growth (Caveda *et al*., 1996). EC junctional stability in the vessel wall is established by cadherins and they also enhance the survival of EC's through promotion of the spread of anti-apoptotic signal of VEGFs (Carmeliet *et al*., 1999). VE-cadherin is, therefore, essential for the maturation of blood vessels (Otrock *et al*., 2007). Of particular interest to this study, is integrin $\alpha_v\beta_3$, which will be discussed in more detail in Chapter 3.

1.2.6 Role of the plasminogen activator/plasmin system in angiogenesis

Plasmin is a protease with broad-range specificity hydrolysing several extracellular proteins, particularly fibrin (as outlined in section 1.1.3.1). The serine proteases, urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA), activate plasminogen. uPA binds to a specific glycosylphosphatidylinositol-anchored cell surface receptor (uPA receptor—uPAR) (Pepper, 2001). Plasminogen activator inhibitor type-1 (PAI-1) plays a crucial role in the regulation of tumour invasion, metastasis, as well as cancer-related angiogenesis (Chorostowska-Wynimko *et al*., 2004; Stefansson *et al*., 2003). Hypoxia is one of the main stimulators of angiogenesis and reportedly increased uPAR (Kroon *et al*., 2000) and PAI-1 (Uchiyama *et al*., 2000) in ECs. *In vivo*, several studies have demonstrated a necessity for the PA/plasmin system in angiogenesis and tumour cell invasion (Bajou *et al*., 1998; Stefansson *et al*., 2003; Otrock *et al*., 2007).

1.2.7 Role of interferons (IFN) in angiogenesis

IFN-α and IFN-β are type I IFN proteins possessing anti-tumour activity. Their actions results in down-regulation of oncogene expression, inducing tumour suppressor genes which, consequently, results in anti-proliferative activity (Nicolini *et al*., 2006). The anti-proliferative and anti-adhesive actions of IFN-α have been demonstrated by Maemura *et al*. (1999) in MCF-7 breast carcinoma cells. IFNγ, also known as immune IFN or type II IFN, is primarily produced by cluster of differentiation 4+ (CD4+), T helper cells (Th1), cluster of differentiation 8+ (CD8+), and natural killer (NK) cells. In cancer xenografts, the anti-proliferative action of IFNγ, probably due to

enhanced cell death by up regulation of some caspases and an anti-angiogenic activity, has been discovered (Farrar and Schreiber, 1993; Boehm *et al*., 1997; Bach *et al*., 1997; Makrillia *et al*., 2009).

1.2.8 Tumour angiogenesis and metastasis

Developing tumours require new vasculature as they grow in order to ensure a constant supply of nutrients and oxygen and to allow for the elimination of metabolic waste (Sooriakumaran and Kaba, 2005). Choy and Rafii (2001) suggested that angiogenesis is also a prerequisite for tumour progression. The angiogenic process in solid tumours is crucial for advanced tumour growth and progression to a metastatic state. It has been proposed that microvessel density (MVD) is an indicator of biological aggressiveness and metastatic potential in many primary tumours (Weidner *et al*., 1993; Iakovlev *et al*., 2012).

Inhibiting the angiogenic process could enhance the effects of chemotherapy and radiation by limiting the tumour to a dormant state of low metastatic potential; hence, interest in antiangiogenic therapies has increased dramatically. Angiogenesis is initiated in quiescent endothelial cells following a shift in the balance between endogenous angiogenesis-inhibitory factors and angiogenesis-promoting factors (Table 1.1). A shift in the equilibrium to a pro-angiogenic state occurs at an early to mid-stage in tumour development. This leads to activation of an ‘angiogenic switch’ and, consequently, the formation of new vasculature (Hanahan and Folkman, 1996). Growth factors up-regulated during angiogenesis, in particular basic fibroblast growth factor (bFGF) and VEGF, have been identified and may also be important as therapeutic targets and/or molecular indicators of disease stage (Weidner *et al*., 1993). VEGF is a key mediator of both normal and abnormal angiogenesis due to its capacity to stimulate virtually every step in the angiogenic process (Beckner, 1999; Schweighofer *et al*., 2009).

Blood vessels are endothelial-lined tubes that provide tissues with oxygen and nutrients and remove their metabolic waste products while also producing a spectrum of growth factors (Table 1.2) with paracrine effects on surrounding cells (Zhang *et al*.,

2009). Thus these vessels may support both normal and malignant tissue growth by providing a sufficient blood supply and by secreting growth factors (Carmeliet *et al*., 1996).

Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) promotes angiogenesis (Clavel *et al*., 2007). VEGF is a stimulator of endothelial cell proliferation *in vitro* and can induce neovascularization *in vivo* (Cursiefen *et al*., 2004). It also promotes inflammation (Dvorak *et al*., 1995; Cursiefen *et al*., 2004). The actions of VEGF are mediated by three specific receptors. One such receptor, soluble VEGF receptor (VEGFR)-1 (sflt-1) consists of an extracellular domain, made up of seven Ig-like domains, a transmembrane domain and an intracellular kinase domain (Kearney *et al*., 2004; Kaplan *et al*., 2005). VEGFR-1 is mainly expressed by endothelial cells and, like VEGF, is upregulated by hypoxia (Clavel *et al*., 2007). As well as hypoxia, VEGF is regulated by many growth factors and cytokines at pre-transcriptional, transcriptional, and post-transcriptional levels (Carmeliet *et al*., 1999; Reinmuth *et al*., 2001; Schweighofer *et al*., 2009). Those molecules that do not stimulate angiogenesis directly, but can modulate angiogenesis by altering VEGF expression in specific cell types and exerting indirect angiogenic or anti-angiogenic effects. Molecules that can initiate VEGF production include fibroblast growth factor 2 (FGF-2), FGF-4, platelet derived growth factor (PDGF), tumour necrosis factor (TNF), transforming growth factor b (TGF-β), insulin growth factor-1 (IGF-1) and IL-6 (Becker *et al*., 2004; Sooriakumaran and Kaba, 2005; Sun *et al*., 2005).
Angiopoietins, unlike other angiogenic factors, are not mitogenic for endothelial cells (Clavel *et al*., 2007). Ang-1 is believed to be involved in the maintenance and stabilisation of mature vessels by promoting interactions between endothelial cells and surrounding extracellular matrix (Clavel *et al*., 2007).

As previously mentioned, angiogenesis is initiated by the release of proteases that allow degradation of the basement membrane and migration of endothelial cells into the interstitial space. Endothelial cell proliferation then occurs with eventual differentiation into mature blood vessels. Each of these processes is tightly regulated through the complex interplay of endogenous factors that promote and inhibit angiogenesis (Table 1.1). In the normal state endothelial cells are usually quiescent, with doubling times as long as seven years, but in the malignant state this growth

rate is accelerated, with doubling occurring as rapidly as every 7 to 10 days (Jaffe *et al*., 1973; Gupta *et al*., 2002). This ‘‘angiogenic switch’’ that promotes and facilitates ‘‘neo-angiogenesis’’ or new blood vessel development is necessary for tumours to grow beyond a diameter of approximately 3 mm (Folkman, 1985). These new neoplastic capillaries differ not only in their growth rate, but also in their structural integrity. They frequently lack a smooth muscle wall, and have irregular leaky basement membranes, which may facilitate tumour cell leakage into the circulation and increase the potential for metastatic spread. Several agents targeting angiogenesis have been developed and are being evaluated in clinical trials (Shepherd and Sridhar, 2003; Wei *et al*., 2013). These anti-angiogenic factors will be discussed in section 1.2.9.

1.2.9 Inhibitors of angiogenesis

Inhibition of angiogenesis has proven effective in arresting tumour growth and metastasis. Various steps in the angiogenic cascade is targeted by cancer therapies. These include inhibitors of MMPs, drugs that block endothelial cell proliferation and endogenous inhibitors of angiogenesis and will be discussed in this section.

1.2.9.1 Matrix metalloproteinase inhibitors

One of the first steps in angiogenesis is the degradation of the extracellular matrix and basement membrane. This is accomplished by proteases such as the matrix metalloproteinases (MMPs). The MMPs belong to a family of endopeptidases that are capable of degrading basement membranes and components of the extracellular matrix (Binder and Berger, 2002). There is a strong association between increased expression of MMPs and tumour growth, invasion and metastasis (Hidaldo and Eckhardt, 2001). MMPs are expressed by tumour and stromal cells, as well as by proliferating endothelial cells. It has been suggested that MMPs are directly involved in neoangiogenesis by interacting with vascular endothelial growth factors (VEGF) and integrins (Brooks *et al*., 1996; Brooks *et al*., 1998; Shou *et al*., 2001; Shepherd and Sridhar, 2003). It appears that during tumour de-differentiation and metastasis there is over-expression of MMPs in tumours and the surrounding stroma. Increased expression of MMP is associated with a worse overall prognosis in several

malignancies (Khokha and Denhardt, 1989; Kodate *et al*., 1997; Karameris *et al*., 1997; Chambers and Matrisian, 1997; Michael *et al*., 1999; Wick *et al*., 2001; Coussens *et al*., 2002; Baker *et al*., 2002). Nearly 30 MMPs have been identified so far. These have been classified into four main classes according to their substrate specificity. As mentioned before, these classes are collagenases, gelatinases, stromelysins and membrane-type MMPs (Ray and Stetler-Stevenson, 1994; Fortunato and Menon, 2002; Polette *et al*., 2004). Four endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) have also been described (Ylisirnio *et al*., 2000; Bellon *et al*., 2004).

1.2.9.2 Drugs that block endothelial cell proliferation

Endothelial cell migration, proliferation and differentiation are tightly regulated by endogenous angiogenesis inducers (Kieran, 2004). The first class of endogenous inducers targets endothelial cells. Examples of these molecules includes members of the VEGF family and angiopoietins. The second group activates cells other than endothelial cells. This group consists of factors such as cytokines, chemokines and enzymes, including fibroblast growth factor-2 (bFGF-2) (Kieran, 2004). The last group of inducers act indirectly by promoting the release of the direct acting factors (Kerbel, 2000). These inducers include TNF-α and TGF-β. Inhibitors of some of these processes have been developed and have entered clinical trials (Wong *et al*., 2009).

1.2.9.3 Endogenous inhibitors of angiogenesis

It has been hypothesized that primary neoplasms are capable of inhibiting the growth of their metastatic lesions by producing tumour-derived inhibitors of angiogenesis such as endostatin and angiostatin (Li *et al*., 2001; Makrilia *et al*., 2009; Wong *et al*., 2009).

The major contributing factors to angiogenesis are outlined in Figure 1.4 as discussed in the previous sections.

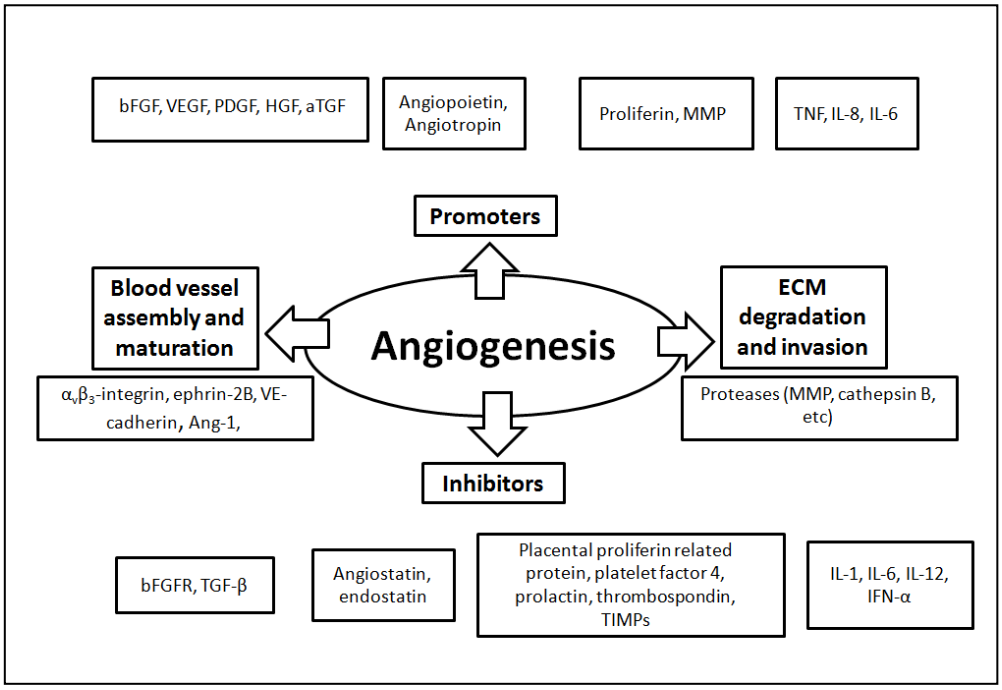


Figure 1.4 An overview of the factors contributing to the process of angiogenesis. Angiogenesis is a process that is tightly regulated by various inhibitors and promoters. This regulation affects blood vessel assembly and maturation as well as ECM degradation and invasion (Wong *et al*., 2009).

1.3 Cancer procoagulant and its role in angiogenesis, inflammation and metastasis

Two common complications in cancer are thrombosis and disseminated intravascular coagulation (DIC). The ability of nearly all types of cancer cells to activate the coagulation system, results in a prothrombotic state in patients with malignancy (Rickles and Falanga, 2009). The pathogenesis of the prothrombotic state in cancer is said to be highly complex and multifactorial. These prothrombotic factors in malignancy include the production of procoagulants (i.e., tissue factor (TF) and cancer procoagulant (CP)) by tumours, as well as production of inflammatory cytokines (Falanga *et al*., 2009). Another factor involves the interaction between tumour cells and monocytes/macrophages and platelets in the blood and endothelial cells. Various other mechanisms of thrombus promotion include responses of the host to the tumour (i.e. inflammation and angiogenesis), decreased levels of inhibitors of coagulation, and impaired fibrinolysis (De Cicco, 2004).

A continuous process that takes place in cells is the shedding of the cell membrane. This process may be a critical factor in determining various behaviour characteristics of cancer cells such as a loss of adhesion, tissue invasion and a hypercoagulable state (Dvorak, 2005). Various substances released by tumour cells possess the ability to directly activate the coagulation system; however, the best characterised are tissue factor (TF) and cancer procoagulant (CP) (Rickles and Falanga, 2001). In addition, tumour cells can release inflammatory cytokines, such as tumour necrosis factor (TNF), interleukine-1 (IL-1), and vascular endothelial growth factor (VEGF), that act on leukocytes and endothelial cells to further enhance the procoagulant activity (Gale and Gordon, 2001). It is also evident that tumour-induced coagulation activation is intrinsically involved in tumour growth, angiogenesis, and metastasis (Hejna *et al*., 1999), which in turn can promote coagulation activation. Cancer procoagulant can directly activate factor X (Mielicki *et al*., 1999). Lee (2002) has also shown that CP can induce dose-dependent platelet activation by a mechanism that appears to be similar to that of thrombin. Although CP is found almost exclusively on malignant cells, its role as a tumour marker or as a factor predictive of clinical thrombosis has not been successfully demonstrated, with the exception of the acute promyelocytic leukaemia in which CP expression in blast cells parallels their degree of malignant transformation and response to all-*trans*-retinoic acid (Falanga *et al*., 1995). Low Ah Kee *et al*. (2012) showed that CP caused a marked increase in the protein expression of the pro-angiogenic cytokine, VEGF, in MCF-7 and E14 cells, implicating a role for CP in angiogenesis.

Previous *in vitro* studies have shown that CP may stimulate the adhesion of platelets to collagen and fibrinogen associated with the production of thromboxane A_2 and activation of phosphatidyl-inositol kinase (Olas *et al*., 2001). CP also acts as a stimulator of the platelet secretory process by inducing secretion of adenine nucleotides and proteins from blood platelets in a dose-dependent manner. It seems possible that CP, like thrombin, can stimulate blood platelets using proteolytically activated receptor-1 (PAR-1) (Olas *et al*., 2001; Low Ah Kee, 2012). Since this receptor is very common on the various cells surface it suggests that CP may stimulate a diverse group of cells including cancer cells. It is known that cell adhesion to the extracellular matrix (ECM) components and other cells is pivotal for cell migration, which plays an important role in invasion and metastasis of malignant

cells. A receptor commonly expressed by cells is integrin $a_v b_3$, which conducts the interactions between cancer cells and host cells necessary for metastasis. This receptor has also been involved in the formation of capillary-like structures during tumour angiogenesis (Olas *et al*., 2001).

Kamocka *et al.* (2005) showed that anti-CP antibody influenced the growth of MCF-7 breast cancer cells and their adhesion to vitronectin. It seems that binding of the CP antigen by the antibody decreased the cell adhesion ability to vitronectin, which, in turn, lead to decrease in viability of the cells. They suggested that CP can play an important role in the growth of cancer cells by regulation of the cell adhesion processes. They also suggested that the procoagulative properties of CP possibly play merely a secondary role in malignant cells proliferation, since no correlation has been found between the level of serum CP activity and the activation of clotting cascade in breast cancer patients (Kamocka *et al*., 2005). The pro-adhesive properties of CP could play an important role in a metastatic spread of cancer as well as in primary tumour growth (Olas *et al*., 2001).

2. THE ISOLATION AND PARTIAL CHARACTERISATION OF CP

2.1 Introduction

Cancer procoagulant (CP) is an enzyme that is found in tumour and foetal tissue, but is completely absent from normal tissue (Falanga and Gordon, 1985a). It is a direct activator of factor X and has been isolated from amnion-chorion membranes (Falanga and Gordon, 1985b) as well as from extracts and cells from human melanoma (Donati *et al*., 1986).

2.1.1 The isolation and properties of CP

CP was first isolated by Falanga and Gordon (1985a) from tumours produced by injection of V2 carcinoma cells into the thigh muscle of young white rabbits. The authors employed two procedures. In the first, CP was purified by benzamidine-Sepharose affinity chromatography, gel filtration chromatography, and phenyl-Sepharose hydrophobic chromatography. In the second procedure, antiserum was raised against the purified protein and was used to prepare an immunoadsorbent column. Tumour extracts were purified by immunoaffinity chromatography followed by p-(chloromercuri) benzoate affinity chromatography (Falanga and Gordon, 1985a). CP, purified by both procedures, was homogeneous on the basis of analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing. Falanga and Gordon (1985a) determined the apparent molecular weight of purified CP (68 kDa) and its isoelectric point (4.8). The authors also found that CP was able to directly activated factor X, in the absence of factor VII, and was inhibited by 1mM iodoacetamide and 0.1mM mercury which are classic cysteine proteinase inhibitors. Activation of factor X is inhibited by cysteine proteinase inhibitors such as iodoacetamide and Hg^{2+} (Gordon and Cross, 1981). Furthermore, CP has other cysteine proteinase characteristics including binding to *p*-(chloromercuri) benzoate and optimum activity at about pH 6.5 (Gordon and Cross, 1981). Carbohydrate analysis revealed less than 1 mol of hexose or sialic acid/mol of protein, indicating that CP contains no detectable carbohydrates. Amino acid composition analysis (Table 2.1) showed that serine (19.1%), glycine (18.77%), and glutamic acid (12.5%)

were the prevalent amino acids. Overall, CP contains about 40% hydrophobic amino acid residues. The amino acid composition of cancer procoagulant was substantially different to other known factor X activating proteinases or other cysteine proteinases including cathepsin B (Falanga and Gordon, 1985b). Due to its hydrophobic nature, CP associates with proteins that are present in tissue extracts and serum. Its activity is thought to be stabilized in a lipid environment, while high salt concentrations are not ideal (Gordon, 1994). In particular, salts of divalent cations such as $CaCl_2$, $HgCl_2$, $MgCl_2$ and $MgSO_4$, at a concentration of 333mM, strongly inhibit CP activity, while salts such as NaCl and choline chloride had negligible inhibitory effect on CP (Low Ah Kee, 2011).

Table 2.1 Amino acid composition of CP. (Adapted from Falanga and Gordon, 1985a).

Amino acid	% of total residues	residues/mol of protein*
lysine	8.1	53
histidine	3.9	26
arginine	2.3	15
aspartic acid	7.1	46
threonine	5.1	33
serine	19.1	125
glutamic acid	12.5	82
proline	3.6	24
glycine	18.7	123
alanine	6.4	42
cysteine	1.2	8
valine	3.1	20
methionine	1.7	11
isoleucine	2.6	17
leucine	3.4	22
tyrosine	2	13
phenylalanine	2.2	14
tryptophan	ND	ND
Total	100	674

*Based on a molecular weight of 68 kDa.

In addition to its ability to activate factor X, it has been suggested that CP is selectively associated with the malignant phenotype (Donati *et al*., 1986). These authors also reported the enzymatic and immunological identification of CP in extracts and cells from human melanoma. Using factor IX and factor VII deficient

plasmas, they showed that CP activity was independent of both the intrinsic and extrinsic pathways of blood coagulation. It was also observed that CP activity was detectable in extracts and cell suspensions from all the patients studied and was higher in extracts from metastases than from the primary tumours (Donati *et al*., 1986). Benign melanocytic lesions, however, did not express CP. In patients with acute non-lymphoid leukaemia, CP activity was detected in bone marrow cell extracts. The authors detected CP activity during the active disease, decreased during partial remission and disappeared during complete remission. Upon relapse, two to five months prior to the reappearance of malignant cells in the bone marrow, CP activity was again present, suggesting that CP is produced before appearance of the malignant phenotype and could be an indicator of the malignant phenotype. Kozwich *et al*. (1994) discovered the potential of a CP enzyme-linked immunosorbent assay (ELISA), which could measure CP concentration in serum, as an aid in diagnosing early stage malignancies. These authors suggested that this early detection, made possible by the CP ELISA, could significantly improve the survival rate of cancer patients. Mielicki *et al*. (1999) demonstrated that CP activity is elevated in the serum of early stage breast cancer patients and drops to baseline in the advanced stage of the disease. There was no evidence of activation of blood coagulation in the analysed cancer patients and there was no correlation between CP activity and any of the examined parameters characterising the clotting system in these patients. The authors concluded that although there was a possibility that the level of clotting activation was below the detection range of the assay employed, they rather suggested that the pro-coagulative properties of CP were not significant for the activation of blood coagulation in cancer patients *in vivo*.

Olas *et al.* (2001) suggested that, since it is a direct activator of coagulation factor X, CP may activate blood platelets and could, possibly, be partly responsible for the modified hemostatic function of platelets from cancer patients. Their results showed that CP may activate blood platelets *in vitro*. It stimulates platelet adhesion to collagen and fibrinogen as well as the production of thromboxane A_2. They were also able to show that CP induced the secretion of adenine nucleotides and proteins from blood platelets in a dose-dependent manner *in vitro*. They deduced that the presence of adenine nucleotides and proteins in the extracellular medium after treatment of platelets with CP together with a lack of lactate dehydrogenasae (LDH)

activity indicated that CP is able to stimulate the platelet secretory process. The authors also observed that CP alone could not induce platelet aggregation; neither could it modify thrombin-induced platelet aggregation, even in the presence of fibrinogen. Kamocka *et al*. (2005) suggested, therefore, the proadhesive properties of CP observed by Olas *et al*. (2001) play an important role in the metastatic spread of cancer as well as in primary tumour growth. The *in vitro* effects of anti-CP antibody on the growth of MCF-7 breast cancer cells and on the cells adhesion to vitronectin were shown to result in a 6–18% decrease in cell viability as well as a reduction in the cells' adhesion to vitronectin. The authors proposed that the possible mechanisms of the interactions between CP and malignant cells include the regulation of the cell adhesion processes to ECM components, especially to vitronectin. (Kamocka *et al*., 2005). Low Ah Kee *et al*. (2012) showed that the steady-state mRNA level of *L1cell adhesion molecule* (*L1CAM*) in MCF-7 breast cancer cells and E14 mouse embryonic stem cells (MESCs) were increased by CP, while an increase in *angiogenin* mRNA was seen in MDA-MB-231 breast cancer cells. The authors also showed that production of vascular endothelial growth factor (VEGF) protein increased in MCF-7 breast cancer cells and E14 MESCs, but decreased in MDA-MB-231 breast cancer cells. CP could, therefore, potentially play a part in the modulation of angiogenesis in cancer as well as have a role in vascular development during embryonic development.

2.2. Introduction to the study

Tumour invasion and metastasis represents a major threat to breast cancer patients. Metastasis is a complex process that involves a cascade of events including angiogenesis, local invasion, and intravasation. The organised breakdown of the ECM components by proteinases occurs at several stages within the metastatic cascade and represents an integral part of the metastatic process. The MMPs and the serine proteinases, plasminogen activators (PAs) are the proteinases primarily involved in ECM degradation.

The hunt for a procoagulant from malignant tissue started over a quarter of a century ago when it became increasingly clear that experimental data, which had accumulated over the years, suggested a role for fibrin in malignant disease. Increased activation of the coagulation system in malignant disease further supported this notion and soon led to the discovery of cancer procoagulant (CP), an enzyme that is derived from tumour and foetal tissue, but not normal tissue (Falanga and Gordon, 1985a). CP, a direct activator of factor X, has since been isolated from amnion-chorion membranes (Falanga and Gordon, 1985b) as well as from extracts and cells from human melanoma (Donati *et al*., 1986).

Research suggests that CP is produced before appearance of the malignant phenotype and could be an indicator of the malignant phenotype. Kozwich *et al*. (1994) discovered the potential of a CP ELISA, which could measure CP concentration in serum, as an aid in diagnosing early stage malignancies. These authors suggested that this early detection, made possible by the CP ELISA, could significantly improve the survival rate of cancer patients. Mielicki *et al*. (1999) demonstrated that CP activity is elevated in the serum of early stage breast cancer patients and drops to the baseline in the advanced stage of the disease.

This investigation aimed to isolate CP from amnion-chorion membranes (Chapter 2) and study some properties of the enzyme using the newly developed direct CP assay. The aims and objectives of this study are listed below.

2.2.1 Aims

1. To investigate the physicochemical properties of CP using new synthetic substrates.

2. To study the effect of CP on the production of inflammatory and angiogeneic cytokines in MCF-7 and MDA-MB-231 breast cancer cells as well as in hTERT-HDLEC endothelial cells.

3. To study the effect of CP on the detachment of MCF-7, MDA-MB-231 and hTERT-HDLEC from gelatin to determine the role of CP in metastasis and angiogenesis.

4. To develop of a novel synthetic substrate for the determination of CP activity.

2.2.2 Objectives

1. Physicochemical properties of CP

1.1 Isolation of CP from amnion-chorion membranes.
1.2 Protein determination using the BCA protein assay.
1.3 Determination of CP activity using the chromogenic factor X assay.
1.4 Molecular weight and homogeneity determination using SDS-PAGE.
1.5 Optimum pH and Temperature determation using Boc-QVR-AMC as a substrate.
1.6 Investigation of the effect of calcium chloride concentration on CP activity.

2. Effect of CP on cytokine expression

2.1 Investigation of the effects of CP on the production of TNF-α, IL-6, IL-1β, IFN-α, IFN-γ and PDGF-β by MCF-7, MDA-MB-231 and hTERT-HDLEC cells after 4, 6, 12, 24 and 48 hours.
2.2 Determination of cell viability using the MTT assay.

3. Effect of CP on detachment

3.1 Seeding of MCF-7, MDA-MB-231 and hTERT-HDLEC cells on gelatin-coated plates.

3.2 Labelling of cells with CellTrackerTM Green fluorescent cell marker.

3.3 Exposure of labelled cells to CP for 6, 12, 24 and 48 hours.

3.4 Determination of the number of detached cells using fluorescence spectroscopy.

4. Synthetic CP substrates

4.1 Design of synthetic fluorescent substrates for CP activity determination.

4.2 Screening of the designed substrates.

4.3 Investigation of substrate and enzyme specificity of the designed substrates.

4.4 Determination of K_m, V_{max}, k_{cat} and k_{cat}/K_m for the designed synthetic substrates.

2.3 Materials and Methods

A total of 36 isolations of CP were performed from amnion-chorion placental membranes using a two-step ion exchange chromatography procedure. CP generated from these isolations was used for experiments discussed in this chapter as well as in chapters 3 and 4. This section discusses the methods and principles used to isolate and characterise CP. Unless otherwise stated, all reagents used in this study were of an analytical grade.

Ethical approval for the use of human tissues (Reference: H13-SCI-BIO-001/Approval) was obtained from the Research Ethics Committee (Human) of the Nelson Mandela Metropolitan University (NMMU) (Appendix A). Amnion-chorion membranes were collected from Netcare Greenacres Hospital (Appendix C), Port Elizabeth, from consenting patients. The consent form is shown in Appendix B.

2.3.1 Isolation and purification of cancer procoagulant

2.3.1.1 Extraction of CP from amnion-chorion placental membranes

Amnion-chorion membranes were kindly donated by the Neo-natal Ward at the Greenacres Hospital, Port Elizabeth. The membranes were transported on ice and were transferred into a beaker containing an equal volume of 20mM Tris-HCl buffer, pH 7.4. Blood clots were removed and the membranes were transferred to a clean beaker containing an equal volume of 20mM Tris-HCl buffer, pH 7.4. Extraction was completed by periodic swirling of the membranes in the beaker over a period of 1.5 hours. The extract was transferred to a centrifuge bottle and fresh buffer was added to the membranes and the process repeated a further 3 times. All extracts were pooled and centrifuged at 2000 x *g* for 15 minutes.

2.3.1.2 Chromatography

The isolation of CP was performed using liquid chromatography based on the reported properties of CP.

2.3.1.2.1 *Ion-exchange chromatography*

Ion-exchange chromatography employs charge differences found on proteins to separate them. An exchange of ions between the protein and the resin occurs. There are two classes of ion-exchangers, namely weak or strong ion-exchangers. Weak ion-exchangers function over a narrow pH range, while strong ion-exchangers function over a wide pH range. Anion-exchangers bind negatively charged proteins which replace the anions attached to the resin. Cation-exchangers, on the other hand, bind positively charged proteins which replace the cations attached to the resin (Sheehan, 2009). CP has a high ratio of acidic to basic amino acids, leaving it with an overall negative charge at neutral pH. Based on this property, anion-exchange chromatography is an efficient means of isolating CP.

The purification of CP is described in subsequent sections using two weak anion-exchangers according to the method outlined by Low Ah Kee *et al*. (2012).

2.3.1.2.1.1 *DE-52-Cellulose anion-exchange chromatography*

The DE52-cellulose resin (Whatman) is a weak anion-exchanger. The resin was purchased in the preswollen form and was equilibrated as per the manufacturer's instructions prior to use. Chromatographic details are given in Diagram 2.1 (Section 2.3.10).

The supernatant (obtained from Section 2.3.1.1) was applied to a 100ml column containing DE52-cellulose resin previously equilibrated with 20mM Tris-HCl buffer, pH 7.4, at a flow rate of 5ml/hour. A stepwise elution (0.25 and 0.5M NaCl) was performed at 20ml/min. The 0.5M NaCl fraction containing CP activity was collected, pooled and concentrated using ultrafiltration. The column was washed with 5 column volumes of 1M NaCl to remove the protein of interest, followed by 10 column volumes of equilibration buffer (20mM Tris-HCl, pH 7.4) to regenerate the resin. Amicon® Ultra-15 centrifugal filter devices (Millipore) with a molecular weight limit of 10 kDa were used to concentrate samples. The devices were centrifuged using an Eppendorf centrifuge (Merck) 5804 R at 4000 x *g* at 4ºC. The concentrated sample

was washed with 20mM Tris-HCl buffer, pH 7.4 and applied to a DEAE-Sepharose column (Section 2.3.1.2.1.2).

2.3.1.2.1.2 *DEAE-Sepharose® anion-exchange chromatography*

DEAE-Sepharose® fast flow (Sigma-Aldrich), an example of a weak anion-exchanger, was packed and used in conjunction with an AKTA FPLC system as described in Diagram 2.1 (Section 2.3.10).

The concentrated sample from Section 2.3.1.2.1.1 was washed with 20mM Tris-HCl buffer, pH 7.4 and applied to a 50ml DEAE-Sepharose column previously equilibrated with the same buffer. The sample was applied at 0.5ml/min on an FPLC system. A gradient of 0 - 0.5M NaCl in 20mM Tris-HCl buffer, pH 7.4 was applied over 200 minutes. The CP fraction was eluted with 1.5M NaCl in 20mM Tris-HCl buffer, pH 7.4. The active CP fractions were collected, pooled and concentrated via ultrafiltration. Amicon® Ultra-4 centrifugal filter devices (Millipore) with a molecular weight limit of 10 kDa were used to concentrate samples. Concentrated samples were washed with 20mM Tris-HCl buffer, pH 7.4 and purified CP was freeze-dried in 100µg aliquots. The column was washed with 10 column volumes of equilibration buffer (20mM Tris-HCl, pH 7.4) to regenerate the resin.

2.3.2 Bicinchoninic acid protein assay

Bicinchoninic acid (BCA) is a weak acid composed of two carboxylated quinoline rings. This assay is used to quantify the total concentration of protein in a solution. Two molecules of bicinchoninic acid chelate a Cu^{+} ion, and forms a purple complex that is soluble in water and strongly absorbs light at a wavelength of 562nm (Smith *et al*., 1985; Noble and Bailey, 2009). All protein determinations were carried out using the BCA protein assay kit (PIERCE) according the manufacturer's instructions. Briefly, 10µl of sample or standard was added to a 96-well plate. BCA reagent (200µl) was added to the sample and incubated at 37˚C for 30 minutes. The absorbance was measured at 562nm using a BioTek PowerWave XS microtitre plate reader. A calibration curve was constructed using bovine serum albumin (BSA) as a

standard. The protein concentration for all samples was calculated using the calibration curve. (Figure 2.1).

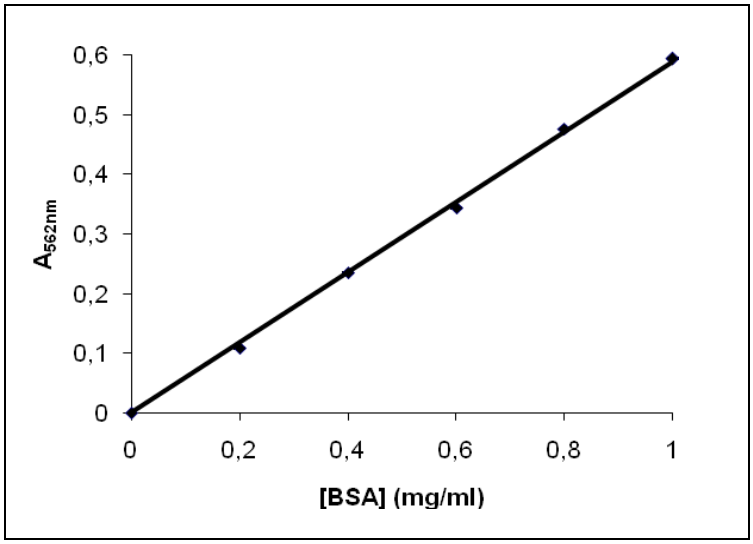


Figure 2.1 BCA assay standard curve. Error bars represent standard deviation of triplicate results (R^2 =0.999).

2.3.3 Three stage chromogenic factor X (FX) activity assay

Factor X activation of samples were quantified by a 3-stage chromogenic assay (Mielicki and Gordon, 1993) modified to a microplate format (Mielicki and Sobolewska, 2001). Briefly, the samples (50µl) were placed in a 96-well microtitre plate, 0.5µg of factor X (5µl) (Haematologic Technologies, Inc, USA) in 15µl buffer I (50mM bis-Tris propane + 25mM $CaCl_2$, pH 6.7) was added and incubated for 30 minutes at 37 °C, thereafter, 5 µg of prothrombin (5µl) (Haematologic Technologies, Inc, USA) and Cephalin (1.2µl) (Sigma, USA) in 13.8µl buffer II (100mM bis-Tris propane + 12.5mM $CaCl_2$, pH 7.8) was added to the well and the plate was incubated an additional 30 minutes at 37 °C, followed by addition of the chromogenic substrate for thrombin (2.5µl) (Sar-Pro-Arg-pNA, Sigma, USA) and 17.5µl distilled water in 14.5µl buffer III (50mM Tris, pH 7.8). The change in absorbance per minute was monitored at 405nm, using a microtitre plate reader (BioTek PowerWave XS, USA). Procoagulant activity (A) was expressed in units [U] or milliunits [mU]. One unit represents the amount of factor-X activator, which is responsible for the cleavage of 1µmol of pNA per minute from the chromogenic substrate under the assay conditions described above (molar extinction coefficient for pNA ε=9905).

2.3.4 Fluorescent synthetic substrate assay

CP activity was also determined using a synthetic substrate, Boc-QVR-AMC [GL Biochem (Shangai) Ltd.] as completed previously in our research group (Low Ah Kee, 2011). 25µl CP (0.4µg/µl) was added to a black 96-well microtitre plate (Nunc), followed by addition of 75µl assay buffer (50mM Tris-HCl, containing 0.277M NaCl, pH 7.4). For the blank, 25µl assay buffer was substituted for enzyme. The plate was incubated at 37ºC for 5 minutes. 75µl (50µM) substrate (G.L. Biochem, China), in assay buffer, was added to the well and the reaction was monitored using a BioTek SynergyMx fluorescence microtitre plate reader for 1 hour at 5 minute intervals at 37ºC. The excitation and emission wavelengths were 355nm and 460nm, respectively. The rate of the reaction was calculated in the initial, linear portion of the progress curve as a change in relative fluorescence per minute (Figure 2.2). Specific activity was calculated from the rate of the reaction as µM AMC hydrolysed (from AMC standard curve, Figure 2.3) per minute per milligram of CP.

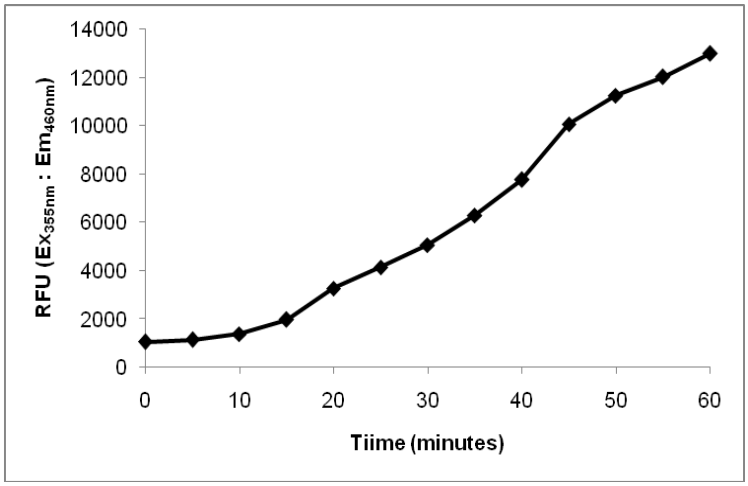


Figure 2.2 Progress curve for the hydrolysis of Boc-QVR-AMC (50µM) by CP (0.4µg/ml).

Figure 2.2 illustrates a typical progress curve for the hydrolysis of Boc-QVR-AMC by CP. The rate of the reaction was calculated between 20 and 30 minutes due to the lag phase observed.

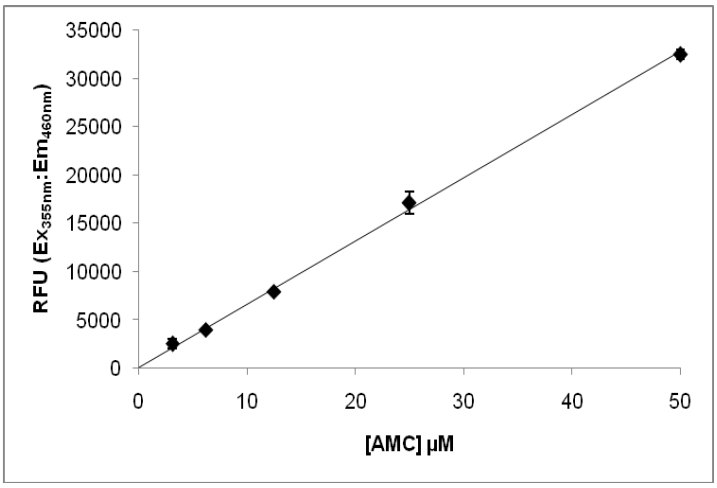


Figure 2.3 AMC standard curve. Error bars represent standard deviation of triplicate results (R^2 =0.998).

2.3.5 Protein visualisation and molecular weight determination of CP

2.3.5.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophores (SDS-PAGE) was used to visualize the homogeneity of the isolated CP fractions and to determine its molecular mass. In electrophoresis, charged molecules are separated in an electric field. SDS, a detergent, disrupts non-covalent protein bonds by binding to the hydrophobic parts of proteins, coating the protein with a negative charge to allow the protein to migrate through the gel based on its molecular mass (Sheehan, 2009).

Samples were electrophoresed on 12% polyacrylamide gels for 1.5 hours under reducing and non-reducing conditions according to the method of Laemmli (1970) The protein standard used was peqGOLD protein marker IV (170, 130, 95, **72**, 55, 43, 34, 26, 17 and **10** kDa). The calibration curve obtained from the preparation of this marker is shown in Figure 2.4. (See appendix D for details).

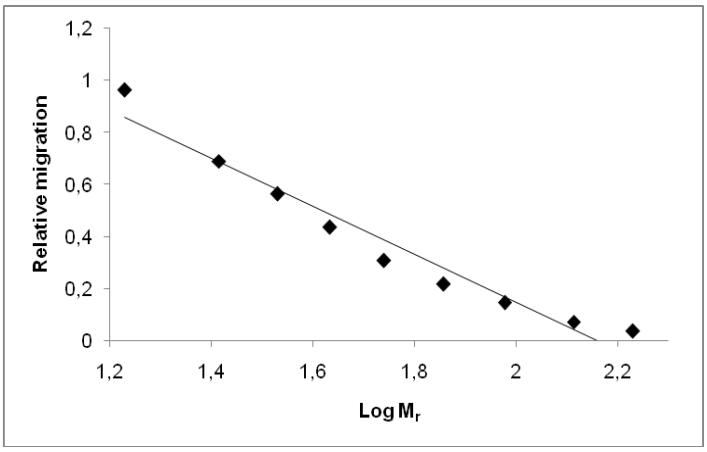


Figure 2.4 SDS-PAGE calibration curve. A 12% resolving gel was prepared. The wide range peqGOLD protein marker IV was used (R^2 = 0.954).

2.3.5.2 Silver stain

Silver staining is a technique that provides a very sensitive tool for protein visualization with a detection limit in the 0.3-10 ng range (Switzer *et al.,* 1979). Protein detection depends on the binding of silver ions to the amino acid side chains, primarily the sulfhydryl and carboxyl groups of proteins (Switzer *et al*., 1979; Oakley *et al*., 1980; Merril e*t al*., 1981; Merril *et al*., 1986), followed by reduction to free metallic silver (Rabilloud, 1990; Rabilloud, 1999). The protein bands are visualized as spots at the site of reduction.

Following Coomassie Brilliant Blue (CBB) staining, the gel was stained with silver nitrate using a mini gel silver stain kit (Fermentas) as per manufacturer's instructions (Figure 2.7).

2.3.6 Delipidation of cancer procoagulant

Protein molecules are retained in solution by water. This occurs because water minimizes the electrostatic attractions between proteins, reducing the strength of the electrostatic attraction by a factor of 80, which is the dielectric constant of water at 0ºC. Solvents, such as acetone, reduce the dielectric constant of water during precipitation, thereby minimising the solvating capacity of water molecules. An increase in solvent concentration results in bulk displacement of water molecules. This allows strong hydrophobic interactions between proteins, causing the

hydrophobic patches of different proteins to interact and aggregate (Mastro and Hall, 1999).

CP was delipidated by adding 9 volumes of ice-cold acetone to 1ml of purified CP (100µg/ml) in a drop-wise manner, while stirring continually. The sample was incubated at -20ºC for 1 hour and centrifuged at 12000 x*g* for 15 minutes using an Eppendorf centrifuge (Merck). The organic phase was decanted into a separate beaker and aspirated to remove acetone. The pellet, containing the delipidated CP, was resuspended in 0.5ml 20mM Tris-HCl, pH 7.8. After aspiration, the lipid fraction (devoid of acetone) was resuspended in 0.5ml 20mM Tris-HCl, pH 7.8. The samples were stored at -20ºC until further analysis.

2.3.7 Lipid detection

To validate the absence of lipid from the delipidated samples, a lipid detection assay was performed by modifying a method used by Alcaraz *et al*. (2004). Briefly, 10% NaOH (5µl) was added to 0.1ml of sample or control. The mixture was incubated at 37ºC for 15 minutes. Following the addition of distilled water (50µl) the mixture was incubated at 37ºC for 15 minutes. 0.5M $CaCl_2$ (1ml) was added and the mixture was centrifuged briefly. The presence of lipids was indicated by precipitation in the sample. A lack of precipitation would be indicative of the absence of lipid from the sample.

2.3.8 The pH optimum of CP

The pH optimum of CP was determined using the direct fluorescent synthetic assay (Section 2.3.4). Activity was tested in the pH range of 1 – 12, using a universal buffer. The universal buffer contained 57mM boric acid, 33mM citric acid, 33mM sodium dihydrogen phosphate and 1M sodium hydroxide. The pH was adjusted to a series of pH values (pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) using 0.1N HCl. CP (25µl; 0.4µg/µl) was added to a black 96-well microtitre plate (Nunc), followed by addition of 75µl assay buffer at the appropriate pH. The plate was incubated at room

temperature for 5 minutes. Boc-QVR-AMC (75µl; 50µM), in assay buffer, was added to the well and the reaction was monitored in a BioTek SynergyMx fluorescence microtitre plate reader for 1 hour at 5 minute intervals. The excitation and emission wavelengths used were 355 and 460nm, respectively.

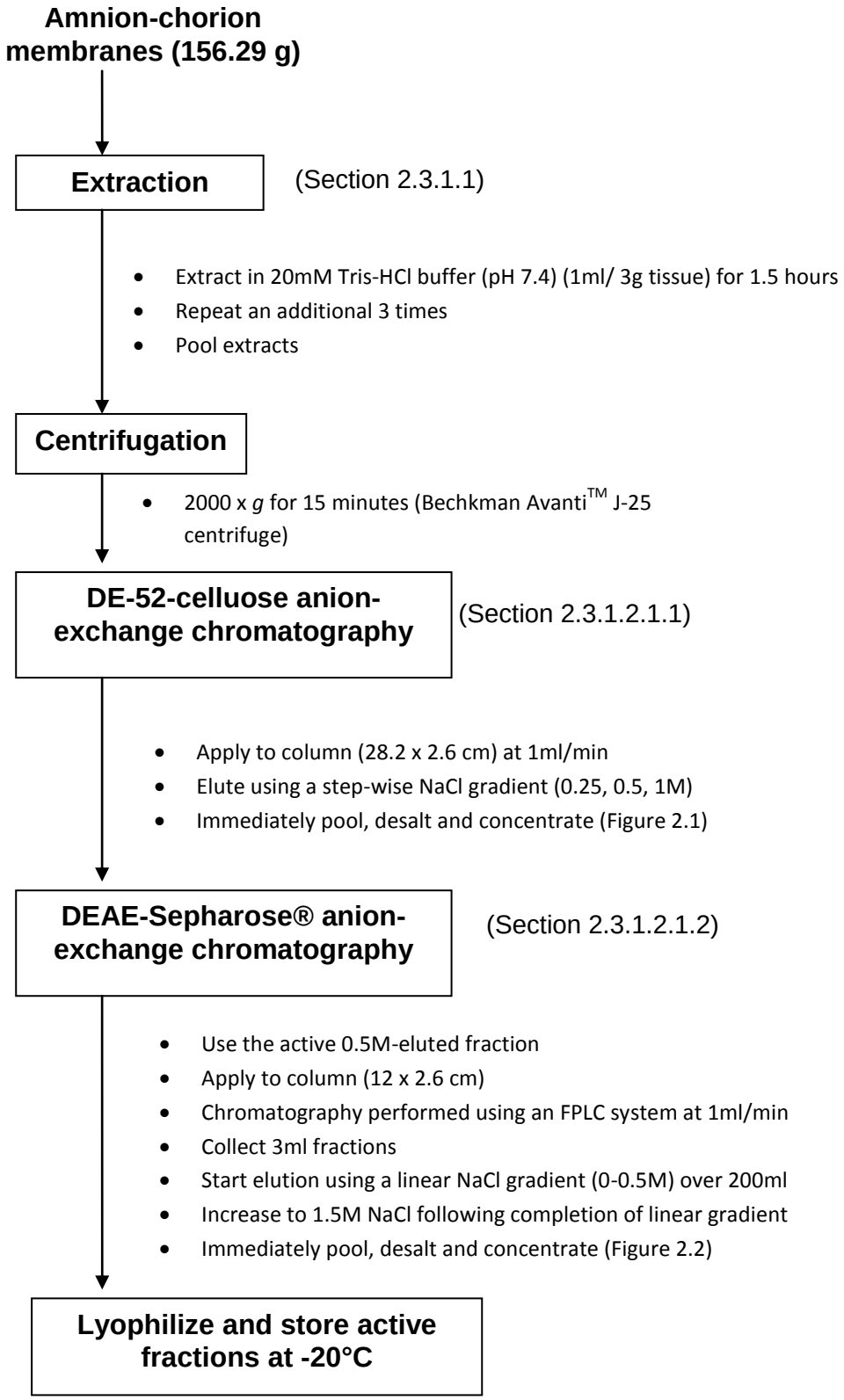
2.3.9 The temperature optimum of CP

The temperature optimum of CP was determined using the direct fluorescent synthetic assay (Section 2.3.4). The assay temperature range used was 30 – 60ºC. CP (25µl; 0.4µg/µl) was added to a black 96-well microtitre plate (Nunc), followed by addition of 75µl assay buffer (50mM Tris-HCl, containing 0.277M NaCl, pH 7.4). The plate was incubated at the appropriate temperature for 5 minutes. Boc-QVR-AMC (75µl; 50µM), in assay buffer, was added to the well and the reaction was monitored at the appropriate temperature in a BioTek SynergyMx fluorescence microtitre plate reader for 1 hour at 5 minute intervals. The excitation and emission wavelengths used were 355 and 460nm, respectively.

2.3.10 Statistics

In all quantification experiments, results are expressed as means±SEM, unless otherwise stated. Statistical differences between two sets of data were determined using a Student's t-test (assuming equal variance), and differences between more than two data sets were determined by one-way ANOVA. $P<0.05$ was considered statistically significant. All statistical calculations were performed using Microsoft Office Excel 2007.

Diagram 2.1 Flow-diagram illustrating a typical isolation of CP from amnion-chorion placental membranes.



2.4 Results and Discussion

The results obtained from the isolation of CP from amnion-chorion placental membranes will be discussed in this section. This section will only show one example of several isolation procedures performed. A total of 36 isolations were performed in this study, to generate enough CP for all subsequent experiments.

2.4.1. Isolation and purification of cancer procoagulant from amnion-chorion membranes

The isolation of CP was performed using amnion-chorion membranes of placenta as outlined in Section 2.3.1. CP-rich fractions were eluted with 20mM Tris-HCl, pH 7.4, containing 0.5M NaCl (Figure 2.5; peak 8). Following desalting, the isolated fraction 8 was passed through a DEAE-Sepharose anion exchanger and a homogenous preparation of CP was eluted using 20mM Tris-HCl, pH 7.4, containing 1.5M NaCl (Figure 2.6).

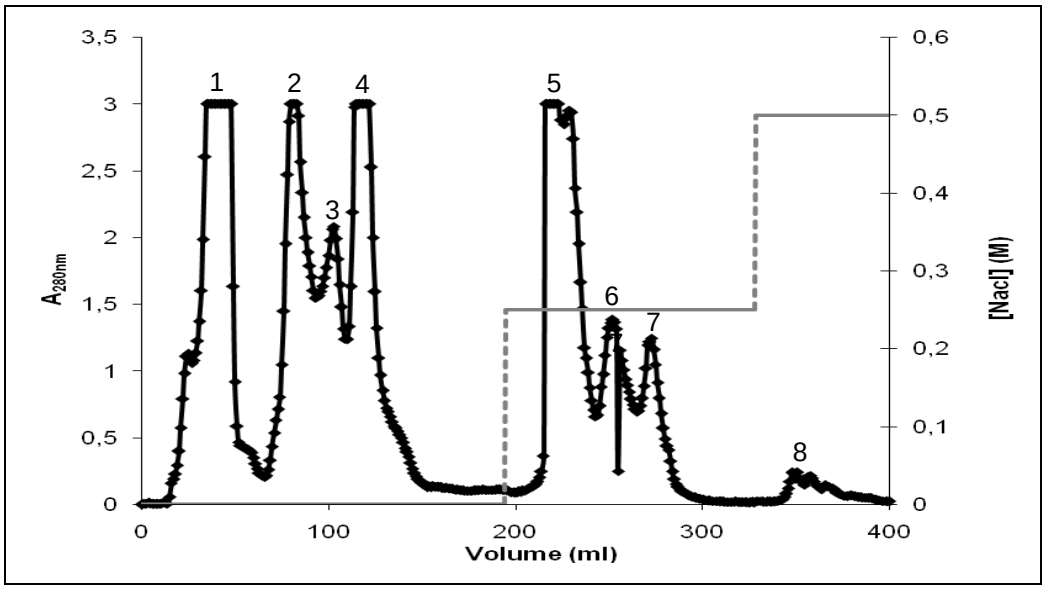


Figure 2.5 An example of a chromatogram obtained from DE-52 cellulose anion exchange chromatography of an amnion-chorion membrane extract. The extract (290ml, 1600.8mg) was applied to the column at a flow rate of 5ml/hour. Elution was performed using a step-wise NaCl gradient (---) at 20ml/hour. Fractions of 7ml were collected and the A_{280nm} (—) monitored. Fractions from peak 8 were pooled, desalted and further purified by DEAE-Sepharose.

Several peaks were resolved by DE-52 cellulose chromatography with peak 8 (Figure 2.5) containing CP activity. It was immediately desalted, concentrated and DEAE-Sepharose chromatography was performed at a flow rate of 0.5ml/min. Unbound protein was eluted using a linear gradient from 0 to 0.5M NaCl, while CP was eluted using a step-wise gradient (Figure 2.6). Peaks obtained after eluting with 1.5M NaCl were pooled, desalted and concentrated and stored as aliquots at -80°C until further analysis.

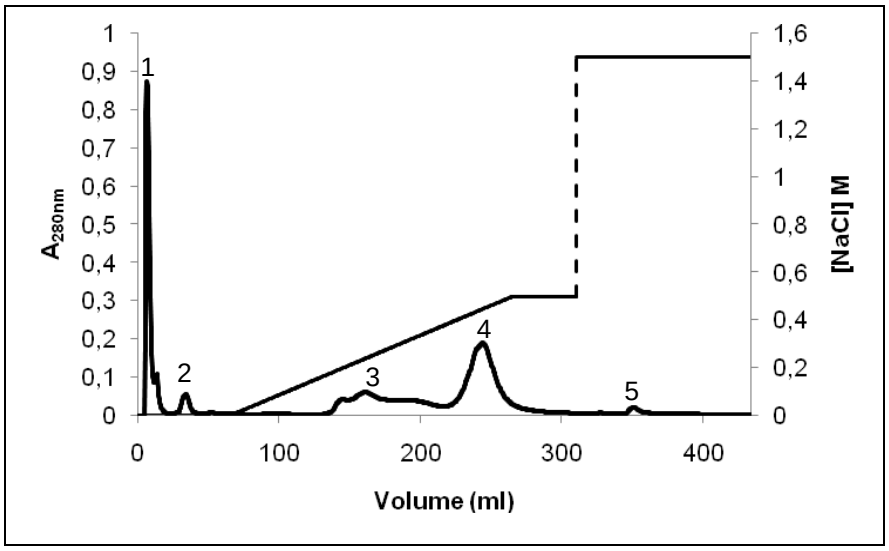


Figure 2.6. An example of a chromatogram obtained from DEAE-Sepharose chromatography of CP obtained from a 0.5M NaCl fraction (peak 8) on DE-52 celluose (Figure 2.5). The 0.5M NaCl eluate from DE-52 cellulose chromatography (6ml, 3.24mg) was applied to a 50ml DEAE-Sepharose column (flow rate of 0.5ml/min). Elution was performed at 1ml/min using a NaCl gradient (0 – 0.5M) followed by an immediate step-wise change from 0.5M to 1.5 NaCl (---). Chromatography was performed on an AKTA FPLC system. Fractions of 3ml were collected and the absorbance at A_{280nm} (—) monitored. Fractions from peak 5 were pooled, desalted, lyophylised and stored at -80ºC.

The absorbance values obtained at 280nm for the active fraction eluted with 1.5M NaCl was consistently low with all purification protocols, despite having FX-activating activity. This can be explained when one considers the amino acid sequence of CP. CP does not contain a high percentage of tryptophan residues and its tyrosine content is only 1.9% of the total amino acid composition (Falanga and Gordon, 1985) therefore explaining the characteric low absorbance values noted for CP at A_{280nm}.

In addition to using the FX assay for determining CP activity, the activity of CP was determined using a direct assay, employing Boc-QVR-AMC as a substrate. The purification table is shown in Table 2.2.

Table 2.2 Purification table for the isolation of CP from amnion-chorion membranes (156.29 g amnion-chorion membranes).

Sample	Volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Total activity (µU)	Specific activity (µU/mg)	Purifi-cation factor	% Yield
Extract	290	5.52	1600.8	91020.65	56.86	1	100
DE-52 (peak 8)	6	0.54	3.24	201.44	62.17	1.09	0.22
DEAE (peak 5)	2.12	0.25	0.53	93.21	175.87	3.09	0.10

As shown in Table 2.2, the yield of CP from DE-52 chromatography was very low (0.22%) and the purification fold (1.09) did not increase by much. There were also more bands present on SDS-PAGE (Figure 2.7). The final product was purified 3.09 fold and resulted in a yield of 0.1%. Even though this yield was very low, it resulted in a fairly homogenous CP preparation (Figure 2.7). The yield could possibly be improved by designing an immunoaffinity column for CP. This low yield, however, is typical for the isolation of CP since a low CP yield of 0.09% was found by Low Ah Kee (2011) using the same isolation procedure. The specific activity obtained in this study was 175.87µU/mg which is slightly higher than the 169.91µU/mg obtained by Low Ah Kee (2011).

The crude extract was extensively purified using two weak anion-exchangers. The molecular weight of the purified CP-fraction was determined by SDS-PAGE (Figure 2.7) to be 68 kDA using the calibration curve (Appendix D).

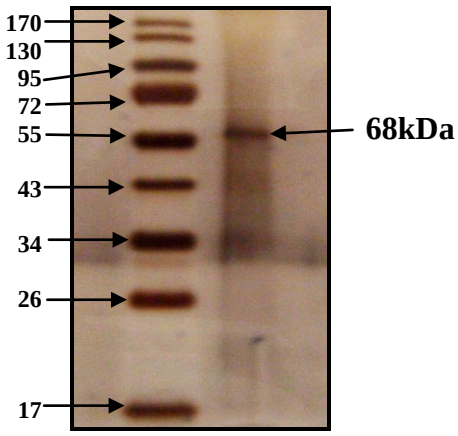


Figure 2.7 Silver-stained non-reducing SDS-PAGE pattern of the purified CP fraction. Molecular masses are shown in kDa. Lane 1, peqGOLD IV protein marker; lane 2, DEAE-Sepharose (1.5M fraction) (non-reducing).

Due to the low yield of CP (Table 2.2), numerous isolations were performed. The purified CP band was too faint to be viewed using the conventional CBB staining protocol. This is due to CP being composed of only 9.3% basic residues (Gordon, 1994) and is, therefore, not strongly stainied with CBB, since CBB binds to two basic residues, namely arginine and lysine. Silver staining was, therefore, used.

2.4.2 pH optimum of CP

The pH optimum of CP (Figure 2.8) was determined using the direct CP assay with Boc-QVR-AMC (50µM) as a substrate.

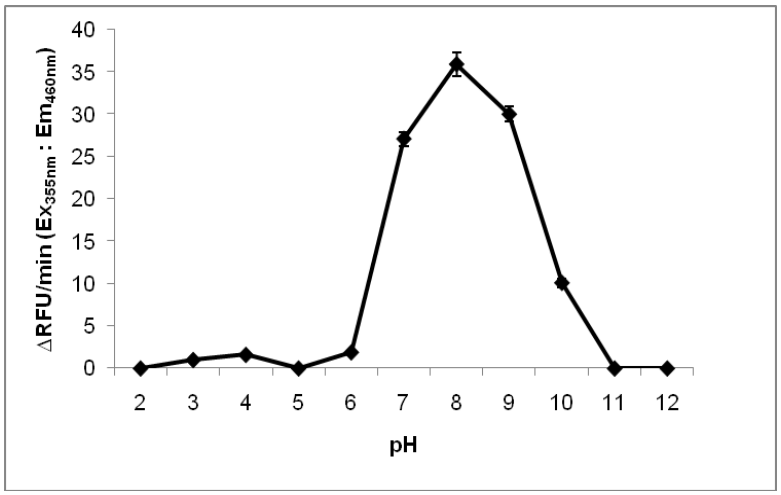


Figure 2.8 The optimum pH for CP activity. Error bars represent the SEM of 3 experiments.

CP exhibited proteolytic activity in the pH range 7-10. It is, therefore, active at physiological pH. The pH optimum of CP was found to be at pH 8. Mielicki and Gordon (1993) showed that CP exhibited a broad pH optimum between 6.29 and 7.25 for the activation of factor X.

2.4.3 Temperature optimum of CP

The temperature optimum of CP (Figure 2.9) was determined using the direct CP assay with Boc-QVR-AMC (50µM) as a substrate.

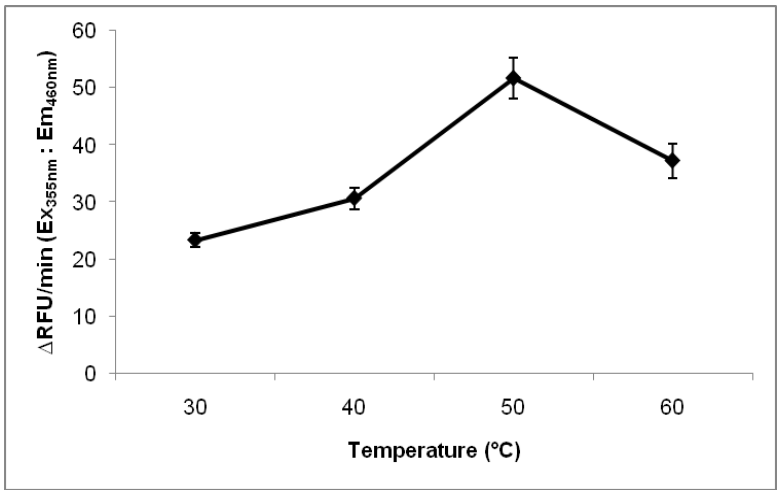


Figure 2.9 The optimum temperature for CP activity. Error bars represent SEM values for 3 experiments.

CP was active over a broad temperature range (30-60ºC). The optimum temperature for CP was found to be 50ºC (Figure 2.9).

2.4.4 Delipidation of CP

Due to the possible presence of lipids on CP, an N-terminal amino acid sequence is not easily obtained and this is further complicated by low yields. Delipidation of CP would, therefore, provide a means of overcoming these difficulties. CP was delipidated as discussed in Section 2.3.6. The activity of CP was determined following delipidation (Figure 2.10).

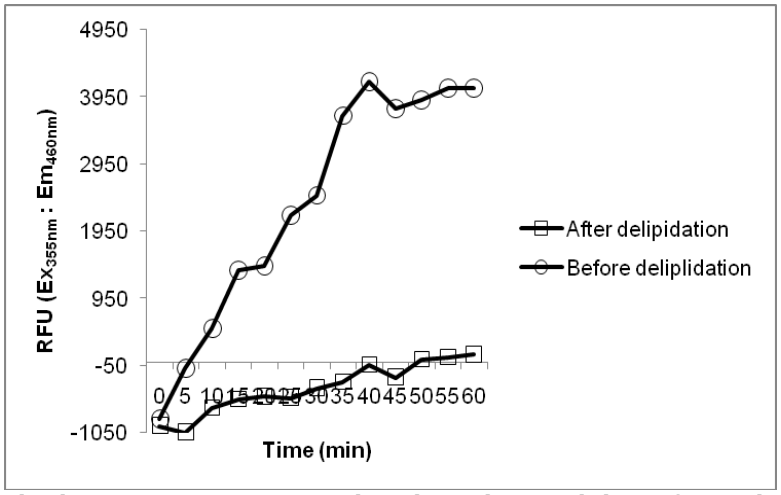


Figure 2.10 Typical progress curves showing the activity of CP before and after delipidation using Boc-QVR-AMC as a substrate.

Figure 2.10 indicates a typical result obtained and highlights that CP activity is drastically reduced upon delipidation. It shows, therefore, that the lipid portion is important for optimal CP activity. The lipid portion of CP was re-introduced into the delipidated solution at various ratios to determine the effect of re-lipidation on CP activity. This procedure was carried out a total of 8 times and similar results were obtained. These results are depicted in Figure 2.11.

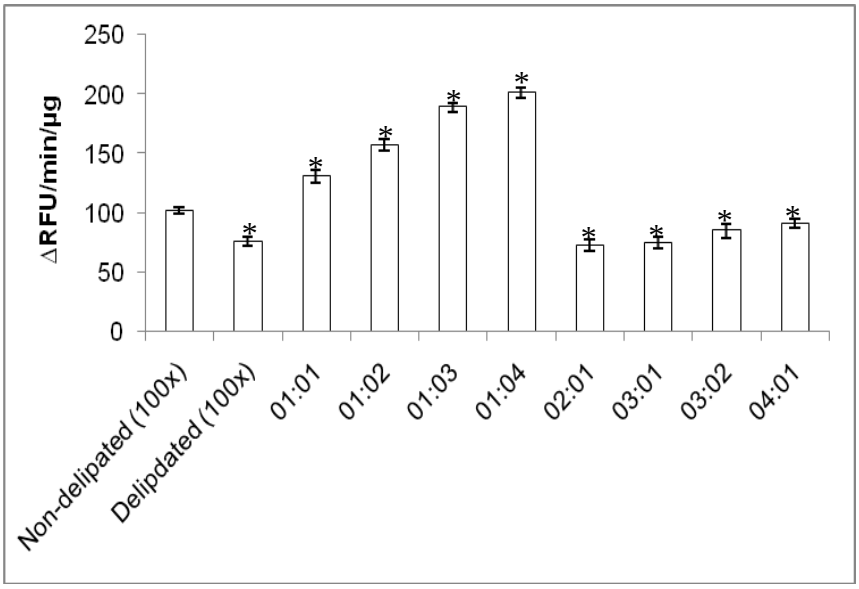


Figure 2.11 Typical results for the activity of delipidated and re-lipidated CP using Boc-QVR-AMC as a substrate. The ratio of protein:lipid is shown. Error bars represent the SEM of 8 experiments. *$p<0.05$.

Figure 2.11 highlights firstly, the significant ($p<0.05$) decrease in CP activity after delipidation and secondly, the increase of activity with an increase in lipid content. The decrease could be due to the loss of the lipid portion of CP (which seems to be important for its activity) causing a conformational change in the protein structure. It could also be due to a loss of protein during the acetone precipitation of CP, or due to aggregation of the protein (Mastro and Hall, 1999). It was noted, however, when the amount of protein was increased, a significant decrease in CP activity was observed compared to non-delipidated CP. This confirms that the presence of lipid on CP is important and enhances its activity *in vitro*.

2.4.5 The effect of calcium chloride concentration on CP activity

CP (0.4µg/µl) activity was determined using the direct fluorescence assay (Section 2.3.4), i.e. Boc-QVR-AMC (50µM). A range of 0 – 25mM calcium chloride was tested and it was determined that CP showed activity even in the absence of calcium chloride, however, CP was optimally active at a calcium chloride concentration of 5mM (Figure 2.12). In fact, there was a significant ($p<0.05$) two-fold increase in CP activity as the calcium chloride concentration increased to 5mM. It was observed that even higher concentrations of calcium chloride (25mM) did not affect CP activity significantly ($p>0.05$).

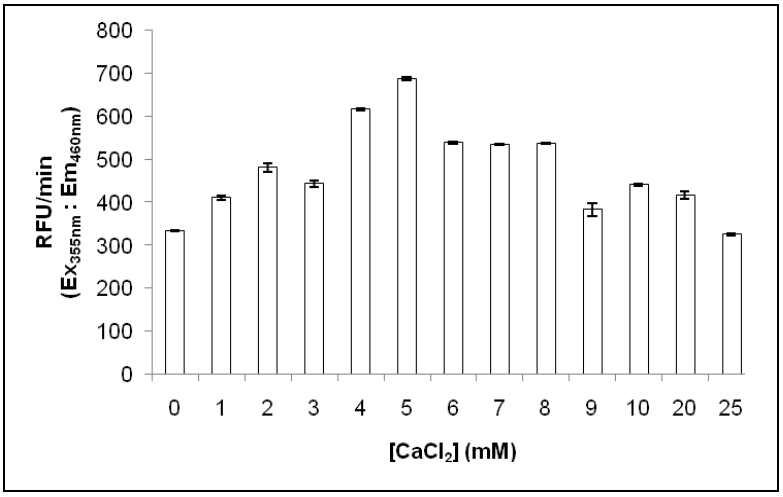


Figure 2.12 The effect of calcium chloride concentration on CP activity. CP (0.4µg/µl) activity using Boc-QVR-AMC (50µM) as the substrate. Error bars represent the SEM of 3 experiments.

2.5 Discussion

A total of 36 isolations were performed using the two-step ion exchange chromatography procedure. The total protein from each isolation varied (0.1 – 0.54mg) depending on the amount of amnion-chorion membrane available. The percentage yield was consistent throughout all 36 isolations. The low yield obtained could be due to the prolonged time period that the amnion-chorion membranes are stored at 4ºC following collection from the patient. It was necessary to wait until a sufficient mass of membranes are collected before commencing the isolation procedure and, therefore, this was unavoidable. This could have negatively influenced the yield of CP. In addition, Low Ah Kee *et al*. (2012) illustrated that production of vascular endothelial growth factor (VEGF) protein increased in MCF-7 breast cancer cells and E14 MESCs, but decreased in MDA-MB-231 breast cancer cells. This is noteworthy and linked to the isolation yields as CP could, therefore, potentially play a part in the modulation of angiogenesis in cancer as well as have a role in vascular development during embryonic development. It could, therefore, be hypothesised that CP activity in the amnion-chorion membranes could, possibly, be much higher during the developmental stages of the foetus (during the first trimester) since angiogenesis is crucial during this period and would be a better source of CP. To investigate this possibility, one would need to monitor the activity of CP throughout a pregnancy and correlate it to the angiogenic process, however, it should be noted that this is not feasible for ethical reasons. CP is usually extracted from the amnion-chorion membrane and it is not possible to obtain the placenta during pregnancy. An alternative would be to use the placenta of aborted foetuses; however, numerous human ethical concerns need to be considered. Additionally, another possible reason for the low yield is the high concentration of salt used to elute CP from the DE-52 and DEAE-Sepharose columns (0.5M and 1.5M NaCl, respectively). Perhaps the high salt concentration disrupts the ionic interactions and interferes with protein folding (Sarraga *et al*., 2006). Low Ah Kee (2011) however showed that CP activity is inhibited by divalent metal ions, but is not affected by NaCl. Nevertheless, the author did not examine the effect of NaCl concentrations higher than 333mM. One could possibly substitute the DE-52 ion exchange step with an immunoaffinity chromatography step such as an E-64 (cysteine protease inhibitor)

column to avoid the use of salt. This would be more specific than ion-exchange chromatography and would also eliminate the need for the high salt concentration (1.5M NaCl) to elute CP from the column. Low Ah Kee (2011), however, obtained a better purification of CP using the two-step ion-exchange chromatography procedure outlined in this study than with the procedure involving an E-64 column. However, the purification of CP with an E-64 affinity column could be improved if the affinity step were to be preceded by delipidation, as it is possible that the lipid portion of CP influences binding of CP to the E-64 resin. Furthermore, lipid present on CP may influence accurate protein determination, resulting in the low yield observed.

Despite the low yield of CP throughout the isolations, it should be noted that the CP preparation resulted in a 68 kDa band as visualized using silver staining, under non-reducing conditions. These results are consistent with that of Low Ah Kee (2011) and Falanga and Gordon (1985a) and other studies in our research group. As mentioned previously, CBB staining of CP is usually weak due to the very low amount (9.3%) of basic residues (arginine and lysine) (Gordon, 1994). Silver staining was, therefore, used since silver ions bind to the amino acid side chains, primarily the sulfhydryl and carboxyl groups of proteins followed by reduction to free metallic silver and is consequently more sensitive (Switzer *et al*., 1979). It was found that a considerable amount of smear was present following silver staining, suggesting that the preparation is not completely homogenous and might be contaminated by other proteins which could jeopardise enzymatic kinetic studies.

CP showed activity in the range pH 7 – 10 and the pH optimum of CP was determined to be at pH 8. This differs slightly from published data which reports a pH optimum of 6.5 (Gordon and Cross, 1981). The method used to assay for CP activity in our study was a newly developed direct synthetic substrate assay for CP as opposed to the conventional factor X assay. The direct assay utilizes the substrate Boc-QVR-AMC and was designed by Low Ah Kee (2011). The pH optimum of 6.5 for CP determined by Gordon and Cross (1981) was carried out using the three-stage factor X assay. Since this multi-step assay depends on other proteases present (factor X and thrombin) which are serine proteases with different pH optima, this could influence the different range of pH optimum values determined for CP. In the direct assay employed in this study, however, these factors do not play a role since it

is absent from the assay. Most of the CP activity assays in this study were completed at pH 7.4. The activity of isolated CP fractions could possibly have been higher if CP activity was assayed at the optimum pH and future assays using Boc-QVR-AMC should, therefore, be carried out at pH 8 instead.

The optimum activity of CP was determined to be 50ºC; however, CP also displayed activity over a broad temperature range (30 – 60ºC). Overall, CP contains about 40% hydrophobic amino acid residues (Falanga and Gordon, 1985). Due to its hydrophobic nature, CP associates with proteins that are present in tissue extracts and serum. Ideally, to determine if the lipid influenced the optimum temperature of CP, the experiment needs in future to be completed on a duplicated fraction. CP activity is thought to be stabilized in a lipid environment, while high salt concentrations are not ideal (Gordon, 1994).

In order to confirm whether a lipid-rich environment stabilises CP activity, CP was delipidated. We found that lipid plays an important role in CP activity (Figure 2.11) as CP activity is drastically reduced upon delipidation. It shows, therefore, that the lipid portion is important for CP activity. The re-lipidation of CP noteably increased CP activity as the ratio of lipid:protein increased, but not when the ratio of protein:lipid increased. This further supports the observation that the presence of lipid plays a major role in the proteolytic activity of CP and its stability. It is possible that the presence of lipid results in a conformational change in CP either at the active site or a binding site of interest, which affects its affinity for the substrate. This should, however, be validated by confirmation and identification of the removed lipid portion using techniques such as mass spectroscopy. In addition, the presence of the lipid portion of CP opens up the possible hypothesis that it's involved in transport of lipids in the blood. Another possibility is that it could mediate cholesterol efflux from endothelial cells which is crucial for angiogenesis efficiency (Fang *et al*., 2013). Quantification and identification of the lipid was not performed in this study and is recommended to be completed using LC-MS or GC-MS analysis. Identification of the lipid by mass spectroscopy could provide further clues as to the function of CP in angiogenesis and metastasis.

It was found that CP activity increased with an increase in calcium chloride concentration up to 5mM. CP showed optimal activity at a calcium chloride

concentration of 5mM, while 25mM calcium chloride did not significantly change the activity of CP. These results are in agreement with those of Gordon and Mielicki (1997) who showed that CP activity was enhanced in the presence of calcium chloride and that CP was optimally active at a calcium chloride concentration of 7mM. In the absence of calcium chloride, CP was still active. This is in accordance with the results of Low Ah Kee (2011). As is evident from this study and from the results of Gordon and Mielicki (1997), even though CP does not require calcium chloride, the presence of this salt enhances its activity.

In this study, CP activity could have been improved by making alterations to the purification procedure. One such alteration could have been to incorporate a reducing agent into all the extraction and chromatography buffers to prevent irreversible oxidation of CP's catalytic cysteine and, therefore, prevent inactivation of of CP. In addition, a protease inhibitor cocktail should have been incorporated into the purification procedure to prevent degradation of CP by other proteases, as well as potential auto-degradation. Furthermore, the chromatography steps could have been preceded by ammonium sulphate precipitation, since including this step has been reported to cause a 50-fold purification of CP (Gordon *et al*., 1985).

3. DESIGNING OF SYNTHETIC FLUOROGENIC CP SUBSTRATES

3.1 Introduction

Due to the difficulties experienced with sequencing of CP to obtain additional information about this enzyme, synthetically synthesised substrates were used to overcome this obstacle. After obtaining sufficient CP, this study was able to further investigate and improve its substrate. The substrate specificity data is presented in this secton to summarise the findings.

3.1.1 Substrates previously designed for CP

The cysteine protease cancer procoagulant (CP) is produced by undifferentiated (foetal) or dedifferentiated (malignant) tissue (Gordon, 1994; Gordon and Cross, 1981). Gordon and Cross (1981), Kozwich *et al*. (1994) and Mielicki *et al.* (1994) have shown the potential of CP as a sensitive and specific early stage tumour marker. Until recently, coagulation factor X was the only known physiological substrate for CP (Gordon and Mourad, 1991). Gordon and Mourad (1991), however, showed that CP activates factor X by cleaving its heavy chain at a different site than other known factor X activators. CP activity is affected by certain divalent cations which act in a region that does not form part of the active site of the enzyme (Falanga and Gordon, 1985b). Not much is known about the active site configuration of CP. There is also a lack of knowledge concerning the preferred substrate structure and kinetic characteristics of the enzyme. Evidence suggests that CP substrate specificity is similar to that of thrombin (Moore, 1992). The author, however, provided no kinetic data and this makes substrate specificity comparison difficult. Various studies employing chromogenic substrates for CP has shown that peptides containing arginine or lysine at the P1 position and proline at the P2 position are amongst the best substrates for CP (Lottenberg *et al*., 1981; Huseby and Smith, 1980; Claeson, 1994). Despite these cleavage sites being characteristic of serine proteinases such as thrombin and trypsin, Mielicki *et al*. (1997) showed that the CP activity is inhibited by classical cysteine proteinase inhibitors such as iodoacetamide and E-64. CP showed no significant action on the Cathepsin B substrate Z-Arg-Arg-

pNA, nor on the papain substrate Ac-Phe-Gly-pNA, which supports the hypothesis that CP does not belong to the papain superfamily (Gordon and Mourad, 1991). It was noted that when proline in the P2 position was substituted by phenylalanine (a hydrophobic amino acid) or valine (pyro Glu-Phe-Lys-pNA or Bz-Phe-Val-Arg-pNA, respectively) only traces of amidolytic activity were detected (Mielicki *et al*., 1997). Thrombin, however, cleaves Bz-Phe-Val-Arg-pNA (K_m=18pM) which is a sequence similar to the one recognized by thrombin in fibrinogen (Lottenberg *et al.,* 1981). Mielicki *et al*. (1997) made a surprising discovery that CP could not cleave the MeO-Suc-Arg-Pro-Tyr-pNA substrate which has an amino acid sequence similar to the cleavage site of factor X by CP: -Lys-Pro-Tyr- (Gordon and Mourad, 1991). They, therefore, concluded that the secondary and tertiary structure of the substrate had an influence on the active site of the enzyme. The differences in the hydrolysis of Bz-Phe-Val-Arg-pNA, fibrinogen and other thrombin substrates by CP and thrombin further supports the importance of the conformation of the active site, in that neither fibrinogen nor Bz-Phe-Val-Arg-pNA, the substrate that mimics the cleavage site of fibrinogen recognized by thrombin, were hydrolyzed by CP. However, other substrates cleaved by thrombin were also cleaved by CP (Mielicki *et al*., 1997).

The Michaelis constant (K_m = 18pM) calculated by Mielicki *et al*. (1997) for CP with chromogenic substrates indicates considerably high affinities, particularly for H-D-Ile-Pro-Arg-pNA and N-p-Tos-Gly-Pro-Arg-pNA, but the calculated catalytic constants, however, indicate that the dissociation of the enzyme-product complex is very slow, resulting in a poor hydrolysis rate. The authors suggested improving the catalytic constants by substituting the chromophore *p*-nitroaniline with other chromophores or fluorophores to increase the velocity of the reaction.

3.1.2 The most commonly used assay for CP activity - FX

The commonly used method available for determining CP functional activity is a three-stage chromogenic assay developed by Mielicki and Gordon (1993). In this assay, CP is mixed with bovine FX $CaCl_2$ in bis-Tris propane buffer (pH 6.7). After 30 minutes of incubation, bovine prothrombin and rabbit brain cephalin are added to the samples. After a further 30- minute incubation at 37°C, thrombin substrate Sar-Pro-

Arg-*p*-nitroanilide is added. The colour development at 405 nm is monitored for 30 minutes. This method for determining CP activity is a lengthy process, involving several incubated steps, a 30-minute recording step and contains many reagents. It is an indirect assay and is dependent on the activities of FX and thrombin. The need for a more direct assay for CP activity exists since CP has been proposed to be used as a tumour marker for early stage cancers (Kozwich *et al*., 1994).

3.1.3 Fibronectin and integrin as CP substrates

Low Ah Kee (2011) demonstrated the proteolytic cleavage of CP on fibronectin, an important ECM component. She showed that CP cleaved fibronectin, but not laminin or type IV collagen. The author determined the CP cleavage sites on fibronectin and noticed that CP cleaved at Ala_{44}/Val_{45} and Ala_{292}/Val_{293} (Figure 3.1). It was also noted that the cleavage sites were in regions rich in Pro and Gln residues.

A second potential physiological substrate for CP could be Integrin $\alpha_5\beta_3$. Integrin $\alpha_5\beta_3$ has been shown to be important for angiogenesis and is, therefore, a potential target for inhibition of tumour growth (Friedlander *et al*., 1995; Brookes *et al*., 1996; Varner and Cheresh, 1996). The first vitronectin receptor to be identified was $\alpha_5\beta_3$ (Pytela *et al*., 1985), but later it was shown that this integrin also binds to fibronectin and several other cell adhesion proteins (Kieffer *et al*., 1991; Charo *et al*., 1990; Wennerberg *et al*., 1996). In all cases the RGD motif in the ligands is of critical importance for binding of $\alpha_5\beta_3$. In each fibronectin subunit, two regions possess cell binding activity. These regions are $III_{9\text{-}10}$ and $III_{14\text{-}V}$ (Figure 3.1). The amino acid sequence RGD is a cell adhesive motif in fibronectin and is located in III_{10} (Figure 3.1). The RGD motif is the most important recognition site for nearly half of all known integrins (Garcia *et al*. 2002).

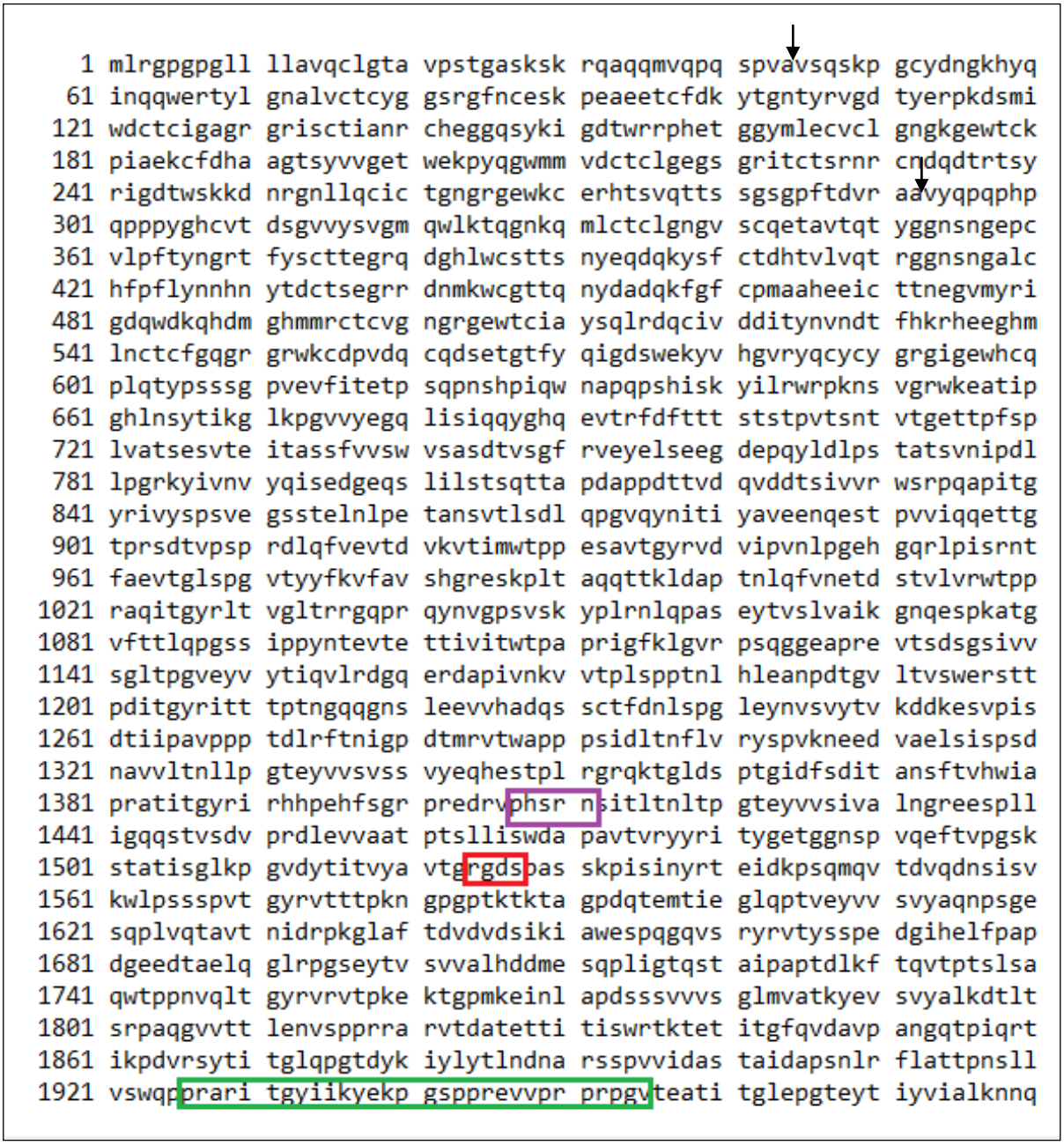


Figure 3.1 Partial fibronectin amino acid sequence showing CP cleavage sites and regions possessing cell binding activity. The sequence was obtained from NCBI protein database, accession number P02751. Arrows indicate cleavage sites obtained from N-terminal sequence analysis by Low Ah Kee (2011). The purple block shows the III_9 domain, red block shows the III_{10} domain containing the RGD cell adhesive motif, the green block encloses the III_{14} domain.

3.1.4 Synthetic fluorogenic substrates

Proteases play a vital role in nearly all biological processes and investigations into these roles are often carried out using peptide-AMC conjugates as fluorogenic substrates to examine protease behaviour (Hong and He, 2010). The use of synthetic fluorogenic substrates is a simple, inexpensive method of detecting enzymatic activity both qualitatively (Irvine *et al*., 1990) as well as quantitatively (Marathe *et al*., 2005; Li *et al*., 2003). 7-Amino-4-methylcoumarin (AMC) (Figure 3.2) can be used as a fluorescence reference standard for AMC-based enzyme substrates.

AMC has a molecular weight of 175.19 g/mol and its fluorescence spectra is not subject to variability due to pH-dependent protonation/deprotonation when assayed near or above physiological pH. This is because aromatic amines, including the commonly used AMC are partially protonated at low pH (less than 5) but fully deprotonated at physiological pH. AMC is widely used to prepare peptidase substrates in which the amide has shorter wavelength absorption and emission spectra than the amine hydrolysis product (Johnson and Spence, 2010).

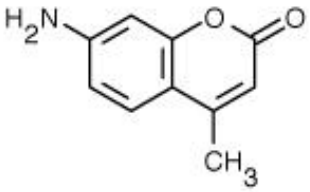


Figure 3.2 The structure of 7-amino-4-methylcoumarin (AMC). The chemical structure was drawn using Chem Sketch.

Synthetic substrates containing an AMC moiety (Ac-Asp-Glu-Val-Asp-AMC) were used by Li *et al*. (2003) and Marathe *et al*., (2005) for the detection of caspase-3 enzyme activity in cell lysates. Reactions were performed for 1 h at 37°C and the release of the fluorogenic AMC moiety was measured at an excitation wavelength of 380nm and emission wavelength of 460nm for detection. AMC has also been used for cell surface staining of primary neurons (Kakiya *et al*., 2012) and as a fluorescent probe for the analyses of glycoproteins, monosaccharides and N-linked oligosaccharides (Yodoshi *et al*., 2008).

3.1.5 Design of synthetic substrates for the determination of CP activity

Previous attempts to design a synthetic substrate for determination of CP activity, to provide a rapid, one-step method for activity determinations has resulted in a novel CP substrate (Boc-QVR-AMC). During these studies, Low Ah Kee (2011) found that CP exhibited C-terminal specificity since it hydrolysed AMC, but not MCA, substrates. Due to a large variation between plots constructed to calculate K_m, k_{cat} and k_{cat}/K_m, Low Ah Kee (2011) could not determine a distinct order of the affinity of CP for the substrates investigated in the study. Of the four plots used (Lineweaver-Burk, Eadie-Hofstee, Hanes and Eisenthal-Cornish-Bowden), only the Eadie-Hofstee and Hanes plots displayed negligible variance. For the Eadie-Hofstee plot, the K_m, k_{cat} and k_{cat}/K_m values for Boc-QVR-AMC were 11.13µM, 58.58min^{-1} and 5262.91$min^{-1}.mM^{-1}$, respectively. For the Hanes plot, K_m, k_{cat} and k_{cat}/K_m for Boc-QVR-AMC was determined to be 11.82µM, 59.32min^{-1} and 5020.46$min^{-1}.mM^{-1}$, respectively. Based on these results, Low Ah Kee (2011) concluded that Boc-QVR-AMC was the best substrate for CP investigated in that study. In the present study, however, it was found that collagenase (a metalloprotease) could also cleave the Boc-QVR-AMC substrate. It was, therefore, necessary to improve the substrate design to increase its specificity for CP. The Boc-QVR-AMC substrate was, therefore, modified by the addition of bulkier residues to examine possible substrates that would, potentially, be less specific to collagenase. These substrates are listed in Table 3.1 below. Substrates were also designed based on two CP cleavage sites on fibronectin (Figure 3.1). These substrates are also listed in Table 3.1. All substrates were synthesized by GL Biochem (Shangai) Ltd (China).

Table 3.1 Synthetic substrates designed for the determination of CP activity.

	Substrate	Mol. Wt.
1	Boc-AVSQSKP-AMC	937.13
2	AVSQSKP-AMC	873.01
3	Boc-AVYQPQP-AMC	1059.22
4	AVYQPQP-AMC	959.10
5	Boc-RGD-AMC	603.66
6	RGD-AMC	503.53
7	Boc-QVR-AMC	658.78
8	Boc-AVR-AMC	601.54
9	Boc-PQVR-AMC	755.90
10	PQVR-AMC	655.78

Substrates 1 and 2 were designed according to the first cleavage site of CP on the fibronectin molecule (Figure 3.1), while substrates 3 and 4 were designed according to the second cleavage site of CP on fibronectin. These cleaveage sites were determined by Low Ah Kee (2011). For substrates 1 and 3, the addition of Boc to the N-terminal end was to introduce a “bulky” residue to test if this would increase the rate of hydrolysis. Low Ah Kee (2011) found that addition of a bulkier reside in the P4 position increased the affinity of the enzyme for the substrate. Substrates 5 (Boc-RGD-AMC) and 6 (RGD-AMC) (Table 3.1) were based on the RGD motif of fibronectin. The RGD motif plays a critical role in cell attachment via its interaction with integrin $\alpha_5\beta_3$ (Kieffer *et al*., 1991; Charo *et al*., 1990; Wennerberg *et al*., 1996). The rationale for substrates 5 and 6 was, therefore, to investigate whether CP could possibly cleave the RGD motif and possibly promote cell detachment by disrupting its interaction with integrin $\alpha_5\beta_3$ (Charo *et al*., 1990). Substrates 7 to 10 were previously designed by Low Ah Kee (2011). The basis of the design for these substrates was the observation that peptides containing arginine or lysine at the P1 position and proline at the P2 position are amongst the best substrates for CP (Lottenberg *et al*., 1981; Huseby and Smith, 1980; Claeson, 1994). Low Ah Kee (2011) identified substrate 7 (Boc-QVR-AMC) as the best synthetic substrate for the determination of

CP activity. This substrate is used as a reference for comparison to the substrates designed in this study.

3.2 METHODS AND MATERIALS

3.2.1 Substrate preparation

The substrates were shipped on dry ice. Each substrate was solubilised in 100% dimethylsulfoxide (DMSO) to a stock concentration of 50mM, taking care to minimize exposure of the substrates to light. The stock solution was divided into 10µl aliquots and stored at -20ºC in the dark. For the substrate assays, the stock solution was allowed to thaw at 4ºC and was diluted to a working concentration of 5mM.

3.2.2 Substrate identification via the direct CP assay

CP activity was assayed using the substrates in Table 3.1 with the method outlined in Section 2.3.4. Substrates that were cleaved by CP were selected as potential candidates in the hope of finding a suitable substrate for the direct assaying of CP activity. All substrates were screened at 50µM (Figure 3.4). The concentration of liberated product was determined from the standard curve of free AMC (Figure 3.3)

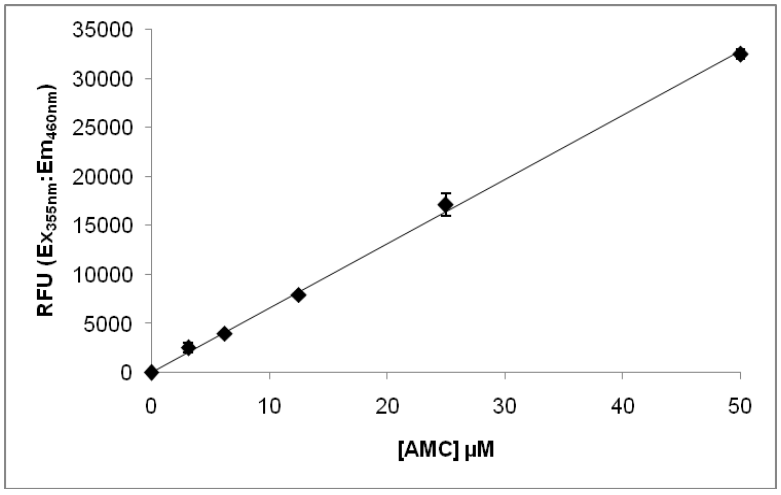


Figure 3.3 AMC standard curve. Error bars indicate SEM of 3 experiments (R^2 = 0.998).

3.2.3 Determination of substrate specificity

CP was purified according to the method outlined in Section 2.3.1. In order to determine whether these substrates were cleaved by proteases other than CP, a representative of each family of proteases was chosen to examine their ability to cleave the substrates listed in Table 3.1. The aspartic protease, pepsin (Sigma), metalloprotease, collagenase (Sigma), cysteine protease, papain (Sigma) and serine proteases, Trypsin and Chymotrypsin (Sigma) were selected for this study. The most suitable substrates for the determination of CP activity were selected based on the specificity data, as well as the kinetic data obtained from sections 3.2.2 and 3.2.3, respectively. The assay method employed was according to the method outlined in Section 2.3.4. In these assays, each enzyme was used at a final concentration of 0.4μg/μl.

3.2.3 Determination of the pH optimum of CP using new substrates

The pH optimum of CP was determined using PQVR-AMC and AVSQSKP-AMC as substrates. The assays were performed according to the method outlined in Section 2.3.8. CP was used at a concentration of 0.4μg/μl. Both substrates were assayed at a concentration of 50μM.

3.3 RESULTS

3.3.1 Substrates cleaved by CP

Screening of the AMC substrates showed that not all the substrates were cleaved by CP (Figures 3.5 to 3.9).

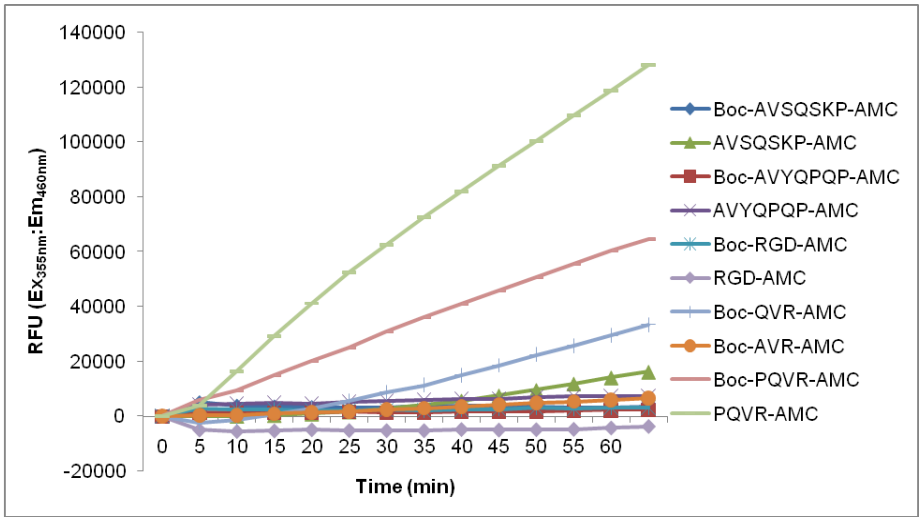
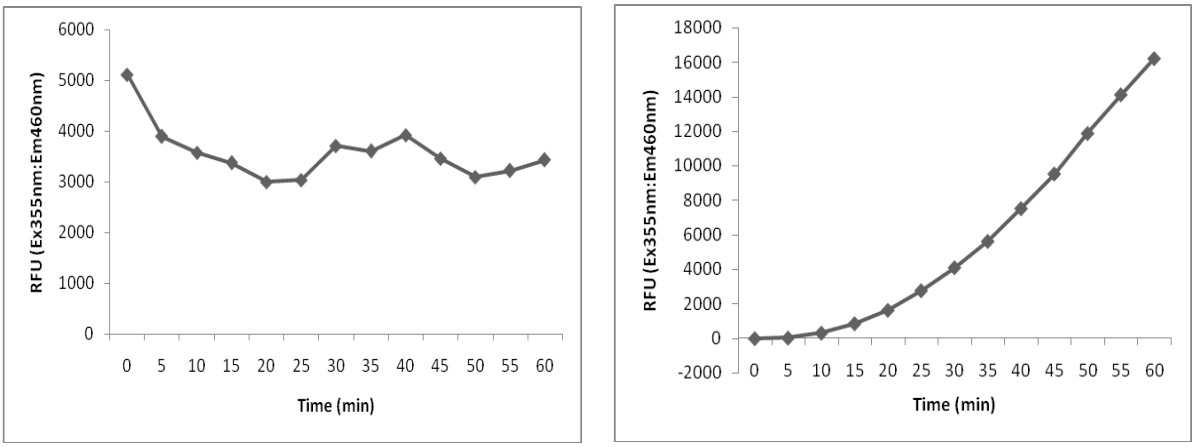


Figure 3.4 Typical progress curves obtained for the screening of AMC substrates using CP (0.4µg/µl) as a catalyst.

Low Ah Kee (2011) showed that CP was able to degrade fibronectin, but not laminin or collagen. The author showed that the CP cleavage sites on fibronectin were Ala_{44}/Val_{45} and Ala_{292}/Val_{293}. The AMC substrates that were designed according to these two cleavage sites on fibronectin are Boc-AVSQSKP-AMC, AVSQSKP-AMC, Boc-AVYQPQP-AMC and AVYQPQP-AMC. Figures 3.4 to 3.9 show typical progress curves obtained for the cleavage of the designed AMC substrates by CP.

The hydrolysis of Boc-AVSQSKP-AMC (Figure 3.5 a) by CP results in a progress curve that starts off with a high relative fluorescence value even in the absence of enzyme, suggesting possible degradation of the substrate. The progress curve has an initial negative slope with a decrease in relative fluoresence intensity, possibly due to quenching of fluoresence over time. Boc-AVSQSKP-AMC was, therefore, not hydrolysed by CP and further kinetic studies were not performed with this substrate. Removal of the Boc residue from the substrate, however, resulted in the hydrolysis of AVSQSKP-AMC (Figure 3.5 b) by CP. The initial relative fluorescence for this the

hydrolysis of AVSQSKP-AMC was low, indicating less degradation of the substrate as compared to Boc-AVSQSKP-AMC. For this progress curve, there was a lag phase of 20 minutes, before fluorescence increased with time. Low Ah Kee (2011) observed similar lag phases with AMC substrates. This lag phase could indicate either allosteric kinetics or that time is needed for the formation of an enzyme-substrate complex. Another possibility could be poor binding of the enzyme to its substrate. It appears that addition of the Boc residue to the substrate inhibited CP activity. Low Ah Kee (2011) observed an improvement in CP activity when Boc was used in the P4 position. This suggests that the positioning of a "bulky" residue in the substrate could be specific to the P4 position. Since initial screening of AVSQSKP-AMC resulted in hydrolysis by CP, this substrate was amongst those chosen for specificity studies (Section 3.3.2).

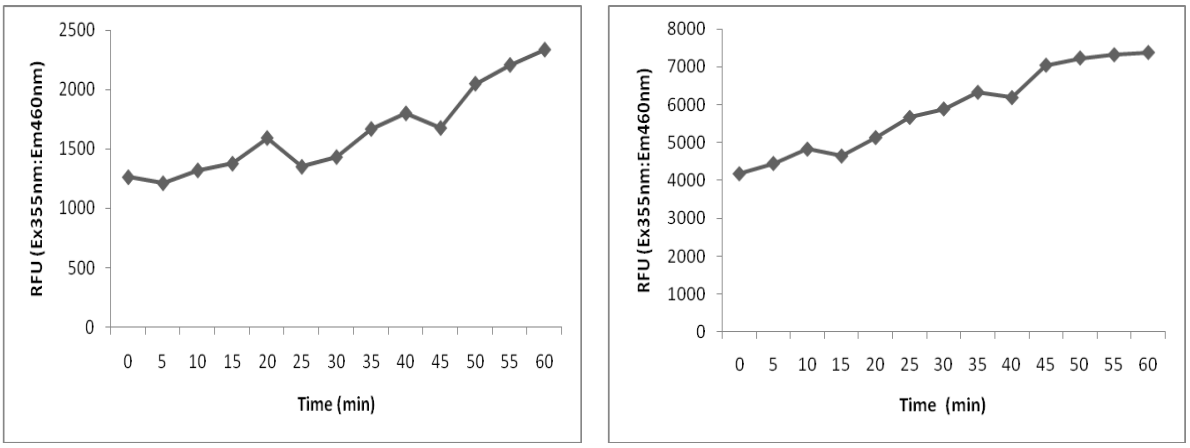


a) b)

Figure 3.5 Progress curve for the hydrolysis of: a) Boc-AVSQSKP-AMC and b) AVSQSKP-AMC by CP (0.4µg/µl).

Boc-AVYQPQP-AMC (Figure 3.6 a) was cleaved by CP. The slow increase in relative fluorescence over time suggests a low affinity of CP for hydrolysing this substrate. The slope increases steadily after 50 minutes. It could be possible that a longer assaying time is needed. However, this is not desired since one of the aims of this study is to design a more rapid assay method for CP. The substrate AVYQPQP-AMC (Figure 3.6 b) was hydrolysed by CP. The removal of the Boc residue from the substrate resulted in a two-fold increase in the reaction rate (Figure 3.10). The specific activity of CP for this substrate, however, was below 50µM AMC/min/mg and was, therefore, not considered for further kinetic studies. However, difference in the

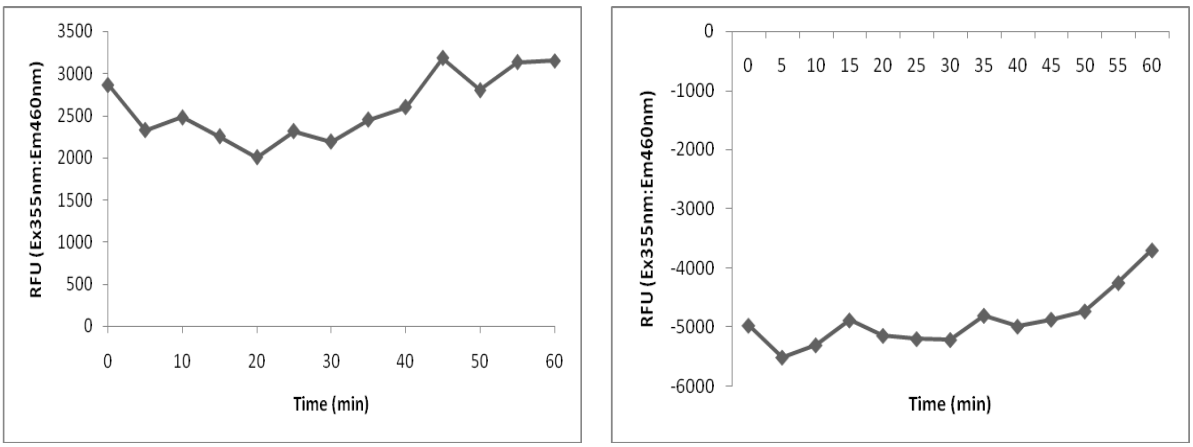
rate of hydrolysis of these two substrates provides further evidence that the positioning of the Boc in the substrate is of critical importance for CP activity.



a) b)

Figure 3.6 Progress curve for the hydrolysis of: a) Boc-AVYQPQP-AMC and b) AVYQPQP-AMC by CP (0.4µg/µl).

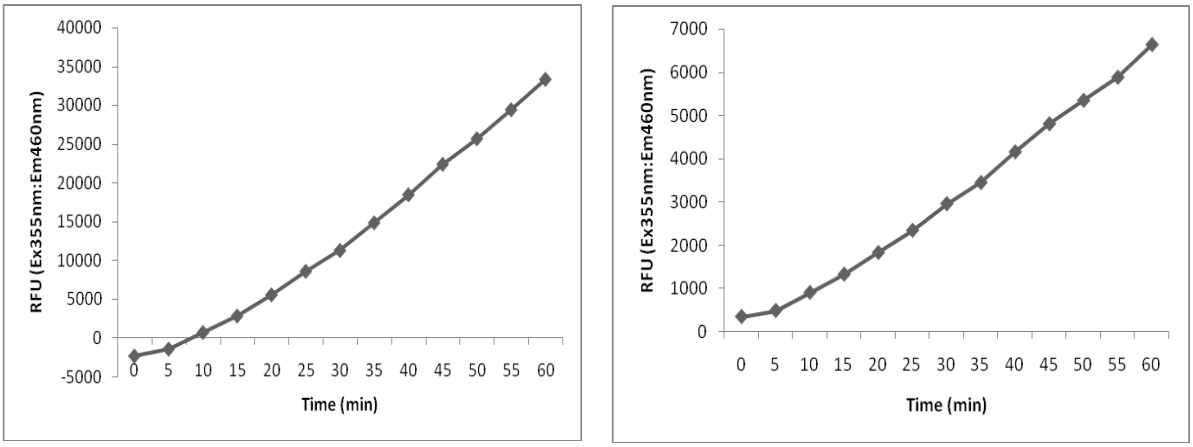
Boc-RGD-AMC (Figure 3.7 a) and RGD-AMC (Figure 3.7 b) were not hydrolysed by CP within 60 minutes. The initial relative fluorescence for RGD-AMC showed a negative value. The blank sample (substrate without enzyme) was much higher than that of the test sample (substrate with enzyme). This high value for the blank, and the negative values for the test sample indicates complete degradation of the substrate without enzyme hydrolysis. This could suggest that the substate RGD-AMC is unstable without a blocking group such as Boc.



a) b)

Figure 3.7 Progress curve for the hydrolysis of: a) Boc-RGD-AMC and b) RGD-AMC by CP (0.4µg/µl).

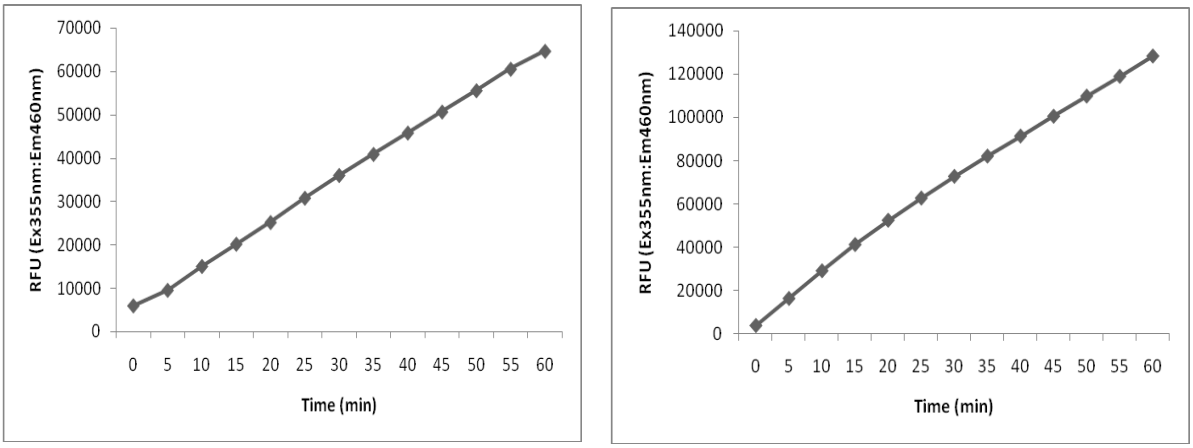
Boc-QVR-AMC (Figure 3.8 a) was, as expected, hydrolysed by CP. There was a 10 minute lag phase before the relative fluorescence steadily increased. Boc-AVR-AMC was also hydrolysed by CP, although at a slower rate than Boc-QVR-AMC. Due to the rate of hydrolysis of Boc-AVR-AMC being lower than 50µM AMC/min/mg, this substrate was not considered for further kinetic studies. Boc-QVR-AMC, however, was hydrolysed at a rate of 100µM AMC/min/mg (Figure 3.10) and used for further kinetic studies (Section 3.3.3).



a) b)

Figure 3.8 Progress curve for the hydrolysis of: a) Boc-QVR-AMC and b) Boc-AVR-AMC by CP (0.4µg/µl).

The final two substrates tested were Boc-PQVR-AMC (Figure 3.9 a) and PQVR-AMC (Figure 3.9 b). These substrates proved to have the highest hydrolysis rates (Figure 3.10). CP cleaved Boc-PQVR-AMC at a rate of 163µM AMC/min/mg and PQVR-AMC at 332µM AMC/min/mg. The addition of Boc to PQVR-AMC resulted in a 2 fold reduction in the rate of hydrolysis of the substrate. Furthermore, for Boc-PQVR-AMC hydrolysis, a lag phase was observed, while no lag phase was observed for PQVR-AMC.



a) b)

Figure 3.9 Progress curve for the hydrolysis of: a) Boc-PQVR-AMC and b) PQVR-AMC by CP (0.4µg/µl).

The substrate specificity data of CP for cleavage of each substrate is summarized in Figure 3.10 along with a comparison to literature. The rates of the reactions were calculated over the linear portion of the progress curves, for example 10 – 20 minutes and converted to a rate per minute (ΔRFU/min). The amount of AMC (µM) released per minute was extrapolated from the AMC standard curve (Figure 3.3) and expressed per milligram of enzyme. As can be seen, AVSQSKP-AMC, Boc-QVR-AMC, Boc-PQVR-AMC and PQVR-AMC showed good cleavage by CP while the other substrates showed little (AVYQPQP-AMC and Boc-AVR-AMC) or no cleavage (Boc-AVYQPQP-AMC, Boc-AVSQSKP-AMC, Boc-RGD-AMC and RGD-AMC) by CP.

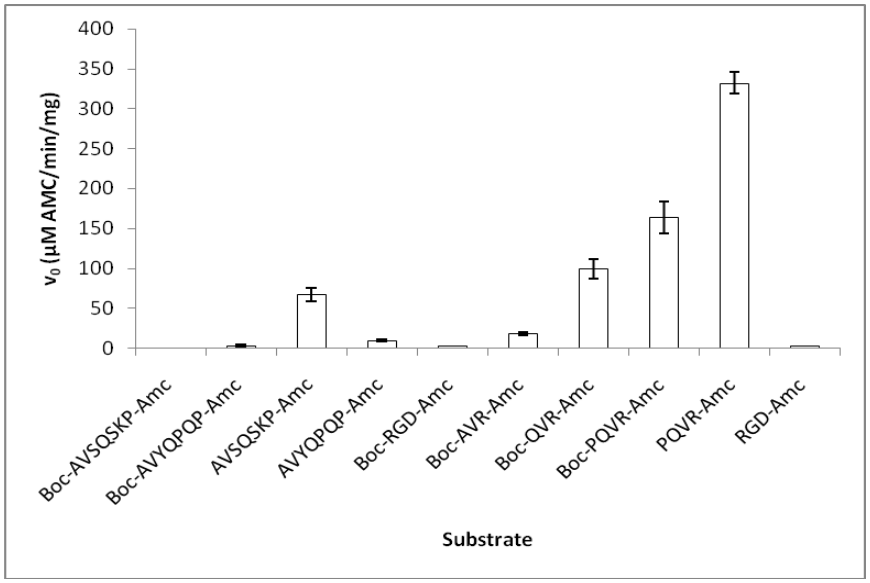


Figure 3.10 A comparison of the specific activity of the synthetic fluorescent substrates hydrolysed by CP (0.4µg/µl). Error bars represent SEM values for 3 experiments.

As can be seen from Figure 3.10, only four substrates were effectively hydrolysed (v_0>50µM AMC/min/mg) by CP. These substrates are AVSQSKP-AMC, Boc-QVR-AMC, Boc-PQVR-AMC and PQVR-AMC (70, 100, 163 and 332µM AMC/min/mg, respectively). Both substrates that were designed according to the cleavage site on fibronectin were hydrolysed. However, addition of a Boc group to the substrate results in a drastic decrease in enzyme activity. AVSQSKP-AMC was hydrolysed at a faster rate than AVYQPQP-AMC. Boc-QVR-AMC, Boc-PQVR-AMC and PQVR-AMC were also hydrolysed by CP. PQVR-AMC was hydrolysed at twice the rate at which Boc-PQVR-AMC was hydrolysed and three times the rate at which Boc-QVR-AMC was hydrolysed. The substrates that were based on the RGD motif of fibronectin (Boc-RGD-AMC and RGD-AMC) were not cleaved by CP. These substrates were designed to investigate whether CP could possibly cleave at the RGD motif, which plays a critical role in cell attachment via its interaction with integrin $\alpha_5\beta_3$ (Kieffer *et al*., 1991; Charo *et al*., 1990; Wennerberg *et al*., 1996). Cleavage of this RGD site, would disrupt it's interaction with integrin $\alpha_5\beta_3$, thereby promoting cell detachment (Charo *et al*., 1990). From the results above, only AVSQSKP-AMC, Boc-QVR-AMC, Boc-PQVR-AMC and PQVR-AMC were selected for further characterisation as potential novel synthetic substrates for CP.

3.3.2 Substrate specificity

3.3.2.1 In silico cleavage of CP substrates

The next step in identifying a suitable substrate for CP involved the specificity of the substrates for CP. To test this, a variety of proteases were screened for their ability to cleave the potential substrates for CP using the ExPASy prediction tool. This data is summarized in Tables 3.2 – 3.6. It should be noted, however, that since this is an *in silico* prediction tool, it would still be required to provide experimental evidence that these enzymes could be cleaving the substrates *in vitro*, since enzyme-substrate interactions are dependent on several factors such as pH, ionic strength, tertiary and quaternary structure and active site configuration to name but a few. Those enzymes predicted to cleave the substrates of interest, were tested *in vitro* depending on availability.

The enzymes listed that are capable of cleaving the sequence AVSQSKP (Table 3.2) all cleave the sequence at different positions from the position of interest (C-terminal proline, 7th position). The only enzyme listed that could cleave at the 7th position (proline) is proline peptidase. All other cleavages would not result in fluorescence, since AMC is attached to the C-terminal proline. It should, therefore, be determined if proline peptidase could cleave AVSQSKP-AMC or Boc-AVSQSKP *in vitro*.

Table 3.2 Enzymes that cleave the sequence: AVSQSKP

Enzyme	Number of cleavages	Cleavage position
LysC	1	6
LysN	1	5
Proline endopeptidase	1	7
Proteinase K	2	1, 2
Thermolysin	1	1

As can be seen in Table 3.3, no enzymes were predicted to cleave the sequence AVYQPQP in the 7th position (C-terminal proline). According to the prediction, chymotrypsin and pepsin would cleave at positions 3 and 2, respectively. These

cleavages, however, would not result in the release of AMC and thereby would not result in the emission of fluorescence.

Table 3.3 Enzymes that cleave the sequence: AVYQPQP

Enzyme	Number of cleavages	Cleavage position
Chymotrypsin	1	3
Pepsin (pH1.3)	1	2
Proteinase K	3	1, 2, 3
Thermolysin	1	1

The sequence AVR was predicted to be cleaved by Arg-C proteinase, clostripain and trypsin in position 3 (C-terminal arginine, Table 3.4). Trypsin was selected to test whether it would cleave AVR *in vitro*.

Table 3.4 Enzymes that cleave the sequence: AVR

Enzyme	Number of cleavages	Cleavage position
Arg-C proteinase	1	3
Clostripain	1	3
Proteinase K	2	1, 2
Thermolysin	1	1
Trypsin	1	3

QVR (Table 3.5) had three enzymes (Arg-C proteinase, Clostripain and Trypsin) that could cleave in position 3 (C-terminal arginine). Trypsin was selected to test whether it would cleave QVR *in vitro*.

Table 3.5 Enzymes that cleave the sequence: QVR

Enzyme	Number of cleavages	Cleavage position
Arg-C proteinase	1	3
Clostripain	1	3
Proteinase K	1	2
Thermolysin	1	1
Trypsin	1	3

The sequence PQVR showed four possible enzymes that could cleave in position 4 (at the C-terminal arginine). These enzymes are listed in Table 3.6. From the list, trypsin was selected for the *in vitro* studies.

Table 3.6 Enzymes that cleave the sequence: PQVR

Enzyme	No. of cleavages	Cleavage position
Arg-C proteinase	1	4
Clostripain	1	4
Proteinase K	1	3
Thermolysin	1	2
Trypsin	1	4

Since the other substrates were not cleaved by CP (Section 3.3.1), they were not included in the *in silico* or *in vitro study*.

3.3.2.2 In vitro cleavage of substrates by enzymes

Russell's viper venom (RVV, 0.75mg/ml), thrombin (0.5μg/ml), trypsin (0.25mg/ml), collagenase (0.1mg/ml) and pepsin (0.1mg/ml) were tested along with CP for their ability to cleave the substrates in question. These enzymes were assayed according to the method outlined in Section 2.2.4. Figures 3.11 to 3.15 show typical progress curves obtained for the hydrolysis of the AMC substrates by the various enzymes tested.

RVV, thrombin and trypsin did not hydrolyse any of the four selected CP substrates (Figures 3.11 – 3.13). For RVV (Figure 3.11) and thrombin (Figure 3.12) it was observed that Boc-PQVR-AMC and PQVR-AMC showed a negative rate as well as negative relative fluorescence values. The reason for the negative rate may be attributed to fluorescent quenching of the substrate and the negative relative fluorescent values could be caused due to degradation of the substrate even in the absence of enzyme. A negative rate was also observed for trypsin cleavage of Boc-QVR-AMC and Boc-PQVR-AMC (Figure 3.13). The relative fluorescence for these substrates started at 41 000 and 44 000 RFU, respectively. Fluorescence quenching was observed and there may have been substrate degradation.

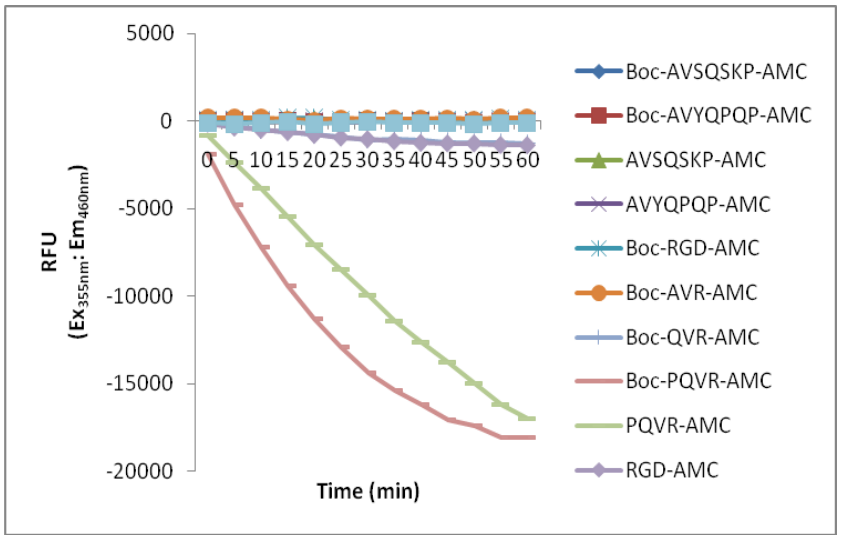


Figure 3.11 Progress curves illustrating the cleavage of the AMC substrate library by RVV (0.75mg/ml).

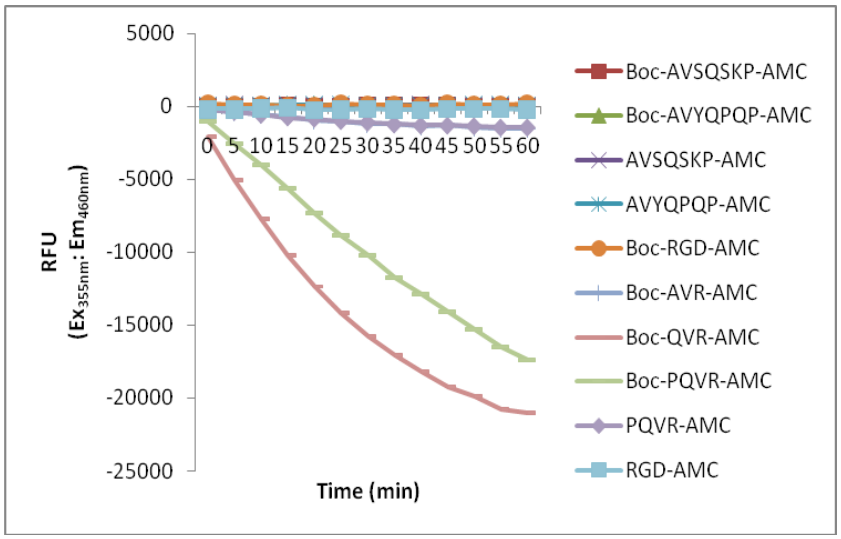


Figure 3.12 Progress curves illustrating the cleavage of the AMC substrate library by Thrombin.

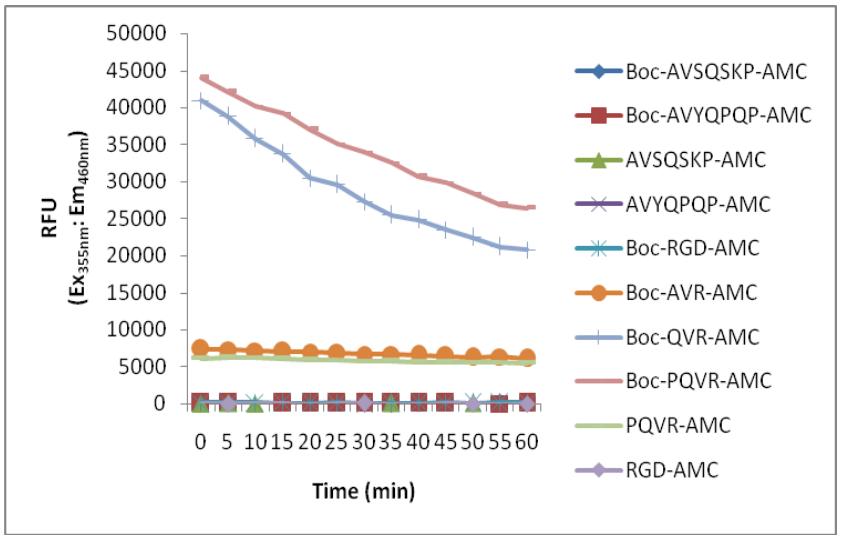


Figure 3.13 Progress curves illustrating the cleavage of the AMC substrate library by Trypsin.

Collagenase and pepsin, however, were capable of hydrolysing some of the substrates (Figure 3.14 and 3.15). For the hydrolysis of Boc-QVR-AMC and Boc-PQVR-AMC by collagenase, activity was observed within the first 5 minutes. After 10 and 30 minutes for Boc-QVR-AMC and Boc-PQVR-AMC, respectively, the activity decreased. This could be due to the enzyme being saturated with substrate, followed by fluorescence quenching over time, substrate inhibition or substrate depletion. The progress curve would be linear between 0 and 15 minutes for the hydrolysis of Boc-QVR-AMC and Boc-PQVR-AMC by collagenase. Boc-AVR-AMC was also hydrolysed, but at a lower rate. The reaction reached a plateau after 10 minutes, indicating saturation of the enzyme with substrate. It was observed that Boc-QVR-AMC and Boc-PQVR-AMC were hydrolysed at a high rate by pepsin (Figure 3.16), however, this rate was lower than that for collagenase. This indicates that these substrates are excellent substrates for collagenase. Pepsin also hydrolysed Boc-AVR-AMC, but at a lower rate as compared to Boc-QVR-AMC and Boc-PQVR-AMC.

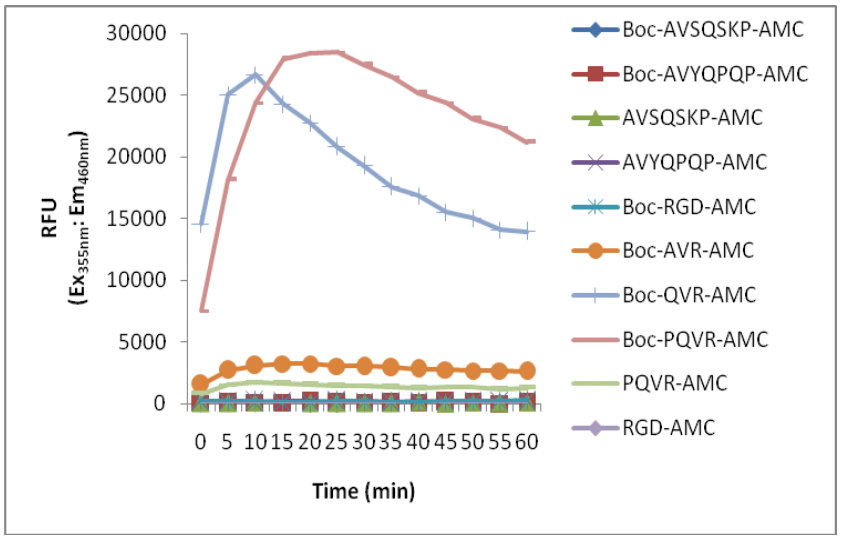


Figure 3.14 Progress curves illustrating the cleavage of the AMC substrate library by Collagenase.

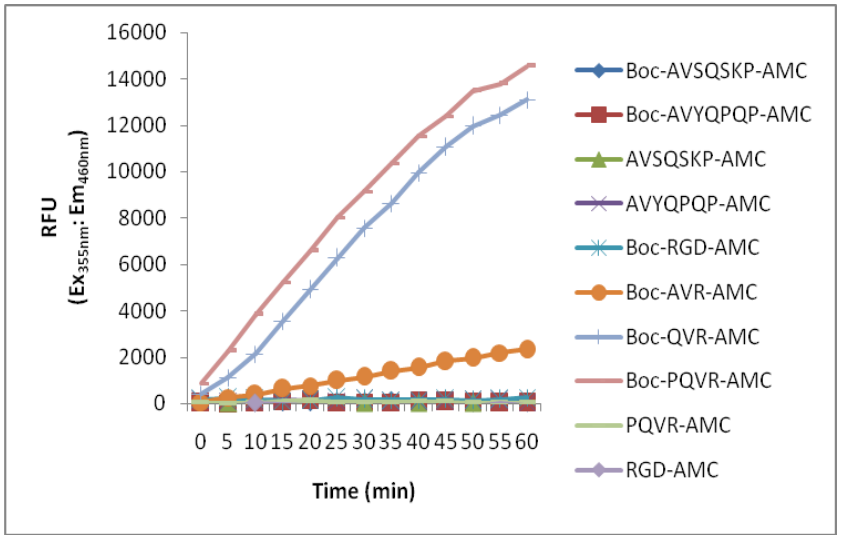


Figure 3.15 Progress curves illustrating the cleavage of the AMC substrate library by Pepsin.

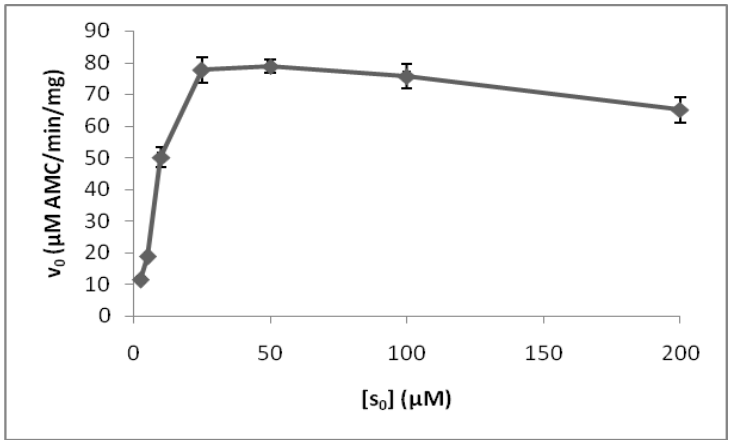
Boc-AVR-AMC, Boc-QVR-AMC and Boc-PQVR-AMC were hydrolysed by both collagenase (Figure 3.14) and pepsin (Figure 3.15). These substrates were, therefore, excluded from the list of potential substrates for CP. The two remaining substrates, PQVR-AMC and AVSQSKP-AMC were cleaved by CP but were not hydrolysed by any of the enzymes tested and were, therefore, selected for further kinetic characterisation (Section 3.3.3). To investigate their potential as substrates to detect CP activity, these substrates were further assayed at various concentrations to establish whether or not cleavage by CP follows Michaelis-Menten kinetics. This data, along with other kinetic data (K_m, V_m, K_{cat}, K_{cat}/K_m) is reported in Section 3.3.3.

3.3.3 Kinetics

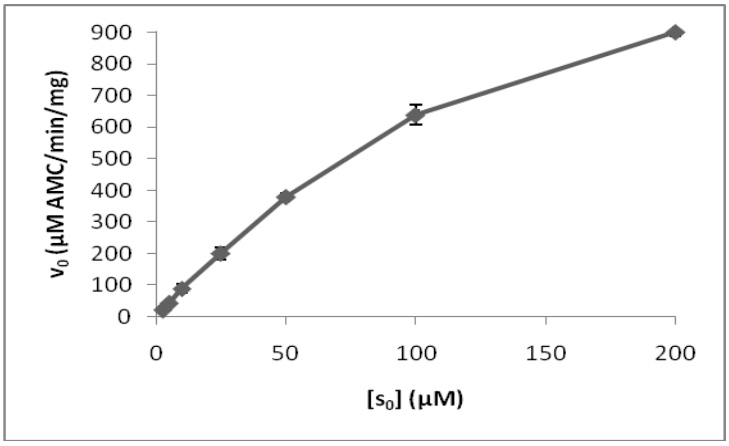
The suitable substrates identified in Section 3.3.2 were further analysed to determine various kinetic parameters such as the Michaelis-Menten constant (K_m), maximum velocity (V_m), enzyme turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m). The reaction rates were calculated from the linear portion of the curve. For example: the rate of change of fluorescence was measured between 10 and 20 minutes and plots of initial velocity (v_0, μM AMC/min/mg) versus initial substrate concentration ([s_0], μM) (Figure 3.16) were constructed.

Michaelis-Menten plots were constructed for each substrate.

a)



b)



c)

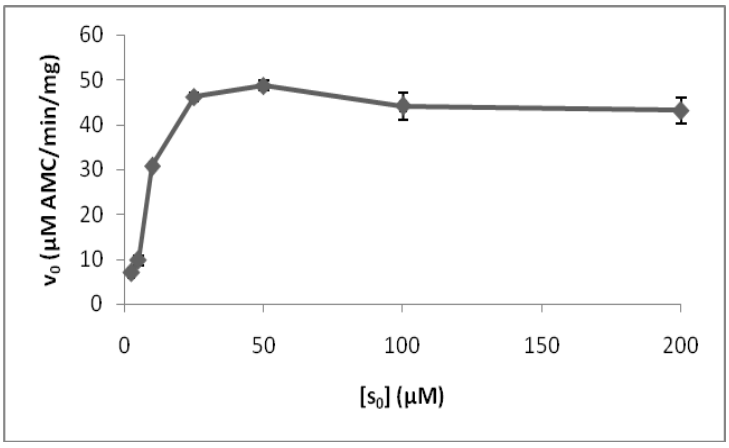
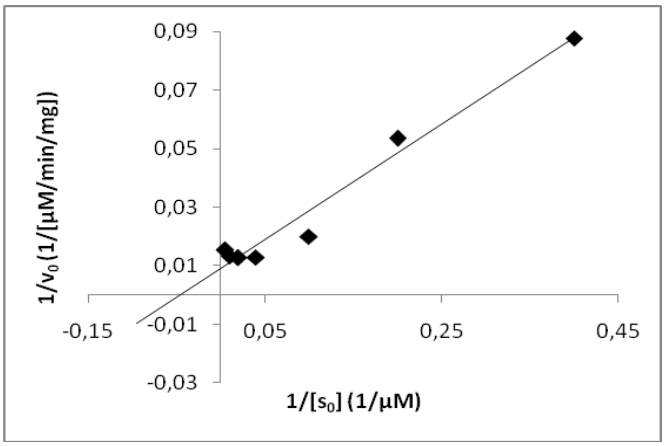


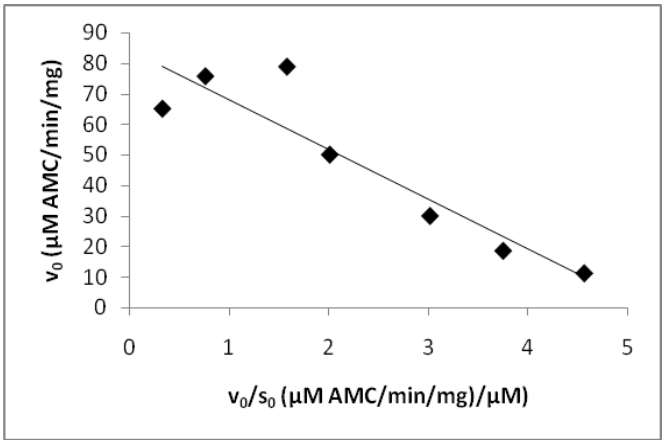
Figure 3.16 Michaelis-Menten plot showing v_0 vs [s_0] for the hydrolysis of: a) Boc-QVR-AMC; b) PQVR-AMC and c) AVSQSKP-AMC at constant CP concentration (0.4µg/µl). Error bars represent the SEM of 3 experiments.

The data from the Michaelis-Menten plots were used to calculate kinetic constants using the following plots: Lineweaver-Burk, Eadie-Hofstee and Hanes (Figures 3.17 – 3.19; Tables 3.7 – 3.9).

a)



b)



c)

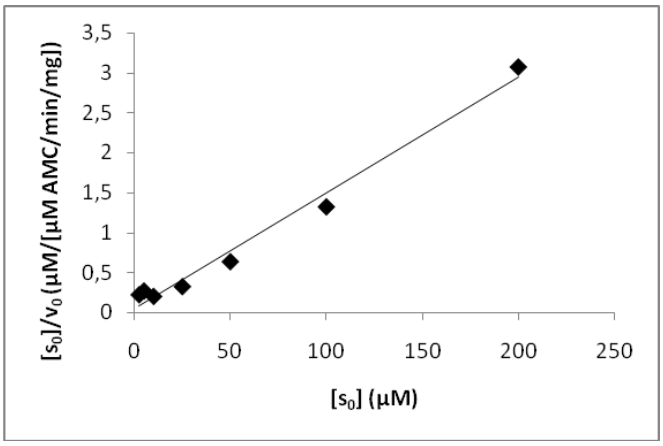
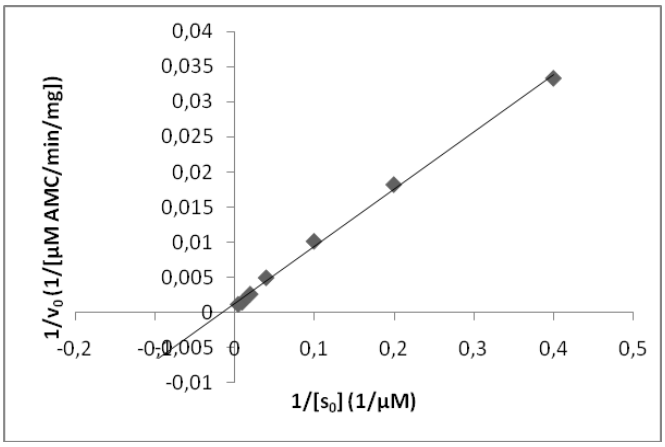
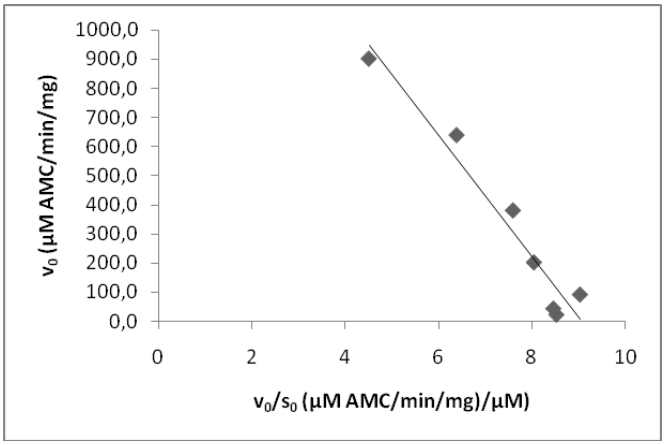


Figure 3.17 Secondary plots for Boc-QVR-AMC hydrolysis using CP (0.4µg/µl) as a catalyst. a) Lineweaver-Burk (R^2=0.970); b) Eadie-Hofstee (R^2=0.853) and c) Hanes (R^2=0.982) plots.

a)



b)



c)

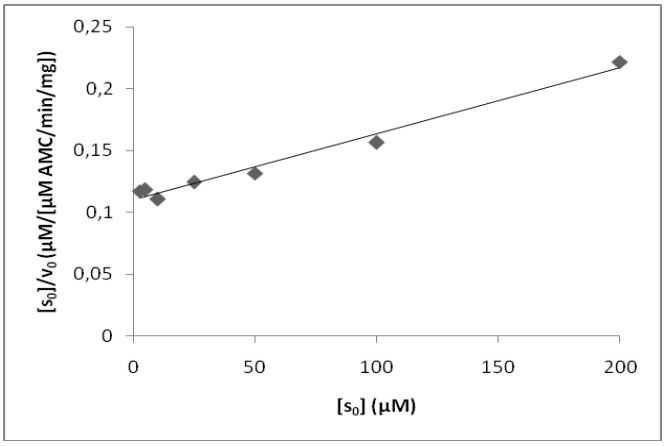
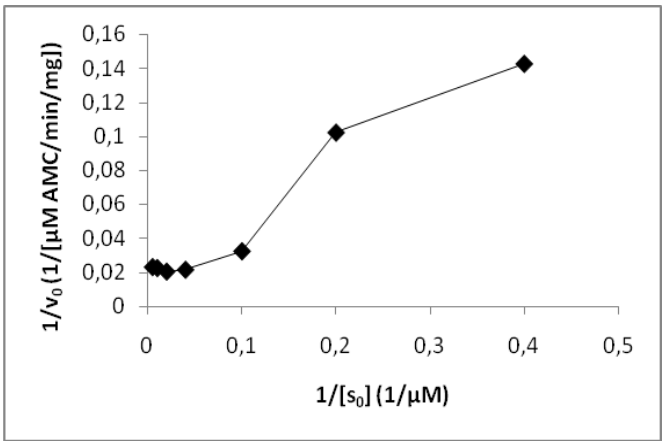
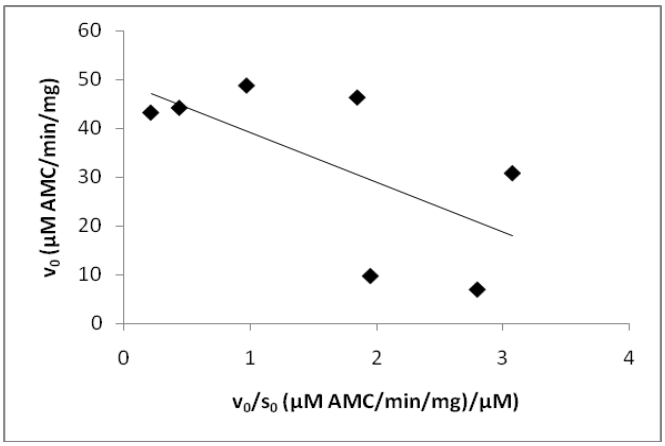


Figure 3.18 Secondary plots for PQVR-AMC hydrolysis using CP (0.4µg/µl) as a catalyst. a) Lineweaver-Burk (R^2=0.997); b) Eadie-Hofstee (R^2=0.945) and c) Hanes (R^2=0.979) plots.

a)



b)



c)

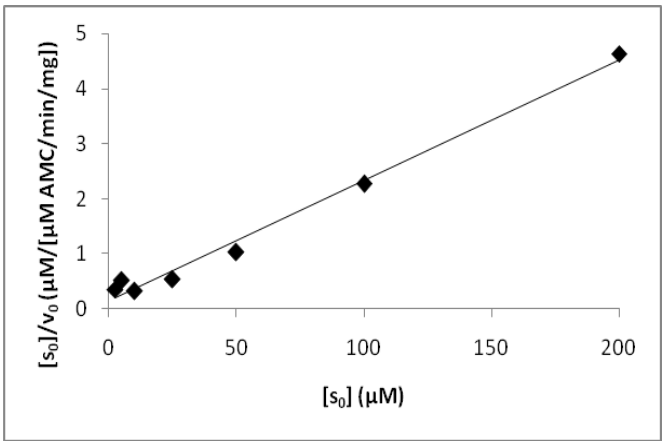


Figure 3.19 Secondary plots for AVSQSKP-AMC hydrolysis using CP (0.4µg/µl) as a catalyst. a) Lineweaver-Burk (R^2=0.944); b) Eadie-Hofstee (R^2=0.416) and c) Hanes (R^2=0.988) plots.

Table 3.7 K_m (μM) values for each substrate calculated using different plots

Substrate	Lineweaver-Burk	Eadie-Hofstee	Hanes
Boc-QVR-AMC	24.6±3	10.2±0.5	3.6±0.05
PQVR-AMC	81±5	207±6	193±6
AVSQSKP-AMC	21.5±2	10.2±0.8	7.3±0.1

The K_m values obtained for the substrates investigated did not vary greatly when compared using the three different plots (Table 3.7). From all the plots, it could be seen that the affinity of AVSQSKP-AMC for CP was very similar to that of Boc-QVR-AMC, the current substrate used in our lab to assay for CP activity (Low Ah Kee, 2011). The affinity of both AVSQSKP-AMC and Boc-QVR-AMC for CP was much higher than that of PQVR-AMC. When comparing AVSQSKP-AMC (Figure 3.16 c) and Boc-QVR-AMC (Figure 3.16 a), it can be seen that the maximum velocity (V_{max}) for these two substrates is reached at a similar substrate concentration. The V_{max} for AVSQSKP-AMC and Boc-QVR-AMC is reached at a much lower substrate concentration than for that of PQVR-AMC (Figure 3.16 b).

Table 3.8 Catalytic constant (k_{cat}; $minute^{-1}$) values for each substrate calculated using different plots.

Substrate	Lineweaver-Burk	Eadie-Hofstee	Hanes
Boc-QVR-AMC	21.3±3	13.9±0.7	12.1±0.2
PQVR-AMC	170.1±11	320.6±9	293.7±9
AVSQSKP-AMC	11.3±1	8.4±0.6	8.1±0.1

Table 3.8 shows the k_{cat} value for each substrate. The value of k_{cat} was determined by dividing V_{max} by total enzyme concentration (E_0). It can be seen that hydrolysis of PQVR-AMC by CP produced the highest k_{cat} value in all the plots. The lowest k_{cat} value was obtained for AVSQSKP-AMC. When examining the maximum v_0 value for PQVR-AMC and AVSQSKP, it can be seen that PQVR-AMC has the highest maximum v_0 and AVSQSKP has the lowest maximum v_0, which confirms the results obtained for k_{cat}.

Table 3.9 Catalytic efficiency (k_{cat}/K_m; $minute^{-1}.mM^{-1}$) values for each substrate calculated using different plots.

Substrate	Lineweaver-Burk	Eadie-Hofstee	Hanes
Boc-QVR-AMC	860±1	1360±1.4	3330±6
PQVR-AMC	2100±2	1540±1.5	1520±1.5
AVSQSKP-AMC	530±0.5	820±0.8	1100±1.1

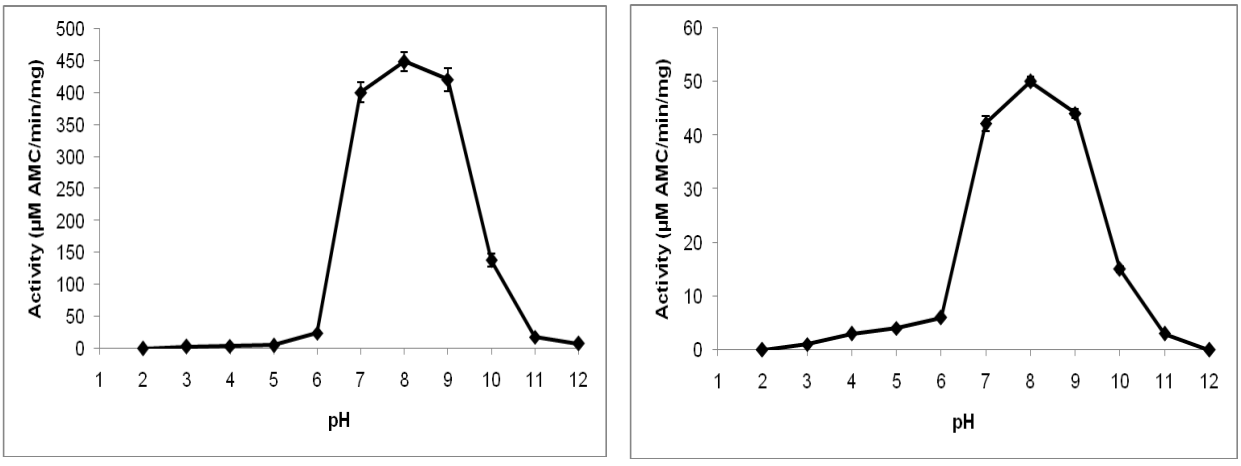
Determination of CP specificity was made possible through comparison of the catalytic efficiencies (Table 3.9). Although CP has a higher specificity constant for Boc-QVR-AMC as compared to AVSQSKP-AMC, Boc-QVR-AMC was cleaved by enzymes other than CP, whereas AVSQSKP-AMC was not. PQVR-AMC showed the highest specificity constant in all the plots except the Hanes plot. PQVR-AMC was also not cleaved by the other proteases that were investigated. It is evident, therefore, that two novel substrates for CP are reported in this study, namely, PQVR-AMC and AVSQSKP-AMC. Low Ah Kee (2011) observed that CP requires a "bulky" amino acid in the P4 position. This is evident from the results of the present study since the bulky Boc residue in the P4 position of Boc-QVR-AMC has a higher catalytic efficiency (Table 3.9) compared to that of AVSQSKP-AMC. When comparing the catalytic efficiencies, it also appears that the shorter length substrates such as Boc-QVR-AMC and PQVR-AMC are better CP substrates than longer length substrates such as AVSQSKP-AMC. In the P1 position, Boc-QVR-AMC and PQVR-AMC both have an arginine residue. CP preferentially cleaves substrates with arginine in the P1 position. The hydrolysis of AVSQSKP-AMC, a substrate designed according to the CP cleavage site on fibronectin, was surprising since it is not known that CP cleaves substrates with proline in the P1 position. However, the hydrolysis rate and the catalytic efficiency for this substrate were lower than that of Boc-QVR-AMC and PQVR-AMC. Perhaps a better substrate would be one with alanine in the P1 position, since this is the preferred cleavage site of CP in the fibronectin molecule.

Mielicki *et al*. (1997) tested the effect of CP on chromogenic substrates. These substrates were: H-D-Ile-Pro-Arg-pNA (K_m = 27±5, k_{cat} = 0.0082±0.0005); N-p-Tos-Gly-Pro-Arg-pNA (K_m = 61±6, k_{cat} = 0.0073±0.0003); H-D-Ala-Pro-Arg-pNA (K_m = 109±8, k_{cat} = 0.0132±0.0007); N-p-Tos-Gly-Pro-Lys-pNA (K_m = 153±12, k_{cat} = 0.0129±0.0009); pyroGlu-Pro-Arg-pNA (K_m = 306±11, k_{cat} = 0.0119±0.0009). The rate of hydrolysis of all the chromogenic substrates examined by these authors was low (V_{max} ranged from 4.18 to 7.5nM/s). As hypothesised by Mielicki *et al*. (1997), there was a vast increase in the hydrolysis rate and sensitivity of fluorogenic AMC substrates used in this study as well as in the study by Low Ah Kee (2011) as compared to the chromogenic pNA substrates used in the study by Mielicki *et al*. (1997). The K_m values for the substrates hydrolysed by CP during the study by Low

Ah Kee (2011) ranged from 11.82 to 393µM, while the k_{cat} values were in the range of 59.32 to 274.66 minute^{-1} (as determined using the Hanes plot) (Table 3.10). Comparing the pNA substrates to the AMC substrates, it is evident that AMC is a better leaving group for the substrates designed for CP, since the catalytic efficiencies for the AMC substrates were orders of magnitude higher than those of the pNA substrates. In the study by Mielicki *et al*. (1997) a CP concentration of 600µg/ml was used, whereas in the present study and that of Low Ah Kee (2011), CP was used at a concentration of 0.4µg/µl (400µg/ml), providing further evidence of the increased sensitivity of AMC substrates as compared to pNA substrates. In the present study, the K_m values for the designed CP substrates ranged from 3.6 to 193µM and the k_{cat} values from 12.1 to 293.7 minute^{-1}. These results were in a similar range to that obtained by Low Ah Kee (2011). A comparison of the K_m, k_{cat} and k_{cat}/K_m obtained in this study to that obtained by Low Ah Kee (2011) will be discussed in Section 3.4 (Table 3.10)

3.3.4 pH optimum of CP using PQVR-AMC and AVSQSKP-AMC as substrates

The optimum pH of CP was determined to be 8 using both PQVR-AMC and AVSQSKP-AMC (Figure 3.20). These results are in agreement with those obtained for Boc-QVR-AMC in Section 2.4.2 (Figure 2.8).



a) b)

Figure 3.20 The optimum pH for CP activity. a) PQVR-AMC (50µM); b) AVSQSKP-AMC (50µM). Error bars represent the SEM of 3 experiments.

3.4 Discussion

Mielicki *et al*. (1997) found that CP only hydrolysed di- and tri-peptide substrates. Low Ah Kee (2011), however, showed that substrates with greater than two or three amino acids could also be hydrolysed by CP. The author showed that the substrates used by Mielicki *et al*. (1997) all contained a non-bulky amino acid, Ala, in the P4 position. Low Ah Kee (2011) reported the importance of a bulky amino acid in the P4 position of CP substrates. Mielicki *et al*. (1997) also deduced that it is essential for Pro to occupy the P2 position in substrates designed for CP, since it was observed that CP preferentially cleaved peptides that have Arg or Lys in the P1 position and Pro in the P2 position. This finding supported the reports of Gordon and Mourad (1991) in which they found that Pro in the P2 position was required for the cleavage of FX by CP. Low Ah Kee (2011), however, demonstrated that it is not important for Pro to occupy the P2 position, since CP was able to readily cleave substrates such as Boc-QVR-AMC and PSQVR-AMC. Based on these findings, this study investigated the cleavage of synthetic substrates, greater than three amino acids in length, designed according to CP cleavage sites on fibronectin. The AMC substrates that were designed according to two CP cleavage sites on fibronectin (Low Ah Kee, 2011) are Boc-AVSQSKP-AMC, AVSQSKP-AMC, Boc-AVYQPQP-AMC and AVYQPQP-AMC. Upon examining the two cleavage sites on fibronectin, it was observed that both cleavage sites contained a Pro residue on the C-terminal portion at position 6 following the cleavage point (P6`). Similarly, the first cleavage site of CP on the FX heavy chain also contains a Pro residue in P6`. It is possible that this C-terminal Pro plays a role in substrate recognition, thereby contributing to CP specificity. In this study it was found that CP was able to cleave AVSQSKP-AMC. This is surprising since it is not known that CP cleaves Pro residues. Proline aminopeptidases are known for cleaving N-terminal proline residues from a substrate (Turzynski and Mentlein, 1990), however, CP exhibits C-teriminal specificity (Low Ah Kee, 2011) and it is, therefore, unlikely that CP is a proline aminopeptidase. Boc-AVYQPQP-AMC, AVYQPQP-AMC and Boc-AVSQSKP-AMC, however, were not cleaved. Low Ah Kee (2011) also designed a substrate (Boc-QSPVR-AMC) based on the fibronectin cleavage site of CP, however, this substrate was not cleaved. This could, possibly, be due to the lack of Pro in the P6 position of this substrate.

Table 3.10 A comparison of the catalytic efficiency (minute^{-1}.mM^{-1}) of fluorogenic substrates for CP.

	This study		Low Ah Kee (2011)	
	Eadie-Hofstee	Hanes	Eadie-Hofstee	Hanes
Boc-QVR-AMC	1360	3330	5262.91	5020.46
PQVR-AMC	1540	1520	1219.38	1212.84
AVSQSKP-AMC	820	1100	*ND	*ND
LPAPR-AMC	*ND	*ND	614.68	601.34
PSQVR-AMC	*ND	*ND	447.73	432.54
QVR-AMC	*ND	*ND	269.34	257.29

*ND=not determined

The amino acid sequence RGD is a widely occurring cell adhesive motif that was first discovered in fibronectin (Pierschbacher and Ruoslahti 1984) and is located in III_{10}. The RGD motif in fibronectin and other cell adhesion proteins is the most important recognition site for many integrins (Garcia *et al*. 2002). Regions close to the RGD site contribute to the contact surface between ligands and integrins (Johansson *et al*. 1987; Aota *et al*. 1991). The purpose for designing the substrates Boc-RGD-AMC and RGD-AMC was to test whether CP could cleave the RGD sequence and, thereby, promote cell detachment and, consequently, lead to metastasis. Cleavage of this site would cause a decrease in cellular adhesion to the ECM. It was found, however, that CP could not cleave the substrates based on the RGD motif. Boc-RGD-AMC and RGD-AMC could, therefore, not be used as a substrate for CP, indicating that this may not be its physiological role.

The substrates that were designed based on the ability of CP to cleave Arg were all cleaved. Mielicki *et al*. (1997) found that CP preferentially cleaved peptides having Arg or Lys in the P1 position. Low Ah Kee designed the following substrates based on CP's ability to cleave Arg or Lys: QVR-AMC, Boc-QVR-AMC, PQVR-AMC, LPAPR-AMC and PSQVR-AMC. Due to its high catalytic efficiency value, the author chose Boc-QVR-AMC as the substrate of choice for CP. In this study, however, it was found that Boc-QVR-AMC was not the best substrate for CP, since it could also be cleaved by collagenase. We, therefore, decided to further investigate PQVR-

AMC, since it showed the second highest catalytic efficiency value. PQVR-AMC, along with AVSQSKP-AMC was not cleaved by any of the enzymes tested in this study. Low Ah Kee (2011) demonstrated the importance of a bulky amino acid in the P4 position by including a Boc group to the QVR-AMC substrate. In this study it was found that addition of the Pro in the P4 position instead of Boc, however, prevents cleavage of the substrate by collagenase and other proteases tested in this study.

In general, progress curves showed a lag phase for most of the substrates in this study. For PQVR-AMC hydrolysis, however, no lag phase was observed. Low Ah Kee (2011) noticed a lag phase for the AMC substrates and postulated that it could be due to an inhibitory effect of the salt used in the assay, since it has been shown that divalent metal ions considerably decreased CP activity. Upon removal of salts from the assay buffer, the author noted a decrease in the time of the lag phase although it was not completely eliminated. Although the presence of Ca^{2+} is required for FX activation (Gordon and Mielicki, 1997) it was shown by Low Ah Kee (2011) that Ca^{2+} is not essential for CP catalytic activity, since the author showed that Boc-QVR-AMC was hydrolysed by CP in the absence of salts. In this study, however, it was shown that even the hydrolysis of Boc-QVR-AMC by CP is optimal at a Ca^{2+} concentration of 5mM, supporting the results of Gordon and Mielicki (1997) that showed the enhancing effects of Ca^{2+} on CP activity. Complete removal of salt is, therefore, not recommended. In this study, the lag phase for PQVR-AMC hydrolysis was decreased by an increase in pre-incubation time at 37ºC of CP with the assay buffer. Possible explanations for the observed lag phases could be that CP undergoes very slow conformational changes before cleaving the substrate or that the substrate requires multiple cleavages at various sites before the critical cleavage of the residue adjacent to the AMC group occurs. Due to a limited amount of CP, only Ca^{2+} concentration was investigated and it is recommended that other salts in the Hofmeister series (NH_4^+, K^+, Na^+, Li^+, Mg^{2+} and guanidinium), be tested in a future study.

Based on the catalytic efficiency results and the specificity results, this study shows that the best substrate for CP is PQVR-AMC. AVSQSKP-AMC, a novel, alternate substrate was also found to be a good substrate for CP since it has a K_m ranging from 7.3 to 21.5µM, depending on the plot used (Table 3.7). The catalytic efficiency

for this substrate ranged from 530 to 1100$min^{-1}.mM^{-1}$, depending on the plot used. These values may not be as high as those of Boc-QVR-AMC and PQVR-AMC, but the substrate shows better specificity for CP than does Boc-QVR-AMC. One can, however, improve AVSQSKP-AMC in order to increase the catalytic efficiency values obtained for CP. Possible suggestions could include replacing Gln in the P4 position with a bulkier amino acid, since Low Ah Kee (2011) demonstrated that a "bulky" residue is preferred at the P4 position. In addition, a Pro residue could be added to the P2 position, since CP activity was enhanced when Pro was used in the P2 position in the study by Mielicki *et al.* (1997). Finally, substituting the Pro in the P1 position for an Arg or Ala residue could increase catalytic efficiency, since CP prefers cleaving substrates having these amino acids in the P1 position (Low Ah Kee, 2011; Mielicki *et al*., 1997).

When examining the specificity of the P1 position, it can be seen that arginine is the preferred amino acid residue. This is in accordance with Mielicki *et al*. (1997) and Low Ah Kee (2011). C-terminal cleavage of arginine is a characteristic of serine proteases such as trypsin. This is interesting since CP was originally classified as a serine protease, but was later reclassified as a cysteine protease (Gordon, 1994). Low Ah Kee (2011) also found that the highest inhibition of CP was produced by benzamidine and PMSF which are both serine protease inhibitors. Additionally it was found that CP could not cleave the cysteine protease substrates Z-Arg-Arg--pNA and Ac-Phe-Gly-pNA (Mielicki *et al*., 1997). It could, therefore, be possible that CP is not a cysteine protease. Low Ah Kee (2011) found that CP exhibited metalloprotease activity in addition to it being inhibited by serine protease inhibitors. CP could, therefore, be a protease with mixed activity, which could explain the different bands separated on reducing SDS-PAGE gels (Low Ah Kee, 2011).

In summary, it is clear that a shorter length substrate would be a better substrate for CP since the catalytic efficiencies were higher for the shorter substrates. Ideally, a substrate with 4 residues could be designed with particular focus on the P1, P3 and P4 positions (Figure 3.21) since these appear to be the most important sites that affect CP activity. From literature it is known that CP preferentially cleaves arginine (Mielicki *et al*., 1997) or alanine (Low Ah Kee, 2011) in the P1 position. Additionally, in this study it was shown for the first time that CP could cleave a substrate with

proline in the P1 position. Generally, an uncharged amino acid in the P3 position is preferred (Low Ah Kee, 2011). The P4 position is of critical importance to CP activity as was demonstrated by Low Ah Kee (2011). In particular, a bulkier residue in the P4 position such as Boc produces a better substrate in terms of catalytic efficiency. It would be interesting to see whether the addition of Fmoc, which is slightly bigger than Boc, would produce an increase the catalytic efficiency. The C-terminal side of the cleavage sites of CP on fibronectin are rich in bulky amino acids such as glutamine (Low Ah Kee, 2011). It would also be interesting to see if addition of bulky residues to the C-terminal side of the substrate would result in a greater catalytic efficiency. This would be possible to examine using internally quenched fluorescent substrates (Stennicke *et al*., 2000) which are cleaved internally as opposed to the terminal cleavage method used in this study.

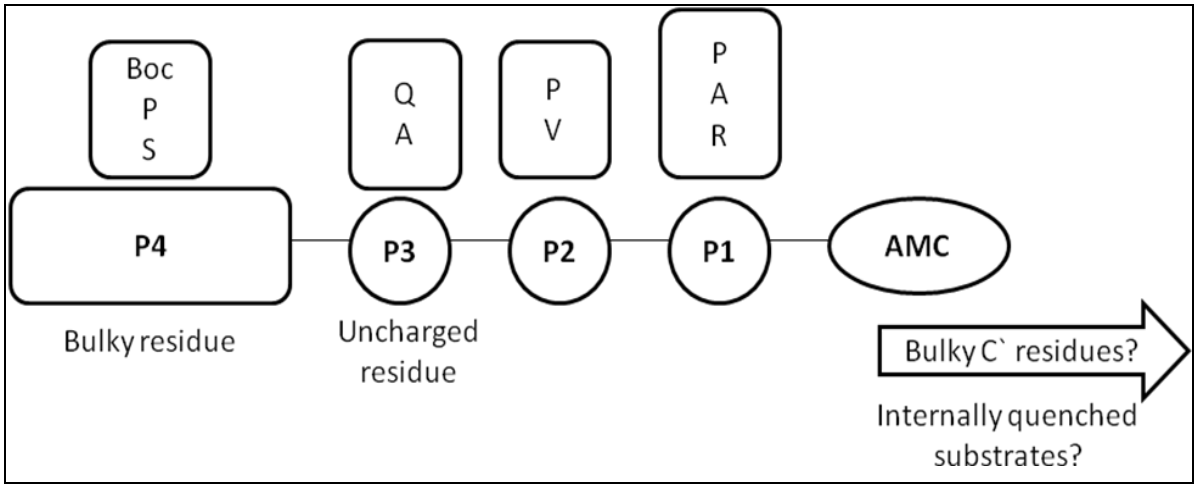


Figure 3.21 A summary of possible future designs for CP fluorescent substrates. The amino acids in the blocks above the P1 – P4 positions are possible residues to be used for each corresponding site.

4. THE EFFECT OF CANCER PROCOAGULANT ON THE EXPRESSION OF PRO- AND ANTI-INFLAMMATORY CYTOKINES IN HTERT-HDLEC, MCF-7 AND MDA-MB-231 CELL LINES

4.1 Introduction

Further attempts to unravel CP's physiological role using a different approach resulted in the investigation of the effects of CP on the production of various inflammatory cytokines by breast cancer and endothelial cells. Crosstalk between tumour cells, blood vessels and infiltrating leukocytes is mediated primarily by cytokines, chemokines, and growth factors. The inflammatory components in the tumour microenvironment lead to enhanced angiogenesis, resistance to hormones in hormone-dependent tumours and inhibition of adaptive anti-tumour immunity (Germano *et al*., 2008). These inflammatory mediators present at the tumour site regulate the survival of tumour cells, proliferation and eventually invasion and metastasis (Figure 4.1).

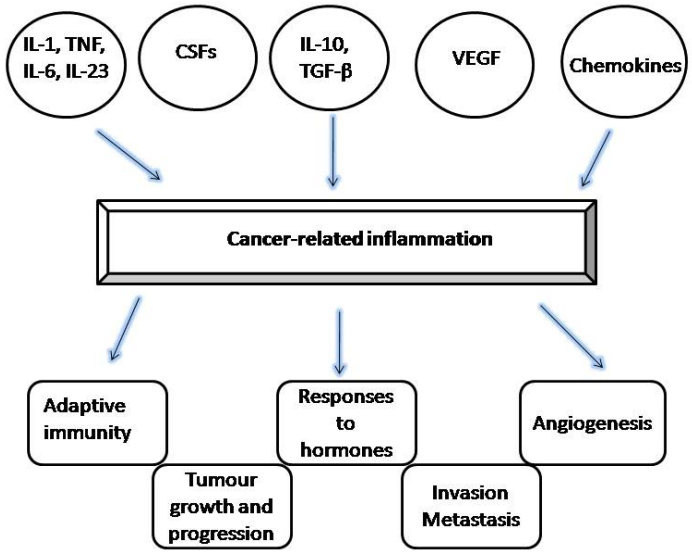


Figure 4.1. The inflammatory mediators in the tumour microenvironment that regulate tumour cell survival, proliferation, invasion and metastasis (Adapted from Germano *et al*., 2008).

It has been shown that cancer and inflammation are linked. Mice deficient in TNF- α were protected against skin carcinogenesis, indicating a genetic link between cancer and inflammation. It has also been demonstrated that TNF-α enhances cancer cell invasion ability, providing early proof that inflammatory cytokines have a pro-tumour effect (Balkwill and Mantovani, 2001). The promotion of tumours by TNF- α can involve different pathways. Firstly, it can have a direct effect on tumour cells at low concentrations of this cytokine; secondly, it can be involved in an interplay with the chemokine system with induction of CXCR4; and lastly, it can stimulate epithelial to mesenchymal transition (Bates and Mercurio, 2003; Kulbe *et al*., 2007; Kulbe *et al*., 2005). Based on these findings, many clinical protocols using TNF-α antagonists have been developed for cancer therapy (Madhusudan *et al*., 2005; Harrison, *et al*., 2007).

4.1.1 Metastasis and cytokines

The metastatic spread of cancer is the leading cause of death in cancer patients (Duffy *et al*., 2008). Metastasis is a complex process involving many sequential steps (Valdivia-Silva *et al*., 2009). It appears to be a non-random, highly organised, multi-directional and organ-selective process (Mundy, 1997; Mehlen and Puisieux, 2006; Faltas, 2012). Metastasis appears to be dependent on complex stroma-stroma interactions at the target organ (Ben-Baruch, 2003; Ben-Baruch, 2006; Lu and Kang, 2007). Similarly, breast cancer causes mortality by metastasizing to many vital organs including bone, lung, brain and liver. Chemokines play an integral part in the regulation of trafficking of the cancer cells and metastasis (Andre *et al*., 2006; Lee *et al*., 2004; Forster *et al*., 1999; Kakinuma and Hwang, 2006). Chemokine receptors are expressed in a non-random manner in breast cancer cells. Many chemokine/receptor pairs are responsible for controlling cell-cell migration (Zlotnik, 2008). Some of the important modulators of the expression of chemokines and their receptors are the pro-inflammatory cytokines such as IL-1, IL-6, IFN-γ and TNF-α. These cytokines have been shown to modulate the expression of CXCR4 and CCR5 in astrocytes (Croitoru-Lamoury *et al*., 2003), CXCR2 in human mesangial cells (Schwarz *et al*., 2002), as well as CX3CR1 in smooth muscle cells (Chandrasekar *et al*., 2003), synovium membrane (Nanki *et al*., 2002), and different epithelial cells

(Matsumiya *et al*., 2001; Fujimoto *et al*., 2001). These effects could, therefore, also be occurring in cancer cells.

4.1.2 Angiogenesis

Angiogenesis, the establishment of blood vessels from pre-existing blood vessels, is an absolute requirement for the growth of normal and tumoural tissues. This process is tightly regulated in healthy adults and occurs during events such as wound healing, inflammation and in female reproductive organs (Kachgal and Putnam, 2011). It also occurs during pathological events such as proliferative retinopathy, rheumatoid arthritis and tumour growth and metastasis (Folkman, 1995a).

Tumourigenesis is a multistep process that begins with loss of normal cell growth control. With respect to angiogenesis, tumour progression can be divided into two phases: pre-vascular and vascular. The pre-vascular phase is characterized by a balance between the rate of tumour cell proliferation and the rate of apoptosis. The vascular phase is initiated by a change in the local balance between positive and negative regulators. This step is characterized by exponential tumour growth caused by a decrease in the rate of tumour cell apoptosis, as well as tissue invasion and the spread of tumour cells. The transition from the pre-vascular to the vascular phase is referred to as the ‘‘angiogenic switch’’ (Fuhrmann-Benzakein, 2000; Karamysheva, 2008).

Various studies have shown the importance of vascular endothelial growth factor (VEGF) and its cognate receptors in tumour angiogenesis, and many strategies have been developed to inhibit tumour growth by interfering with VEGF–VEGF receptor interactions. Other cytokines which have been shown to regulate angiogenesis positively in the experimental setting include fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and hepatocyte growth factor (HGF) (Dvorak *et al.,* 1995). It has been hypothesized that angiogenesis results from induction of a positive regulator such as VEGF and/or loss of a negative regulator. Low Ah Kee *et al*. (2012) has shown that CP caused up-regulation of VEGF in MCF-7 breast cancer cells.

Two common complications in cancer are thrombosis and disseminated intravascular coagulation (DIC). The ability of nearly all types of cancer cells to activate the coagulation system, results in a prothrombotic state in patients with malignancy. The pathogenesis of the prothrombotic state in cancer is said to be highly complex and multifactorial. These prothrombotic factors in malignancy include the production of procoagulants (i.e., tissue factor (TF) and cancer procoagulant (CP)) by tumours, as well as production of inflammatory cytokines. Another factor involves the interaction between tumour cells and monocytes/macrophpages and platelets in the blood and endothelial cells. Various other mechanisms of thrombus promotion include responses of the host to the tumour (i.e., inflammation and angiogenesis), decreased levels of inhibitors of coagulation, and impaired fibrinolysis (De Cicco, 2004).

A continuous process that takes place in cells is the shedding of the cell membrane. This process may be a critical factor in determining various behaviour characteristics of cancer cells such as loss of adhesion, tissue invasion and hypercoagulable state (Dvorak *et al*., 1983). Various substances released by tumour cells possess the ability to directly activate the coagulation system; however, the best characterised are tissue factor (TF) and cancer procoagulant (CP) (Rickles and Falanga, 2001). In addition, tumour cells can release inflammatory cytokines, such as tumour necrosis factor (TNF), interleukine-1 (IL-1), and vascular endothelial growth factor (VEGF), that act on leukocytes and endothelial cells to further enhance the procoagulant activity (Gale and Gordon, 2001). It is also evident that tumour-induced coagulation activation is intrinsically involved in tumour growth, angiogenesis, and metastasis (Hejna *et al*., 1999), which in turn can promote coagulation activation.

Cancer procoagulant can directly activate factor X (Falanga and Rickles, 1999). Lee (2002) has also shown that CP can induce dose-dependent platelet activation by a mechanism that appears to be similar to that of thrombin. Although CP is found almost exclusively on malignant cells, its role as a tumour marker or as factor predictive of clinical thrombosis has not been successfully demonstrated, with the exception of the acute promyelocytic leukaemia in which CP expression in blasts parallels their degree of malignant transformation and response to all-*trans*-retinoic acid (Falanga *et al*., 1998).

The physiological response to tumour-specific antigens and tumour-procoagulant molecules is characterised by the release of potent inflammatory mediators (i.e., TNF-α, IL-1β) from activated macrophages and stimulated T-cells that can further enhance the prothrombotic process (Falanga and Rickles, 1999). In addition to being potent mediators of inflammation, these cytokines can also induce the expression of TF, activate platelets, and down-regulate the protein C pathway (Rickles and Falanga, 2001). In particular, TNF and IL-1 induce TF expression on tumour-associated macrophages, and endothelial expression of leukocytes and cellular adhesion molecules, and platelet activating factor (PAF). Thus, all the steps needed for the promotion of coagulant activity are enhanced. Additionally, these cytokines induce endothelial expression of platelet activator inhibitor 1 (PAI-1), and impair the protein C anticoagulant pathway by the down-regulation of thrombomodulin and endothelial protein C receptor. Consequently, fibrinolytic and anticoagulant activities are decreased. Cytokines are also involved in tumour proliferation through interactions with coagulation and increased tissue factor pathway inhibitor (Gouin-Thibault *et al*., 2001). In essence, inflammatory cytokines may induce both promotion of coagulant activity and suppression of anticoagulant activity (Rickles and Falanga, 2001). Besides the up-regulation of VEGF in MCF-7 cells by CP (Low Ah Kee *et al*., 2012) a connection between CP and inflammatory cytokines has not yet been reported.

4.1.3 Cytokines in breast cancer

Many human cancer studies have demonstrated the accumulation of tumour-associated macrophages (TAM) and have shown that this accumulation is associated with angiogenesis and with the production of angiogenic factors. These factors include the angiogenic cytokines, vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PDGF) (Balkwill and Mantovani, 2001). These TAMs accumulate in hypoxic regions of tumours. Hypoxia triggers a pro-angiogenic condition in these cells (Leek *et al*., 2002; Bingle *et al*., 2002; Murdoch *et al*., 2008). Salcedo *et al*. (1999) showed that many molecules that play a role in the promotion of angiogenesis are expressed by macrophages in low oxygen conditions

and examples of these are VEGF, TNF-α, basic fibroblast growth factor (bFGF), and CXCL8. The *in situ* recruitment of macrophages, therefore, represents an indirect channel of amplifying angiogenesis, together with angiogenic molecules that are produced directly by tumour cells. Cursiefen *et al*. (2004) showed that the HIF-1-dependent chemokine CXCL12 acts as a strong chemo-attractant for endothelial cells that bear CXCR4. HIF-1 has also been reported to be involved in angiogenesis and is regulated at the receptor level by VEGF and bFGF. Schioppa *et al*. (2003) was able to show that the angiogenic program that is established by hypoxia, also depends on the up-regulation of CXCR4 at the protein level by endothelial cells. VEGF and placenta-derived growth factor (PIGF) are potent attractants of monocytes and contribute to TAM recruitment (Barleon *et al*., 1996; Fischer *et al*., 2007). Hematopoietic bone marrow cells that express VEGF1R move to tumour specific pre-metastatic sites where they form a niche favouring the secondary localization of cancer (Kaplan *et al*., 2005). Mast cells and TAM also provide a source of matrix degrading enzymes (MMP) which causes the mobilisation of VEGF from ECM stores (Coussens *et al*., 1999; Noonan *et al*., 2008; Coussens *et al*., 2000). VEGF-C and VEGF-D act on the receptor VEGFR3, mediating lymphoangiogenesis. It was also recently discovered that VEGF-A increases lymphoangiogenesis by recruiting circulating monocytes (Schoppmann *et al*., 2002). Hotchkiss *et al*. (2003) proposed a role for VEGF-C in peritumoural lymphangiogenesis in human cervical cancer and consequent dissemination of cancer cells with formation of lymphatic metastasis. In addition TAM was shown to contribute to the pro-angiogenic process via the production of the angiogenic factor thymidine phosphorylase (TP). TP promotes the migration of endothelial cells *in vitro* and its level of expression is associated with tumour neovascularisation (Azenshtein *et al*., 2002).

Cytokines are low molecular weight glycoproteins, which are rapidly produced and usually secreted by various healthy and diseased cells upon stimulation. They act on a variety of adjacent target cells in an additive, synergistic, or antagonistic manner, depending on the cell type. Cytokines are intercellular mediators that regulate survival, growth, differentiation, and the effector functions of cells (Nicolini *et al.*, 2006). It is, therefore, no surprise that cytokines markedly influences the growth of tumours *in vivo*. Cytokines, however, are also produced by cancer cells and can have both tumour growth-promoting as well as inhibiting activity.

In a study examining the effect of cytokine-mediated inflammatory stimulation, low levels of MCF-7 migration under non-stimulated conditions were observed (Valdivia-Silva *et al*., 2009). This was consistent with previous reports which showed that cancer cells have a weak activity and low motility (Youngs *et al*., 1997; Prest *et al*., 1999). The migration index displayed a significant increase, however, after being stimulated by cytokines. This increase was dependent on the type of pro-inflammatory cytokine used and the receptor it binds to (Valdivia-Silva *et al*., 2009). The authors' data also indicate that stimulation by cytokines in the tumour microenvironment may affect the cancer cells' migration by different mechanisms. Different cells would behave differently under the same cytokine stimulation. In this study, the effect of CP on the production of various cytokines in two different breast cancer cell lines as well as an endothelial cell line was investigated. The author postulates that CP increases the metastatic potential of cancer cells through stimulation of pro-inflammatory and angiogenic cytokine production.

4.2 METHODS

4.2.1 Maintenance of cell lines

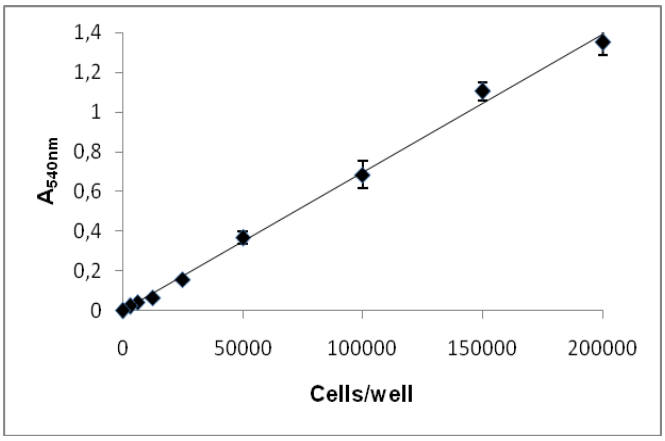
MCF-7 and MDA-MB-231 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS). hTERT-HDLEC cells were grown in Microvascular endothelial cell growth medium (EGM-2MV) supplemented with 10% FBS and growth factors on gelatin (0.1%) covered plates. All cell lines were maintained at 37˚C with 5% carbon dioxide/95% air atmosphere in a humidified incubator.

4.2.2 MTT assay

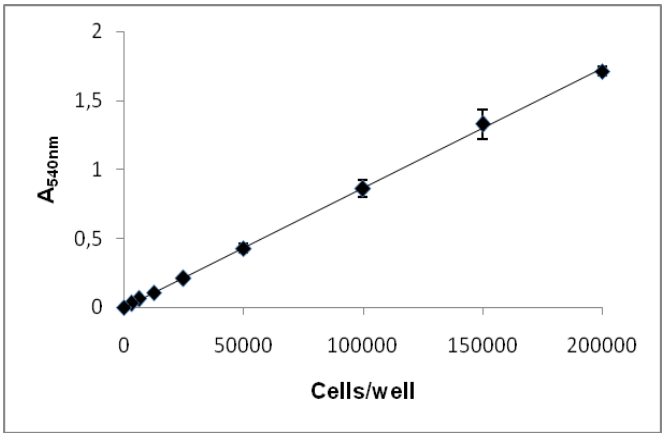
The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT (a yellow, soluble dye) into water-insoluble, purple formazan crystals by living cells, which determines mitochondrial activity.

In this study, a modified method described by Holst-Hansen and Brünner (1998) was used. Following exposure of the different cell lines to the various treatments, the media was removed and 0.5ml MTT reagent (Sigma) at 1mg/ml was added to the wells and incubated at 37ºC for 2 hours. Thereafter, the MTT was removed and 1ml DMSO was added to solubilise the formazan crystals. The amount produced was measured at an absorbance of 540nm using a BioTek XS microplate reader. The number of viable cells was calculated using extrapolation from an MTT standard curve which was constructed for each cell line. Typical MTT standard curves for the various cell lines used are represented in Figure 4.2.

a)



b)



c)

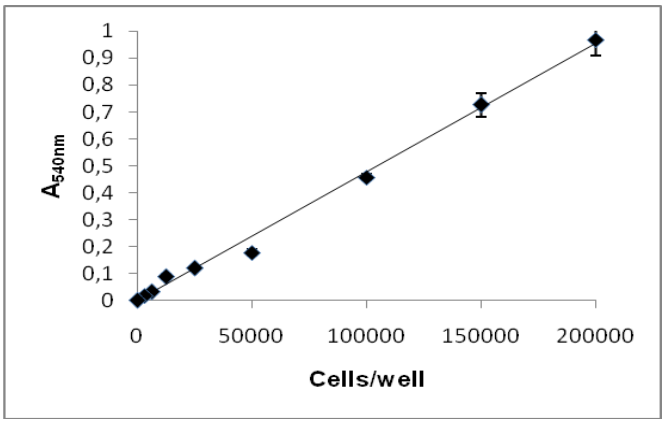


Figure 4.2. MTT standard curves for: a) MCF-7 cells (R^2=0.996, n=3); b) MDA-MB-231cells (R^2=0.999, n=3) and c) hTERT-HDLEC cells (R^2=0.994, n=3). Error bars represent standard error of the mean (SEM).

4.2.3 CellTracker Green fluorescent labelling of cells

Molecular Probes® CellTracker™ fluorescent probes are chloromethyl derivatives that freely pass through cell membranes of live cells and are, through esterase hydrolysis, converted from a non-fluorescent, mildly thiol-reactive compound to cell-impermeant glutathione-fluorescent (5-chloromethylfluorescein) dye adducts. These cell-impermeant dye adducts are possibly produced by glutathione S-transferase-mediated reactions. The cell-impermeant form is passed to daughter cells through several generations, but is not transferred to adjacent cells in the population. Cells that are loaded with CellTracker™ probes are typically fluorescent and viable for extended periods, making these probes excellent long term cell tracers (Bajènoff *et al*., 2008; Johnson and Spence, 2010).

In this study, CellTracker™ Green 5-cloromethylfluorescein diacetate (CMFDA) was used to label the cells. After seeding cells into a microtitre plate, the media was removed and replaced with media (200µl) containing 10µM CellTracker™ Green fluorescent dye (Molecular Probes). Fluorescence was measured using a BioTek Synergy Mx fluorescent microtitre plate reader. Excitation was achieved at 492nm and emission at 517nm.

4.2.4 Effect of CP on the detachment of MCF-7 and MDA-MB-231 cells

MCF-7 and MDA-MB-231 cells were grown to 70% confluence prior to seeding into 48 well microtitre plates (CoStar), coated with 0.1% bovine gelatin (Sigma). The cells were seeded at a density of 15 000 cells/well and incubated at 37˚C overnight to allow for attachment. The media was removed and 10µM CellTracker™ Green fluorescent dye (Molecular Probes) (20µl/well) was added according to the method outlined in Section 4.2.3. The fluorescent dye was removed and the cells were exposed to the various treatments: untreated control (DMEM), 10µg/ml collagenase (Sigma) (positive control) or 1, 10 and 20µg CP (purified from amnion-chorion membranes as per Section 2.3.1). Exposure times were 6, 24 and 48 hours. At each time interval, the media (containing detached cells) was collected in a clean 48-well microtitre plate and fresh media was added to the cells which did not detach.

The plate containing the detached cells was immediately read in a fluorescence plate reader at an excitation wavelength of 492nm and an emission wavelength of 517nm. MTT analysis was also performed and it was determined that the cell viability was above 80% in all treatments across the time period investigated. The results were expressed as the percentage of cells that had detached relative to an untreated control. The data was normalized relative to cell number.

4.2.5 Effect of CP on the motility of hTERT-HDLEC cells.

hTERT-HDLEC cells were grown to 70% confluency prior to seeding into 48 well microtire plates which were previously coated with 0.1% gelatine. The same experimental design was used as described in Section 4.2.4.

4.2.6 Cytokine expression

In an attempt to provide further evidence of CP's involvement in angiogenesis and metastasis, its effect on cytokine expression by MCF-7, MDA-MB-231 and hTERT-HDLEC cells was investigated. In particular, the level of cytokines commonly associated with the promotion (TNF-α, IL-6, IL-1β and PDGF-β) and inhibition (IFN-α and IFN-β) of angiogenesis and metastasis were measured using ELISA kits according to the manufacturer's instructions (eBioscience for TNF-α, IL-6, IL-1β, IFN-α and IFN-β; Komabiotech for human PDGF-β). Briefly, all cell lines were exposed to 10µg/ml CP or 10ng/ml LPS (positive control). Untreated cells served as a control. Media was collected after 6, 12, 24 and 48 hours and stored at -80˚C until cytokine analysis via ELISA. 100µl of media from each time point was used to determine cytokine levels in the media. Values are expressed, normalised to 10 000 cells.

Below are examples of typical standard curves obtained for the cytokine ELISAs.

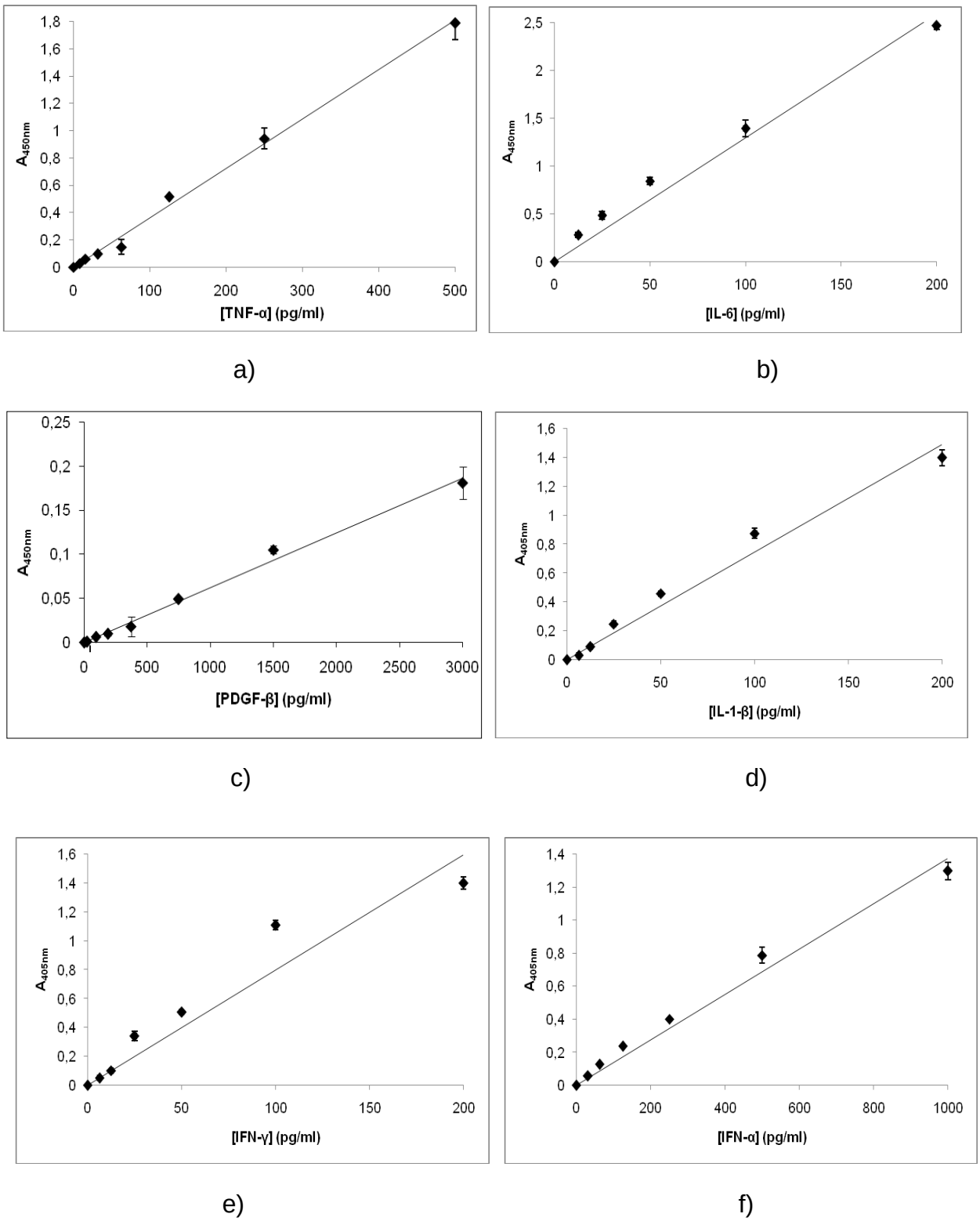


Figure 4.3 Cytokine standard curves of: a) TNF-α (R^2=0.995, n=3); b) IL-6 (R^2=0.974, n=3); c) PDGF-β (R^2=0.990, n=3); d) IL-1β (R^2=0.977, n=3); e) IFN-γ (R^2=0.908, n=3) and IFN-α (R^2=0.981, n=3). Error bars represent the SEM.

4.3 RESULTS AND DISCUSSION

CP (10 and 20µg/ml) induced detachment of MCF-7 cells by 20 and 21%, respectively, after 6 hours. At lower concentrations (1µg/ml) CP did not induce significant changes in cell motility after 6 hours. Although not significant, 1µg/ml CP induced a time-dependent decrease in detachment of MCF-7 cells from gelatin. After 24 hours, however, CP (10µg/ml and 20µg/ml) induced motility of the MCF-7 cells by 25 and 30%, respectively. After 48 hours, the same trend was observed, where CP (10 and 20µg/ml) caused significant increases (29 and 32%, respectively) in cell detachment. This implies that CP promotes the detachment of MCF-7 cells from gelatin *in vitro* in a time- and dose-dependent manner.

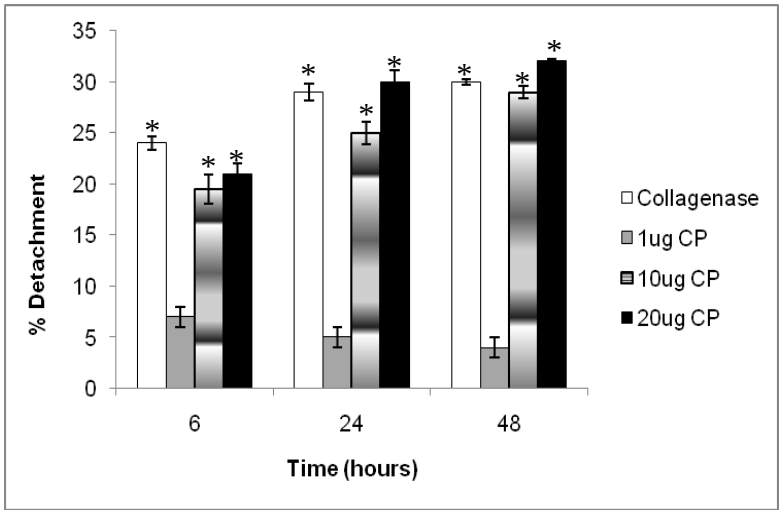


Figure 4.4 The effect of CP on the detachment of MCF-7 cells relative to control cells. Error bars represent the SEM. *, $p<0.05$ (n=3).

Quite evident from the results is the difference in effect of low (1µg/ml) and high (10 and 20µg/ml) CP concentrations on the detachment of the MCF-7 cells from gelatin. While a high concentration of CP promotes detachment of MCF-7 cells, low concentrations seem to cause a decrease in detachment. This decrease in detachment could, possibly, be interpreted as an increase in cell adhesion. There is some evidence pointing to this possibility. CP (at low concentrations) was seen to cause an increase in the adhesion of MCF-7 cells to vitronectin (Kamocka *et al*., 2005). These authors observed that pre-incubation of MCF-7 cells with anti-CP antibody caused a reduction in the adhesion of the cells to vitronectin. The adhesive

properties were completely restored upon further addition of purified CP (0.5–8mg/ml) to the MCF-7 cells pre-incubated with anti-CP antibody. When a high concentration (16mg/ml) of CP was added to the sample, however, the authors observed that the adhesive properties of the cells were only partially recovered. This concentration, however, is not physiologically possible. The positive control, collagenase, induced detachment of MCF-7 cells by 24-30% over the time period tested.

An earlier high level of CP in early stage cancer patients' serum (Gordon and Cross, 1981; Kozwich *et al*., 1994) and low in the advanced stage of cancer (Mielicki and Wierzbicki, 1990) has been reported. Based on the results of the present study, it could be hypothesised that CP has a regulatory role in metastasis, depending on the stage of metastasis and its concentration at each stage. In the initial stages of metastasis CP may be present at high concentrations and its role would, therefore, be to promote detachment from the primary tumour through regulation of various cell adhesion markers as well as the promotion of angiogenesis and in the later stages the expression of CP decreases and promotes cell adhesion at a secondary site for successful colonization of the cells.

It is evident from the results that CP at high concentrations (10 and 20μg/ml) promotes the detachment of less aggressive MCF-7 cells from gelatin, increasing the metastatic potential of MCF-7 cells. The observation that low concentrations of CP exhibit proadhesive properties in MCF-7 cells is consistent with the results obtained in the study by Kamocka *et al*. (2005).

The effect of CP on the metastatic and more aggressive MDA-MB-231 cancer cell line was also investigated. As can be seen from the results, CP significantly increased the detachment of MDA-MB-231 cells at low (1μg/ml) and high (10 and 20μg/ml) concentrations in a time-dependent manner. The lower concentration of CP (1μg/ml) did not significantly increase the detachment of MDA-MB-231 cells from gelatin after 6 hours, but after 24 and 48 hours a significant increase in detachment was observed. The same trend was observed at a CP concentration of 10μg/ml. For the 20μg/ml CP sample, a significant increase in cell detachment was observed as

early as 6 hours. Collagenase also caused a significant increase in cell detachment (20-23%) across the time period studied. MDA-MB-231, a mesenchymal-like cell line, is extremely aggressive and invasive, whereas MCF-7 is a luminal (epithelial)-like cell line that has a relatively low invasive phenotype and potential (Lacroix & Leclercq, 2004; Charafe-Jauffret *et al*., 2006). This would explain why CP-stimulated detachment is higher in the MDA-MB-231 cells than in the MCF-7 cells, since MDA-MB-231 cells, without stimulation, have an inherently high metastatic potential as compared to MCF-7 cells. It was noted from the results that at a low concentration of 1μg/ml of CP, detachment was differently affected in MDA-MB-231 cells as compared to MCF-7 cells. In MCF-7 cells, the detachment from gelatin was found to decrease over time at low concentration, whereas in the MDA-MB-231 cell line, there was an increase in detachment over time. Kamocka *et al*. (2005) noticed a decrease in the viability (16–18%) of MCF-7 cells that were treated with the anti-CP polyclonal antibody. In this study, it was seen that the viability of MCF-7 and MDA-MB-231 cells did not decrease when exposed to both low and high concentrations of CP. Taken together, there is potential that CP exhibits anti-apoptotic characteristics and enhances cancer cell growth and proliferation.

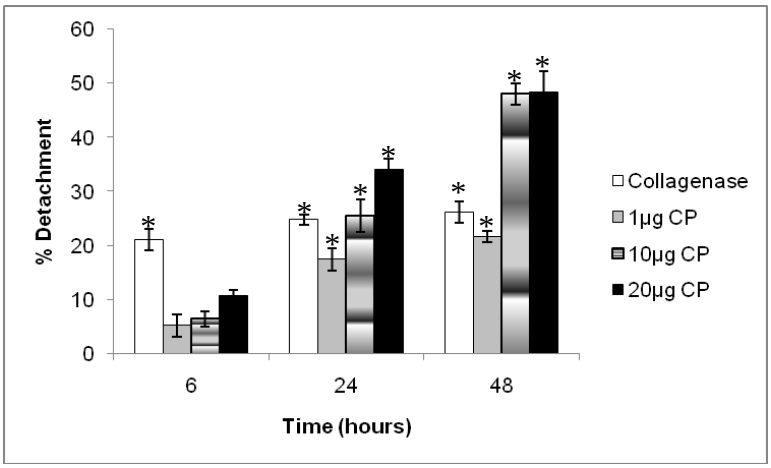


Figure 4.5 The effect of CP on the detachment of MDA-MB-231 cells relative to control cells. Error bars represent the SEM. *, $p<0.05$ (n=3).

CP was also found to increase the motility of hTERT-HDLEC cells *in vitro.* As can be seen from the results (Figure 4.6), the positive control (collagenase) showed increased motility of the cells. This result, however, was only significant after 24 and 48 hours. There were no significant changes in the motility of the cells that were

exposed to 1µg CP (p=0.14) across the time period examined. At a concentration of 10µg/ml, CP significantly increased the motility of hTERT-HDLEC cells by 23% after 6 hours and continued to increase up to 46% after 48 hours (p=0.007). At a higher concentration (20µg/ml) CP had a similar, but more pronounced effect. At this concentration, CP significantly increased motility of the cells to 38% after only 6 hours (p=0.00027).

Endothelial cells play a critical role in angiogenesis, forming the newly sprouting vessels (Wong *et al*., 2009). hTERT-HDLEC cells are lymphatic endothelial cells that have been transfected with a retrovirus containing the coding region of human telomerase reverse transcriptase (hTERT) in order to extend its lifespan (Nisato *et al*., 2004) are involved in lymphangiogenesis (Pegu *et al*., 2008). The results suggest that CP increases the motility of hTERT-HDLEC cells *in vitro* in a time- and dose-dependent manner, providing additional evidence that CP plays a role in angiogenesis by stimulating endothelial cell motility. This could be further confirmed by determining if CP is able to promote tube formation and should be examined in a future study. This possible involvement of CP in tumour angiogenesis was also shown at a genetic level by Low Ah Kee *et al*. (2012). The author provided evidence that CP caused an up-regulation of VEGF in MCF-7 cells. VEGF is a potent promoter of angiogenesis, bringing about the migration of endothelial cells.

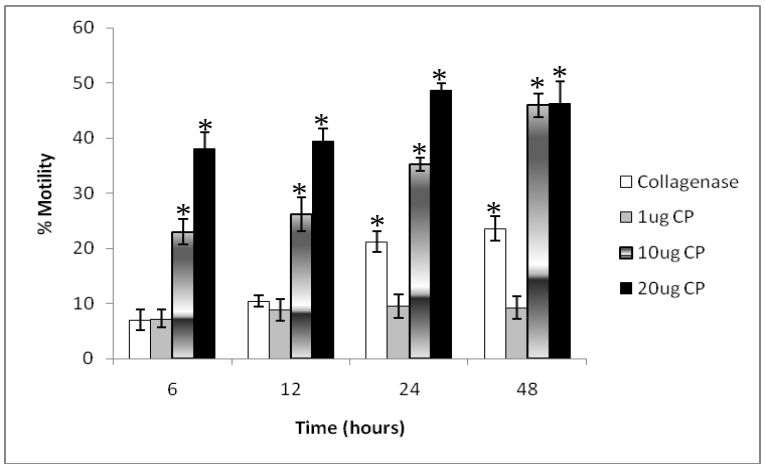


Figure 4.6 The effect of CP on the motility of hTERT-HDLEC cells relative to control cells. Error bars represent the SEM, n=3.

Further, the author also noted that CP's mechanism of action in metastasis and angiogenesis is not through degradation of the ECM, but rather through regulation of various metastatic and angiogenic molecules. CP could not degrade laminin and type IV collagen, only fibronectin (Low Ah Kee, 2011). It did, however, up-regulate the expression of pro-angiogenic and –metastatic markers such as VEGF, RhoC, angiogenin and IL-8 (Low Ah Kee *et al*., 2012). One should not, however, completely eliminate the possibility of CP being involved in the degradation of the extracellular matrix.

4.3.1 Effect of CP on cytokine levels in hTERT-HDLECs, MCF-7 and MDA-MB-231 cells

The effect of CP on the expression of various cytokines by endothelial cell line hTERT-HDLECs and breast cancer cell lines MCF-7 and MDA-MB-231 was investigated in a time study. The cytokines used in this study were chosen due to their involvement as pro- (TNF-α, IL-6, IL-1β and PDGF-β) or anti-angiogenic (IFN-α and IFN-γ) factors in the processes of angiogenesis and metastasis. The cells were exposed to CP over a period of 3 days and the media was collected after 6, 24, 48 and 72 hours. LPS was used as a positive control.

4.3.1.1 TNF-α

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine that can regulate a wide variety of cellular responses including proliferation (Kaiser and Polk, 1997), differentiation (Li and Swartz, 2001), inflammation (Grund *et al*., 2008), and cell death (Marques-Fernandez *et al*., 2013). Although TNF-α was originally characterized to cause hemorrhagic tumour necrosis at high concentrations in many types of cancer, low concentrations of TNF-α seem to increase tumour growth and progression (Nicolini *et al*., 2006). TNF-α binds to TNF-α receptors (TNFRs) that recruit several proteins which function as a platform adapter and trigger different signalling pathways depending on cell type. Particularly in breast cancer, TNF-α has been reported to induce apoptosis or to inhibit *in vitro* proliferation in MCF-7 cells (Purohit *et al.,* 2002), while other breast cancer cell lines have been shown to be resistant to

TNF-α-induced apoptosis. TNF-α is found in many cells such as reactive astrocytes, endothelial cells, infiltrating macrophages and malignant gliomas (Maruno *et al*., 1997; Roessler *et al*., 1995). The TNF-α receptor (TNFR) is expressed by glioma (Chambaut-Guerin *et al*., 2000) and endothelial cells (Slowik *et al*., 1993). TNF-α causes activation and up-regulation of angiogenic factors such as VEGF, thereby indirectly inducing tumour angiogenesis (Ryuto *et al*., 1996; Wong *et al*., 2009). Yoshida *et al*. (1997) was able to show enhancement of VEGF, IL-8 and bFGF production by TNF-α in human microvascular endothelial cells. It was also able to induce tubular morphogenesis *in vitro*.

From the results obtained, when compared to untreated control, CP did not seem to induce the production of any significant amounts of TNF-α in hTERT-HDLEC (Figure 4.7 a) and MCF-7 cell lines (Figure 4.7 b). However, in MDA-MB-231 cells (Figure 4.7 c), CP caused significant up-regulation of TNF-α after 12 hours and this response became more pronounced after 24 and 48 hours. After 72 hours, it was noted that cell viability declined to below 80% (results not shown) in all three cell lines investigated. This could be due to induction of apoptosis triggered by an increase in TNF-α levels or due to cell growth, contact inhibition and this could have resulted in cell death. It should also be noted that the basal levels of TNF-α in hTERT-HDLEC and MDA-MB-231 are considerably lower than those in MCF-7 cells. The amount of TNF-α obtained for MDA-MB-231 and hTERT-HDLEC cells was less than 5pg/ml, which is in agreement with the literature (Blot *et al*., 2003; Pegu *et al*., 2008). The amount of TNF-α obtained from MCF-7 was slightly higher than that found in literature (Sprowl *et al*., 2012; Desai *et al*., 2013). This could be due to the use of DMEM in the present study instead of RPMI media as used in the literature. Initially this experiment was carried out using MCF-7 cells and the time period chosen was 24, 48 and 72 hours. However, it was noted from the results that earlier time periods (4 and 6 hours) were needed. Due to limited amounts of CP, however, earlier time periods were not used for the MCF-7 cells.

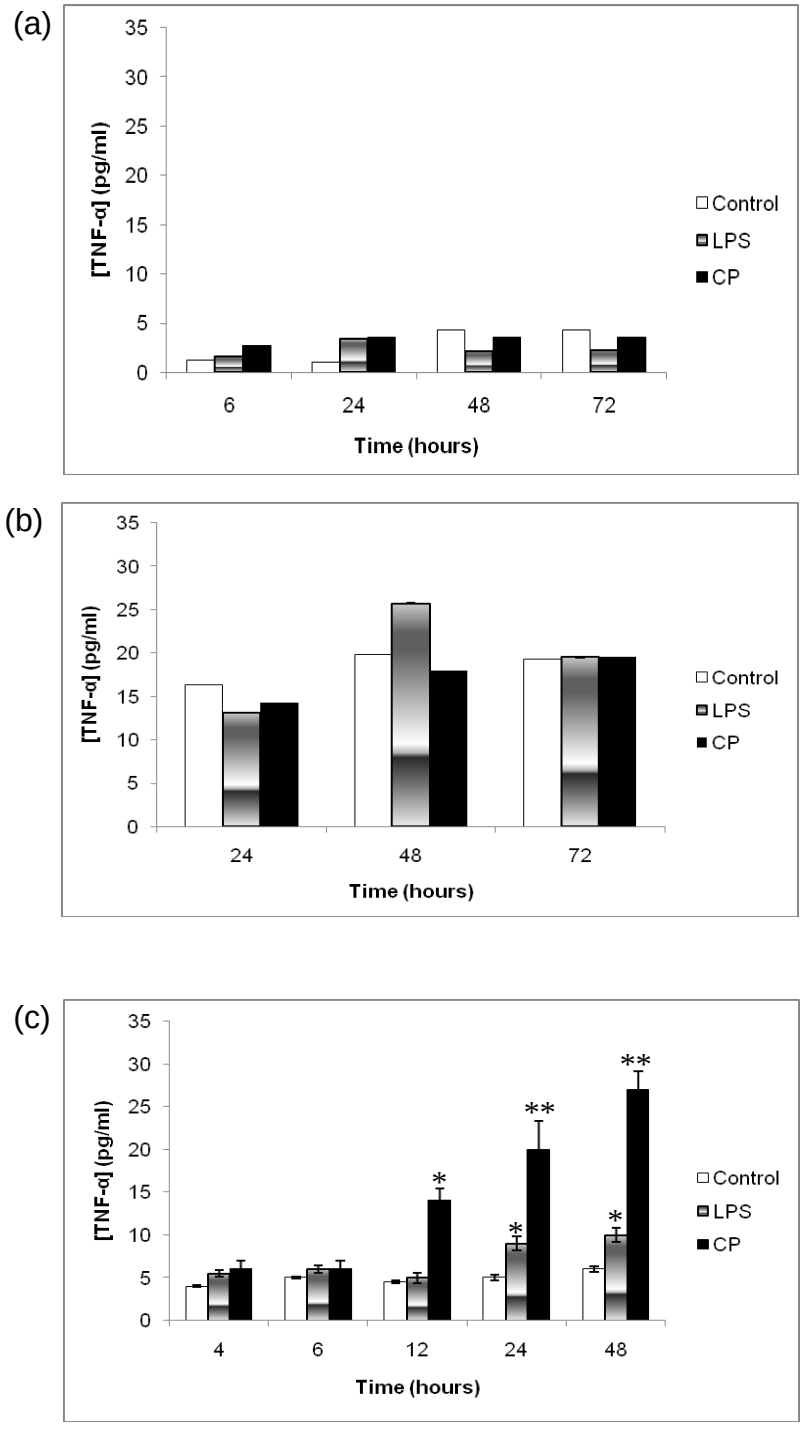


Figure 4.7 The effect of CP on the expression of inflammatory cytokine, TNF-α. (a) hTERT-HDLEC, (b) MCF-7, (c) MDA-MB-231. Qualitative methods were used to determine the expression of untreated control (□), LPS (10ng/ml)-treated cells (positive control) (▤) and CP (10µg/ml)-treated cells (■), normalised to 10 000 cells. Error bars represent SEM values of 3 experiments. *$p<0.05$ and **$p<0.001$ relative to the corresponding control.

4.3.1.2 IL-6

IL-6 has been found in high concentrations in human breast cancer cell lines and in breast tumour samples. Fibroblasts, macrophages and lymphocytes (mainly Th2 cells) are thought to be an important source of IL-6 (Balkwill and Mantovani, 2001). This cytokine exerts its effects through glycoprotein (gp) 130-mediated activation of signalling pathways (including the JAK/STAT and MAP kinase pathways) resulting in the transcriptional regulation of genes involved in cell proliferation, survival and differentiation (Heinrich *et al*., 1998; Tu *et al*., 2012). IL-6 promotes tumour growth by up-regulating anti-apoptotic and angiogenic proteins in tumour cells. Increased IL-6 production also increases estradiol-17b hydroxysteroid dehydrogenase (17b-HSD) type I which converts oestrone (E1) to the biologically active oestrogen, oestradiol (E2) (Purohit *et al*., 2002). Therefore, it has been hypothesized that IL-6 and IL-1β stimulate proliferation of breast cancer cells through oestrogen production by activating steroid-catalyzing enzymes in the tissue. IL-6 may favour proliferation and metastasis of cancer cells, development of osteolysis and humoral hypercalcemia, and it has also been suggested to be a cachectic factor in cancer patients (Nicolini *et al*., 2006).

IL-6 has been reported to be an important inflammatory cytokine with growth-promoting and anti-apoptotic activities (Ishihara and Hirano, 2002; Lin and Karin, 2007; Naugler and Karin, 2008). It also serves as an effector signal of activated NF-κβ in the promotion of neoplasia. Klein *et al*. (1989) demonstrated a pro-tumoural role for IL-6 in multiple myeloma (MM) where both an autocrine loop of IL-6 production as well as a paracrine loop by bone marrow stromal cells has been reported. To support this pro-tumoural role for IL-6, Lattanzio *et al*. (1997) was able to show that IL-6 deficient mice displayed resistance to the developing of murine plasmocytoma. An alternative pathway of connection between IL-6 and cancer, having NF-κβ as a linker, has been recently suggested (Annunziata *et al*., 2007; Keats *et al*., 2007). The presence of a specific polymorphism in the IL-6 promoter region and, consequently, high levels of IL-6 is a predisposing genetic factor that contributes to a deterioration in the prognosis of breast cancer (Berger, 2004), strengthening the observation that IL-6 plays a key role in breast cancer (Berger,

2004; Zhang *et al*., 2013). Chung and Chang (2003) reported that colon cancer patients produce high levels of IL-6, but low levels of IL-6 receptor in inflamed colon and colon cancer have been observed, suggesting that IL-6 may preferentially activate through trans-signalling rather than via the classical receptor pathway. Colitis-associated-cancer (CAC) provides a clear association between IL-6 production and cancer. It has been found that TGF-β reduces the formation of cancer through inhibition of IL-6 trans-signaling (Becker *et al*., 2004).

It was noted that the IL-6 level for the control after 24 hours in hTERT-HDLEC and MCF-7 cells (Figure 4.8 b) was low (7.2 pg/ml and 15 pg/ml, respectively). Although not significant, the level of IL-6 for the MCF-7 control decreased with time. Both these observations are in accordance with literature (Desai *et al*., 2013) which showed that IL-6 levels were low after 24 hours and below the detection range after 48 hours. Waage *et al*. (1989) calculated the half-life of IL-6 to be 103±27 min. IL-6 was detected in patient serum up to 36 hours. The authors also noted that IL-6 was secreted into the serum later than TNF-α. The short half-life of IL-6 could, therefore, be responsible for the decreases of IL-6 levels in the MCF-7 and hTERT-HDLEC control cells over time.

We also found that CP caused significant up-regulation of IL-6 in hTERT-HDLEC cells as early as 6 hours (Figure 4.8 a). In MCF-7 cells, CP had no significant effects on the levels of IL-6 (Figure 4.8 b). However, in the metastatic cell line, MDA-MB-231, CP caused significant up-regulation of IL-6 as early as 4 hours, with the effect most pronounced at 24 hours (Figure 4.8 c). This early expression of IL-6 in hTERT-HDLEC and MDA-MB-231 cells is not surprising, since IL-6 is involved in the transcriptional regulation of genes that are responsible for processes such as cell proliferation, survival and differentiation, as well as the up-regulation of various angiogenic proteins in tumour cells (Heinrich *et al*., 1998; Tu *et al*., 2012). MCF-7 cells are a non-metastatic cell line, while MDA-MB-231 cells are metastatic (Lacroix & Leclercq, 2004; Charafe-Jauffret *et al*., 2006). hTERT-HDLEC cells are endothelial cells that, upon stimulation by various factors, has the ability to migrate (Pegu *et al*., 2008). Considering the migratory capacity of these cell lines, it becomes clear that the cells associated with migration (hTERT-HDLEC and MDA-MB-231) are also the cells that are significantly affected by CP stimulation to produce the inflammatory

response observed. This suggests that CP-induced IL-6 is also involved in the CP-induced migratory process of these cells. Another major difference between MCF-7 and MDA-MB-231 cells is the expression of oestrogen receptors (ER) on the cell surface. MCF-7 cells are oestrogen receptor positive, while MDA-MB-231 cells are oestrogen receptor negative (Hsieh *et al*., 2010). It is known that increased expression of IL-6 causes an increase in the expression of estradiol-17b hydroxysteroid dehydrogenase type I which converts oestrone (E1) to the biologically active oestradiol (E2) (Purohit *et al*., 2002). This results in the promotion of breast cancer cell proliferation. Since MCF-7 cells are capable of taking up exogenous oestrogen, due to the presence of the oestrogen receptors on their cell surface, the activation of estradiol-17b hydroxysteroid dehydrogenase type I is not required and hence, the production of IL-6 would not be needed. However, since MDA-MB-231 cells are ER negative, they require the activation of estradiol-17b hydroxysteroid dehydrogenase type I by IL-6 for cell proliferation to occur (Purohit *et al*., 2002). The results, therefore, indicate that CP may enhance IL-6 expression in MDA-MB-231 and hTERT-HDLEC cells, possibly to promote cell proliferation.

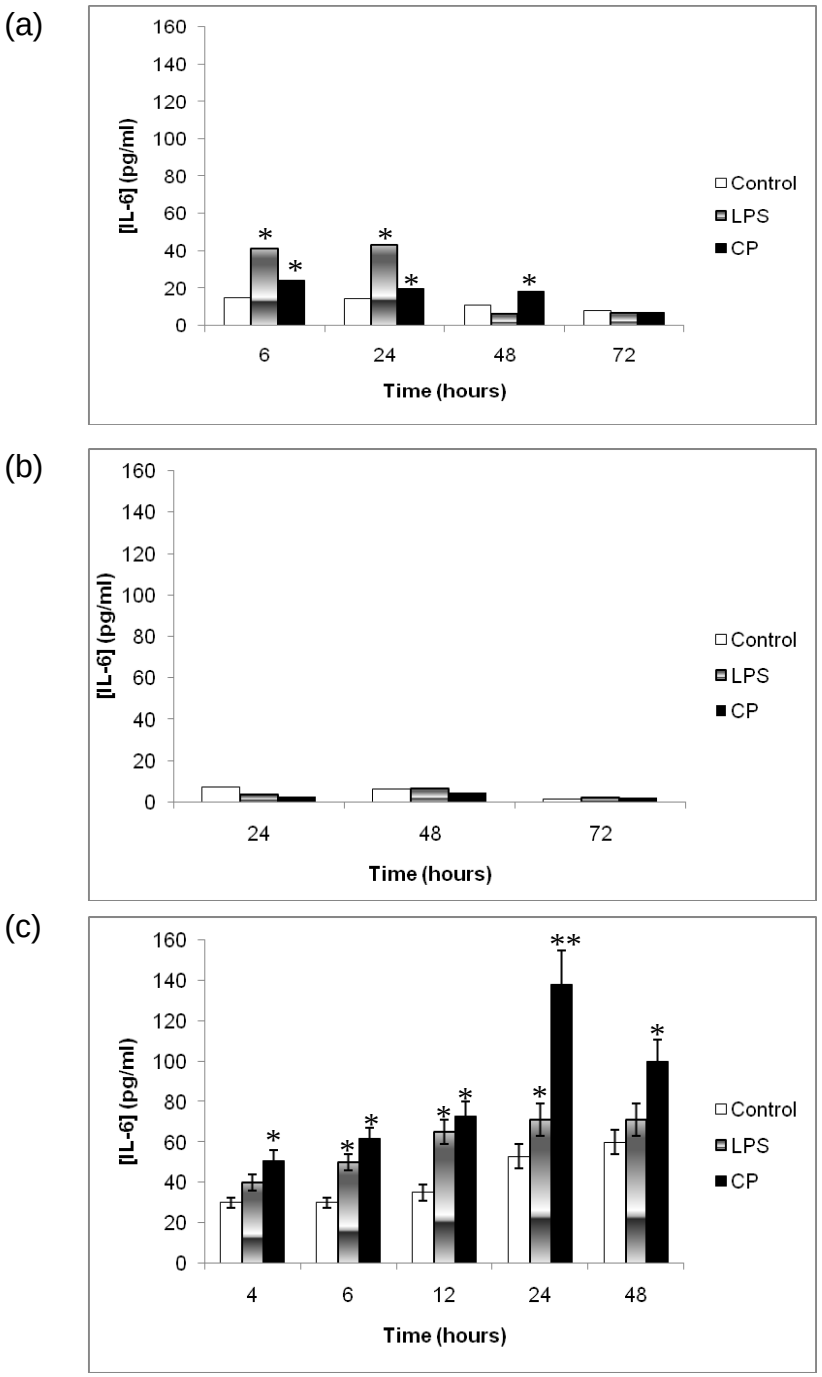


Figure 4.8 The effect of CP on the expression of inflammatory cytokine, IL-6. (a) hTERT-HDLEC, (b) MCF-7, (c) MDA-MB-231. Qualitative methods were used to determine the expression of untreated control (☐), LPS (10ng/ml)-treated cells (positive control) (▤) and CP (10µg/ml)-treated cells (■), normalised to 10 000 cells. Error bars represent SEM values of 3 experiments. *p<0.05 and **p<0.001 relative to the corresponding control.

4.3.1.3 PDGF-β

PDGF belongs to the vascular endothelial growth factor family and has been associated with deterioration in prognosis and metastatic spread (Relf *et al*., 1997). The family of platelet-derived growth factor (PDGF) consists of four gene products that form five dimeric isoforms. In particular, PDGF-β is thought to stimulate tumour growth through tumour stromal cells expressing the PDGF-β receptor in a paracrine fashion through its homodimeric isoform, PDGF-BB (Fredriksson *et al*., 2004). There is speculation that it may play a critical role in metastatic spread by helping disseminating cells adjust to the microenvironments of distant sites (Zhu *et al*., 2006).

Platelet-derived growth factor acts on several cell types involved in wound healing. It stimulates mitogenicity and chemotaxis of fibroblasts and smooth muscle cells and chemotaxis of neutrophils and macrophages. It also stimulates macrophages to produce and secrete other growth factors of importance for various phases in the healing process. Moreover, PDGF has been shown to stimulate production of several matrix molecules, like fibronectin (Blatti *et al*., 1988) and collagen (Canalis, 1981). Platelet-derived growth factor may also be of importance at later stages of wound healing, since it stimulates contraction of collagen matrices *in vitro* (Clark *et al*., 1989) implicating a role in wound contraction *in vivo*. Moreover, PDGF stimulates the production and secretion of collagenase by fibroblasts (Bauer *et al*., 1985), suggesting a role in the remodelling phase of wound healing.

PDGF-β and platelet-derived growth factor β receptor (PDGFRβ) play an important role in vessel wall development and differentiation (Lindahl *et al*., 1997). The presence of PDGF-β is essential for the recruitment of pericytes and microvasculature maturation. However, VEGF partly mediates the angiogenic effects of PDGF (Guo *et al*., 2003; Wang *et al*., 1999). When PDGFR-β signalling is inhibited, the tumour vasculature is particularly vulnerable to withdrawal of VEGF, leading to endothelial apoptosis as well as vascular regression (Bergers *et al*., 2003). PI3K/Akt, MAPK/ERK and STAT3 signalling mediate the effects of PDGF on angiogenesis (Sun *et al*., 2005). The expression of VEGF can also be induced by PDGF-β via a PI3K/Akt dependent mechanism (Reinmuth *et al*., 2001).

The effect of CP on the expression of PDGF-β was determined. It was found that PDGF-β expression was up-regulated after 24 hours in hTERT-HDLEC (Figure 4.9 a) and as early as 4 hours in MDA-MB-231 cells (Figure 4.9 c), but down-regulated in MCF-7 cells after 24 hours (Figure 4.9 b). The up-regulation of PDGF-β in MDA-MB-231 cells was observed up to 24 hours, with levels returning to normal after 48 hours. This trend was not observed in hTERT-HDLEC cells in which up-regulation was still observed after 48 hours. The results indicate that the inherent levels of PDGF-β in MDA-MB-231 cells was also high (481 pg/ml), although not as high as that found by Yotsumoto *et al.* (2013). These authors reported that MDA-MB-231 cells expressed 700-800 pg/ml PDGF-β. The difference between the results obtained by these authors and that of the present study could be due to the different media used to culture the MDA-MB-231 cells. The present study used DMEM, while Yotsumoto *et al*. (2013) cultured the MDA-MB-231 cells in Leibovitz's L-15 medium.

It is evident from the results of the present study that the CP-induced expression of PDGF-β is differently modulated in MDA-MB-231 and hTERT-HDLEC cells as compared to MCF-7 cells. These results suggest a possible role for CP in wound healing as well as metastatic spread, through induction of PDGF-β expression in hTERT-HDLEC and MDA-MB-231 cells, respectively.

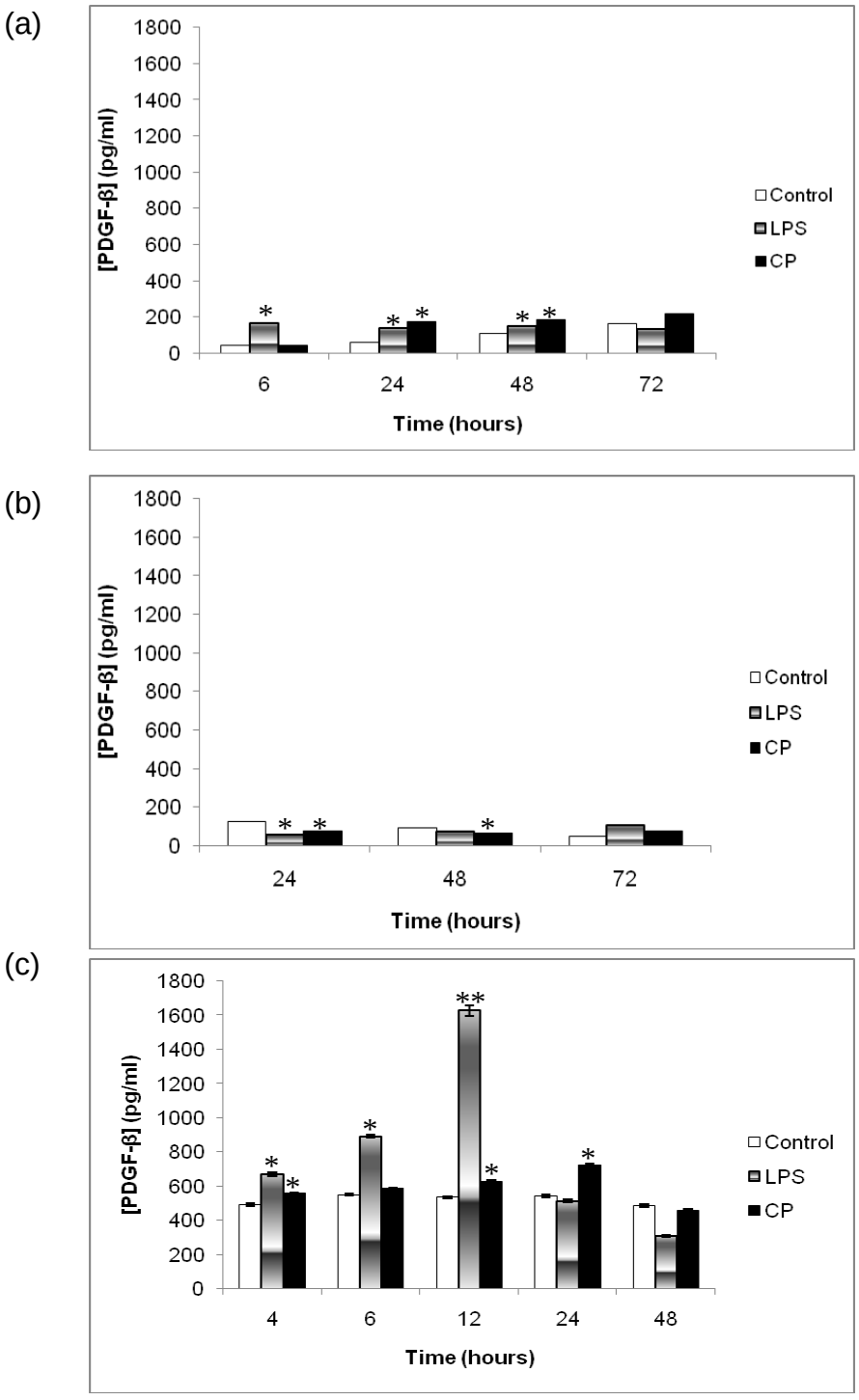


Figure 4.9 The effect of CP on the expression of inflammatory cytokine, PDGF-β. (a) hTERT-HDLEC, (b) MCF-7, (c) MDA-MB-231. Qualitative methods were used to determine the expression of untreated control (□), LPS (10ng/ml)-treated cells (positive control) (▣) and CP (10µg/ml)-treated cells (■), normalised to 10 000 cells. Error bars represent SEM values of 3 experiments. *$p<0.05$ and **$p<0.001$ relative to the corresponding control.

4.3.1.4 IL-1β

Interleukin-1 (IL-1) is a proinflammatory cytokine (Yano *et al*., 2003). Independent researchers discovered this cytokine as endogenous pyrogen (Hadley *et al*., 1966), leukocytic endogenous mediator (Pekarek *et al*., 1972), lymphocyte activation factor (Blyden and Handschumacher, 1977) and *β*-cell-activating factor (Wood, 1979). IL-1 is made up of two different molecules, namely, IL-1α and IL-1β. L-1α and IL-1β are synthesized as precursors without leader sequences (Yano *et al*., 2003). Each precursor has a molecular weight of 31 kD, and are processed to mature forms of 17 kD by specific cellular proteases. The amino acid sequences of IL-1α and IL-1β share a 25% homology. IL-1α is the cell-associated and mainly regulates intracellular events. In contrast, IL-1β is released from cells and is a systemic, hormone-like mediator. Upon binding to their receptors, however, IL-1α and IL-1β exhibit similar biological activities (Dinarello, 1996). A wide variety of cells including leukocytes, fibroblasts, and malignant cells produce IL-1 in response to various stimuli such as bacterial products and cytokines (Dinarello, 1996). Many cell types such as endothelial cells, smooth muscles, epithelial cells, hepatocytes, fibroblasts, keratinocytes, epidermal dendritic cells, T lymphocytes, and malignant cells express receptors for IL-1. IL-1 has two types of receptors (IL-1R); the type I receptor (IL-1RI) transduces signals, whereas the type II receptor (IL-1RII) binds IL-1 without any signal transduction (Colota, 1994). IL-1 is a multi-functional cytokine that plays crucial roles in various biological reactions such as inflammation, haematopoiesis, and reactions in the immune system, endocrine system, and central nervous system (Dinarello, 1996). The effects of IL-1 in malignant diseases are still controversial. Studies have shown that IL-1 increased the proliferation of certain malignant cells via induction of other growth factors, including TNF-α, IL-6, IL-8, and transforming growth factor-β (TGF-β) (Dinarello, 1996; Lachman *et al*., 1987). Contrary to these findings, IL-1 was shown to inhibit growth of melanoma, glioma, meningioma, breast, cervical, thyroid, and ovarian carcinoma. (Onozaki *et al*., 1985; Lachman, *et al*., 1986; Herzog and Collin, 1992; Kilian *et al*., 1991). These inhibitory effects of IL-1 were thought to have been brought about due to the induction of TNF-α, IL-6, and oxygen radicals, diminished polyamine synthesis, increased differentiation and polymerization of F-actin, cell cycle arrest, and production of nitric oxide (Dinarello, 1996). Much

attention has been paid to the regulatory roles of IL-1 in metastasis. It has been reported that endogenous IL-1 promotes metastasis of melanoma cells. This is brought about by increased tumour-cell binding to endothelial cells via induction of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM- 1) (Burrows *et al*., 1991; Scherbath and Orr, 1997). Yano *et al*. (2003) suggested that IL-1β facilitates metastasis of lung cancer via promoting multiple events, including adhesion, invasion and angiogenesis. They showed that exogenous IL-1β enhanced expression of various cytokines (IL-6, IL-8, and vascular endothelial growth factor (VEGF) and intracellular adhesion molecule-1 (ICAM-1) by various lung cancer cells expressing IL-1 receptors. It was also demonstrated that over-expression of IL-1β resulted in increased expression of the cytokines, ICAM-1, and matrix metalloproteinase-2 (MMP-2) (Yano *et al*., 2003).

In the present study, the CP-induced expression of IL-1β by hTERT-HDLEC, MCF-7 and MDA-MB-231 cells were monitored. The results (Figure 4.10) indicate low levels (less than 8pg/ml) of expression of IL-1β in the controls across all cell lines tested. Even LPS did not induce significant expression ($p>0.05$) of IL-1β in these cells. For hTERT-HDLEC (Figure 4.10 a) and MCF-7 (Figure 4.10 b) cells, CP decreased the levels of IL-1β compared to the control, but this decrease was not significant. In the MDA-MB-231 cell line (Figure 4.10 c) CP caused an increase in the level of IL-1β up to 24 hours, but this increase was also not significant. CP, therefore, did not induce a significant change in the expression of IL-1β in any of the cell lines investigated ($p>0.05$). This is at first surprising, since IL-1β increases the proliferation of certain malignant cells (Dinarello, 1996; Lachman *et al*., 1987; Yano, *et al*., 2003). One would, therefore, expect CP to increase IL-1β levels for the promotion of proliferation of tumour cells, since it is thought to be involved in the promotion of angiogenesis and metastasis (Kaplinska *et al*., 2009; Low Ah Kee, 2011; Low Ah Kee *et al*., 2012). However, it was also reported that IL-1β inhibits the growth of melanoma, glioma, meningioma, breast, cervical, thyroid, and ovarian carcinoma (Onozaki *et al*., 1985; Lachman, *et al*., 1986; Herzog and Collin, 1992; Kilian *et al*., 1991) possibly due to the induction of, amongst other factors, TNF-α and IL-6 (Dinarello, 1996). MCF-7 and MDA-MB-231 cells are breast cancer cells and if IL-1β indeed inhibits proliferation of breast cancer, then the lack of induction of expression of IL-1β by CP in these cells would, therefore, be understood.

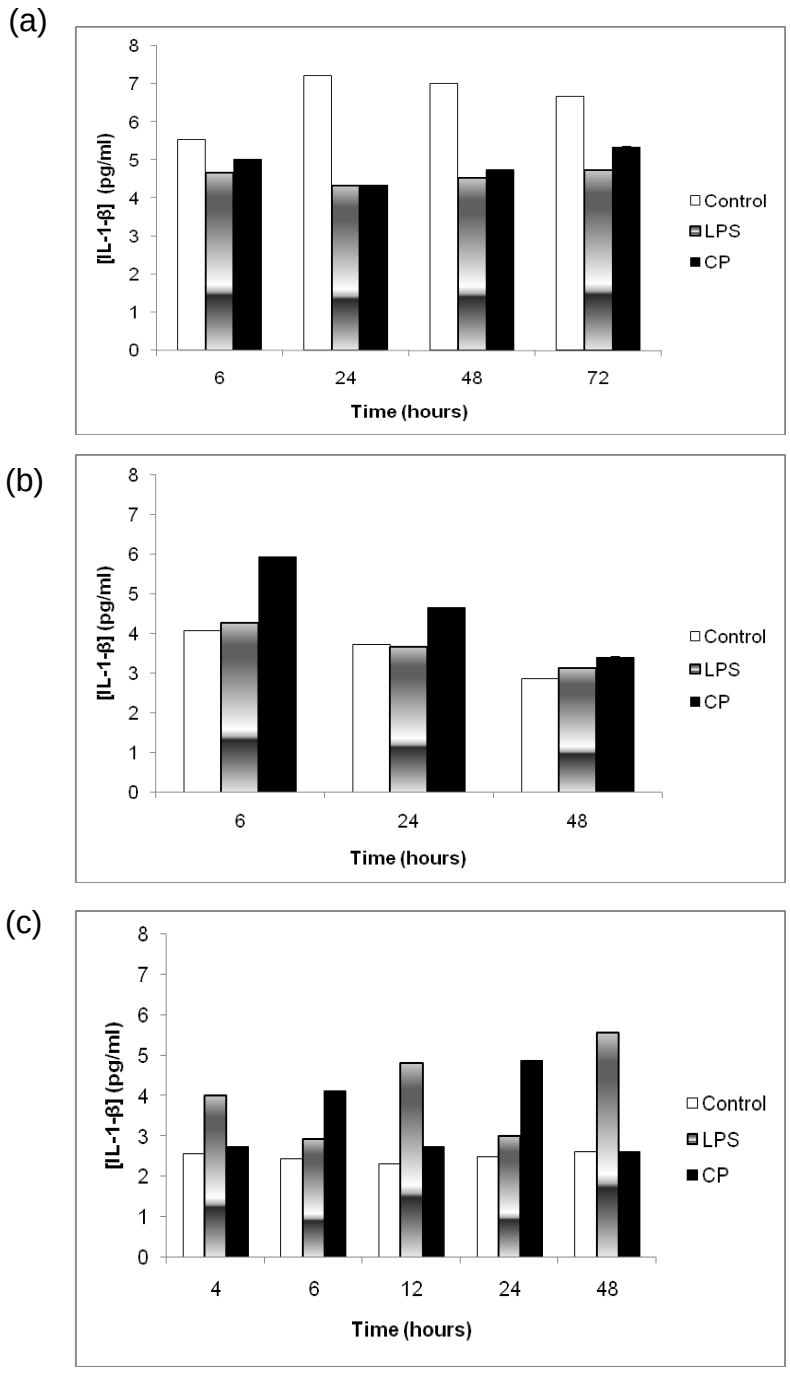


Figure 4.10 The effect of CP on the expression of inflammatory cytokine, IL-1β. (a) hTERT-HDLEC, (b) MCF-7, (c) MDA-MB-231. Qualitative methods were used to determine the expression of untreated control (□), LPS (10ng/ml)-treated cells (positive control) (▤) and CP (10µg/ml)-treated cells (■), normalised to 10 000 cells. Error bars represent SEM values of 3 experiments.

4.3.1.5 IFN- γ

Interferon-γ (IFN-γ) is a cytokine typically associated with cytostatic, cytotoxic and anti-tumour effects during cell-mediated adaptive immune response (Farrar and Schreiber, 1993; Boehm *et al*., 1997; Bach *et al*., 1997). Clinically, it has been used as a treatment for several malignancies, even though it produces mixed results and severe side effects. Despite the overwhelming amount of evidence that implicate a role for IFN-γ in tumour immune surveillance, a few reports suggest that this cytokine, under certain circumstances, may also have pro-tumourigenic effects. (Zaidi and Merlino, 2011). IFN-γ is a member of a family of proteins that was originally identified by their ability to protect cells from viral infection in a non-specific manner (Wheelock, 1965; Isaacs and Lindenmann, 1957). According to structural and functional criteria as well as the stimuli that induce their expression, these proteins have been divided into two classes. The first class is primarily induced in response to viral infection of cells and is referred to as type I IFNs. Type I IFNs are further subdivided into two groups (IFN-α and IFN-β) on the basis of their cellular origin (Pestka *et al*., 1987). The second class is called type II or immune IFN and is now known as IFN-γ, is produced mainly by T lymphocytes, NKT cells and natural killer (NK) cells subsequent to activation with immune and inflammatory stimuli rather than viral infection (Farrar and Schreiber, 1993; Boehm *et al*., 1997). IFN-γ has direct anti-proliferative and anti-metabolic effects on several tumour cells (Nicolini *et al*., 2006). Inhibition of cellular proliferation through IFN-γ-dependent mechanisms has been observed in human fibrosarcomas and murine fibroblasts. These effects were, however, not reported in mutagenised human cells lacking Stat1 or in murine cells derived from Stat1 deficient mice (Chin *et al*., 1996; Bromber *et al*., 1996). IFN-γ was also found to inhibit angiogenesis indirectly through stimulation of angiostatic chemokine secretion (Luster and Ravetch, 1987; Angiolillo *et al*., 1995).

For the experimental conditions completed, CP did not induce a significant expression of IFN-γ by hTERT-HDLEC, MCF-7 and MDA-MB-231 cells. The results (Figure 4.11) indicate low levels (less than 9pg/ml) of expression of IFN-γ in the controls across all cell lines tested. LPS (10 ng/ml) did not induce significant expression ($p>0.05$) of IFN-γ in these cells. For hTERT-HDLEC (Figure 4.11 a) and

MCF-7 (Figure 4.11 b) cells, CP decreased the levels of IFN-γ compared to the control up to 48 hours, but this decrease was not significant. In the MDA-MB-231 cell line (Figure 4.11 c) CP caused an increase in the level of IFN-γ from 6 hours up to 48 hours, but this increase was also not significant. CP, therefore, did not induce a significant change in the expression of IFN-γ in any of the cell lines investigated ($p>0.05$). These results indicate that CP does not promote expression of the anti-tumour cytokine, IFN-γ, in the cell lines tested. Since IFN-γ is indirectly involved in anti-angiogenic pathways (Luster and Ravetch, 1987; Angiolillo *et al*., 1995), it is not surprising that CP did not cause expression of IFN-γ in any of the cell lines tested in this study, since CP promotes angiogenesis (Low Ah Kee, 2011; Low Ah Kee *et al*., 2012).

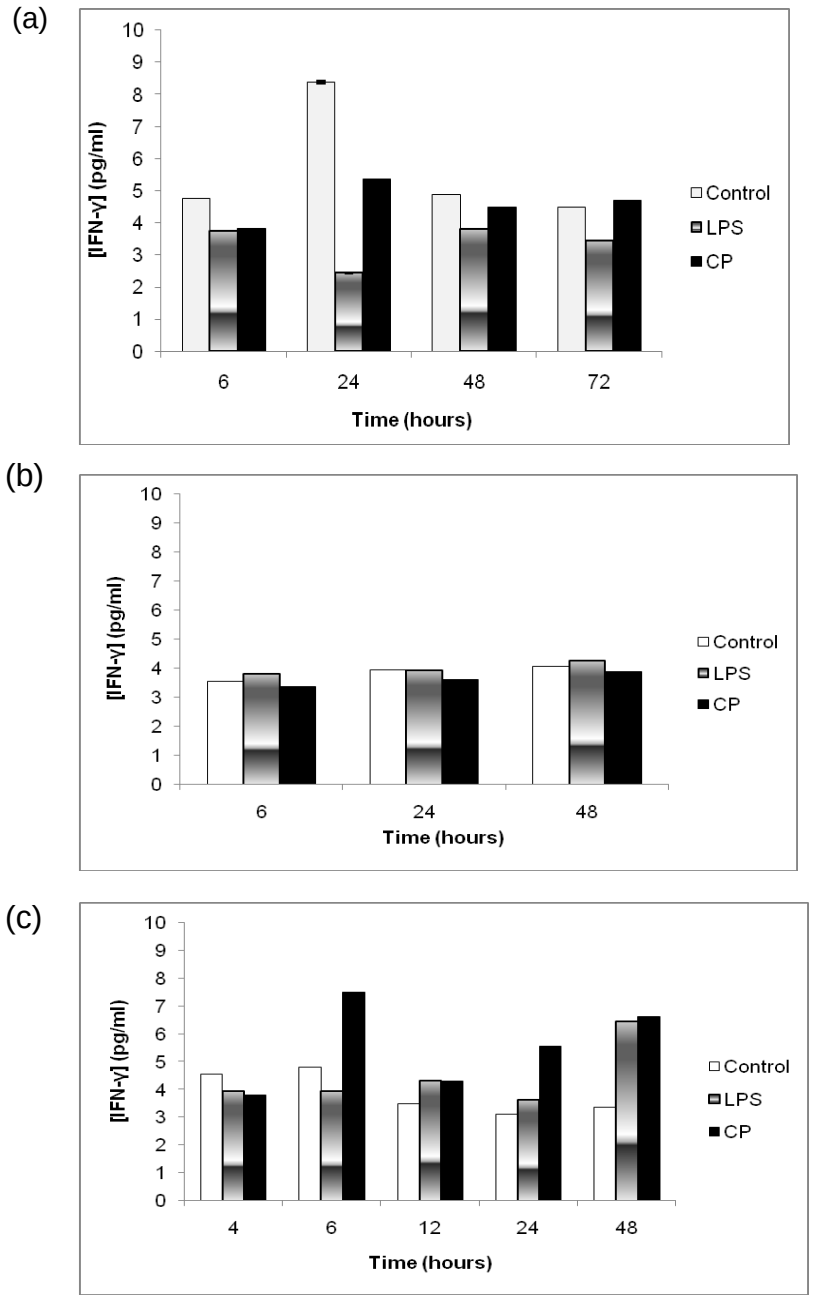


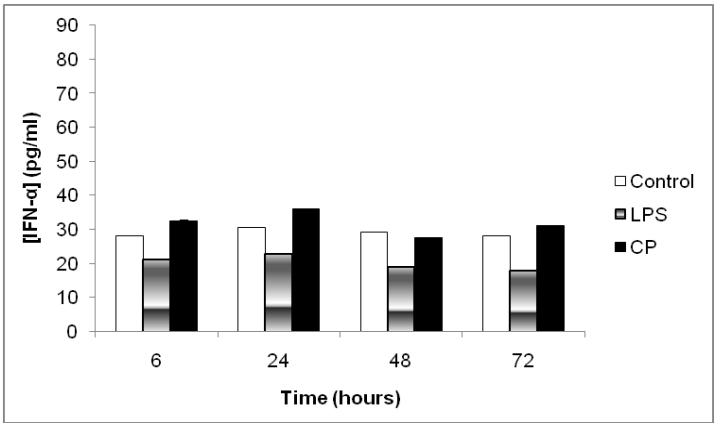
Figure 4.11 The effect of CP on the expression of inflammatory cytokine, IFN-γ. (a) hTERT-HDLEC, (b) MCF-7, (c) MDA-MB-231. Qualitative methods were used to determine the expression of untreated control (□), LPS (10ng/ml)-treated cells (positive control) (▤) and CP (10µg/ml)-treated cells (■), normalised to 10 000 cells. Error bars represent SEM values of 3 experiments.

4.3.1.6 IFN-α

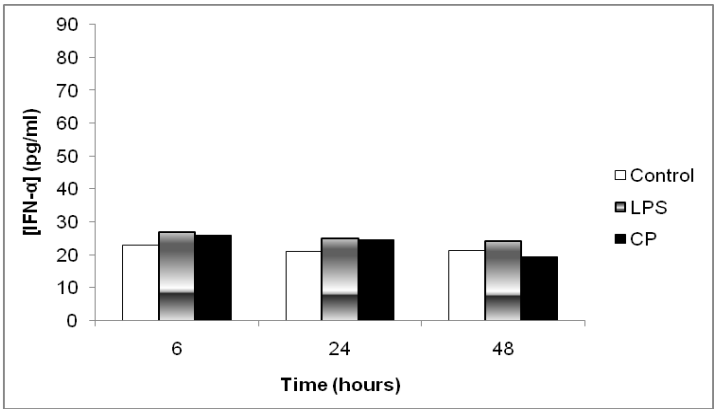
The alpha and beta interferons are type I interferon (IFN) proteins with anti-tumour activity (Nicolini *et al*., 2006). These cytokines down-regulate oncogene expression and induce tumour suppressor genes which result in anti-proliferative activity. Anti-proliferative and anti-adhesive actions of IFN-α have been shown in MCF-7 breast carcinoma cells (Maemura *et al*., 1999). The anti-proliferative effect of IFN-α 2a and 2b on the growth of ZR-75-1 human breast cancer cells was synergistic with that of the anti-oestrogen, toremifene (Martin and Symonds, 2002). In human breast cancer, IFN-α 2a, combined with all-trans retinoic acid (ATRA), did not potentiate the growth inhibition of ATRA (Toma *et al*., 2000).

For the results obtained, CP increased the expression of IFN-α by MDA-MB-231 across the time period examined (Figure 4.12 c). Although this increase was not significant up to 24 hours, there was a significant increase in the expression of IFN-α after 48 hours in MDA-MB-231 cells. Since this cytokine is one with anti-tumour activity (Nicolini *et al*., 2006), anti-proliferative and anti-adhesive actions (Maemura *et al*., 1999), it is surprising that up-regulation was observed in MDA-MB-231 cells. It could be argued that up-regulation of IFN-α after 48 hours, together with the decreased levels of PDGF-β in MDA-MB-231 cells after 48 hours, is due to possible colonisation of a secondary site. Additionally, this observation could also point to a possible time-dependent switch between pro- and anti-angiogenic cytokines. The production of this anti-inflammatory cytokine at this time, could be preparing the cells to colonise the secondary site by up-regulating various adhesion proteins needed for adhesion.

(a)



(b)



(c)

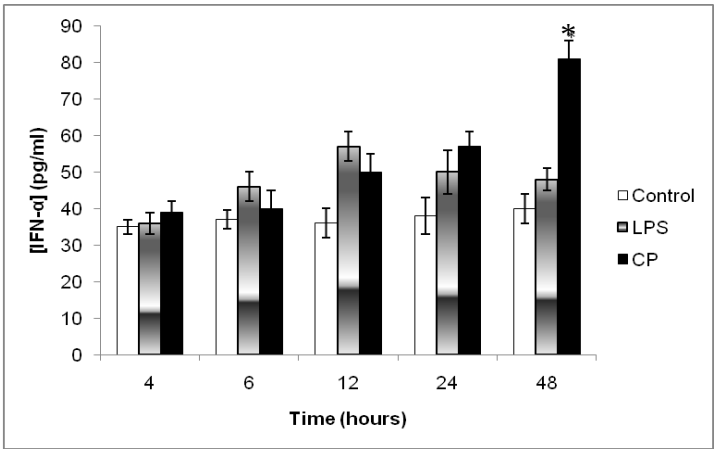


Figure 4.12 The effect of CP on the expression of inflammatory cytokine, IFN-α. (a) hTERT-HDLEC, (b) MCF-7, (c) MDA-MB-231. Qualitative methods were used to determine the expression of untreated control (□), LPS (10ng/ml)-treated cells (positive control) (▤) and CP (10μg/ml)-treated cells (■), normalised to 10 000 cells. Error bars represent SEM values of 3 experiments. $p<0.05$ relative to the corresponding control.

4.4 Discussion

The results obtained in this study suggest that CP promotes the migration of MCF-7 and MDA-MB-231 cells *in vitro* for the time period studied (Table 4.1). CP also produced a pro-inflammatory response in hTERT-HDLEC and MDA-MB-231 cells, but not in MCF-7 cells. CP-induced expression of cytokines is differently modulated in the less aggressive MCF-7 cell line as compared to the metastatic and more aggressive MDA-MB-231 cell line.

Table 4.1 Summary of the effect of CP on cytokine levels of hTERT-HDLEC, MCF-7 and MDA-MB-231 cells.

	hTERT-HDLEC	MCF-7	MDA-MB-231
1. TNF-α	X	X	↑* (up to 72 hours)
2. IL-6	↑ (up to 48 hours)	X	↑* (up to 48 hours)
3. PDGF-β	↑ (up to 72 hours)	X	↑* (up to 24 hours)
4. IL-1β	X	X	X
5. IFN-γ	X	X	X
6. IFN-α	X	X	↑* (at 48 hours)

X, no significant effect; ↑, up-regulation; *, significant effect. Shaded rows represent pro-inflammatory cytokines.

There are marked similarities in the inflammatory response produced by CP in hTERT-HDLEC and MDA-MB-231 cells, which are both associated with a migratory phenotype. The induction of IL-6 production by CP in hTERT-HDLEC and MDA-MB-231 cells further supports the possible involvement of CP in the metastatic spread, as well as proliferation of breast cancer cells, possibly through IL-6-dependent activation of estradiol-17b hydroxysteroid dehydrogenase type I. In addition, the early expression of IL-6 caused by CP in hTERT-HDLEC and MDA-MB-231 cells could imply the existence of a “preparatory phase” prior to angiogenesis and/or metastasis. Furthermore, CP-induced PDGF-β expression in hTERT-HDLEC and MDA-MB-231 cells could point to involvement of CP in wound healing and metastatic spread, respectively.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

CP has been implicated in the process of angiogenesis through up-regulation of VEGF-A in MCF-7 and E-14 cells (Low Ah Kee *et al*., 2012). It has also been detected in many malignant tumours (Falanga and Gordon, 1985a) as well as the amnion-chorion membranes of human placenta (Low Ah Kee *et al*., 2012). This study set out to isolate CP from amnion-chorion membranes and investigate its role in metastasis and angiogenesis, focussing particularly on tumour and endothelial cell detachment from gelatin and on its effects on cytokine expression by tumour and endothelial cells. The study also aimed to design a novel substrate and assay for the detection of CP activity.

CP has been successfully isolated from amnion-chorion membranes using a two-step anion-exchange chromatography procedure (Low Ah Kee *et al*., 2012). The same procedure was used in this study to isolate CP. In the current study, this method produced a 68 kDa protein, the reported molecular mass of CP. This study failed to produce sufficient quantities of purified CP for N-terminal amino acid sequencing analysis. Several isolations are required using this procedure in order to generate sufficient amounts of CP for N-terminal amino acid sequence analysis. This is due to the very low yield of CP obtained using this procedure. Unravelling the N-terminal amino acid sequence would provide a basis for uncovering the DNA sequence of CP through molecular biology techniques. Knowledge of the DNA sequence of CP could allow for silencing of the gene that controls CP expression, consequently leading to further elucidation of its biological functions. Furthermore, CP has been proposed to be used as an early detection cancer marker (Kozwich *et al*., 1994) and information on the primary structure of CP could prove to be a valuable tool in designing such an early detection cancer assay.

To begin to unravel the involvement of CP in metastasis, its effect on the detachment of tumour cells from gelatin was investigated. Detachment of tumour cells from the primary tumour is one of the first and most crucial steps in the process of metastasis. CP promoted the detachment of both MCF-7 and MDA-MB-231 cells from gelatin,

indicating that CP may have a small role in the detachment of tumour cells from the primary tumour. CP was shown to degrade fibronectin and not laminin or type IV collagen (Low Ah Kee, 2011). Perhaps a better attachment substrate for the detachment experiment would have been fibronectin. Nevertheless, we were able to show that CP may have a role in tumour cell detachment from the primary tumour. CP could not degrade collagen (Low Ah Kee, 2011) and it is, therefore, likely that CP does not degrade gelatin either, since gelatin is a hydrolysed form of collagen. The mechanism by which CP promotes detachment, therefore, does not appear to be through the direct degradation of proteins, but rather indirectly through stimulation of the production of various factors such as VEGF-A. A disadvantage of creating an adhesive surface for cell attachement is the ability of cells to remodel the protein (gelatine) on the plate surface either by degradation of the protein or by secreting additional matrix especially if it is a long-term study (Reinhart-King, 2003). In this case, it is advised to perform the experiments using polyacrylamide sheets (Wang and Pelham, 1998). For endothelial cell migration, a better approach would be to use the Boyden Chamber assay in which cell migration is measured based on the number of cells that migrate from a chamber toward a chemotactic agent (Boyden, 1962).

CP activity is highest during the early stages of cancer and decreases during the later stages thereof (Kozwich *et al*., 1994; Mielicki *et al*., 1994). One of the first events during cancer progression is the activation of the inflammatory system. In this study, we were interested in examining the effect of CP on the producton of inflammatory cytokines by tumour cells as well as by endothelial cells. We found that CP caused significant increases in the levels of pro-inflammatory cytokine levels that were also implicated in the process of angiogenesis, such as PDGF-β, IL-6a TNF-α. PDGF-β, a member of the angiogenic cytokine family was differently modulated in MDA-MB-231 and hTERT-HDLECs as compared to MCF-7 cells. CP caused up-regulation of PDGF-β in MDA-MB-231 and hTERT-HDLEC cells (both assocated with a higher migratory capacity), while down-regulation was observed in the MCF-7 cell line (lower migratory capacity), giving a clue as to the importance of this cytokine in metastasis. A future study could look at the effect of blocking the PDGF-β receptor to see if it would affect the detachment of cells caused by CP. The early expression of IL-6 and PDGF-β caused by CP in hTERT-HDLEC and MDA-MB-231 cells could

imply the existence of a “preparatory phase” prior to angiogenesis and/or metastasis. The induction of a “pre-metastatic niche” by the primary tumour is an important step that takes place before metastasis manifests itself (Bonnomet *et al*., 2010). It would be interesting to investigate whether CP plays a role in mediating the establishment of this “pre-metastatic niche”, which is mediated by bone-marrow-derived cells. It was noted that CP did not cause up-regulation of any cytokine in MCF-7 cells (Table 4.1). Perhaps CP exerts its effects on MCF-7 cells in a different manner, such as interaction with the oestrogen receptors or PARs. CP did not cause changes in the expression of anti-inflammatory cytokines such as IFN-α and IFN-γ, except in MDA-MB-231 cells where CP caused an increase in IFN-α levels after 48 hours. This implies that CP exhibits pro-tumoural activity. However, since the anti-tumour IFN-α was up-regulated during later stages of exposure of MDA-MB-231 cells to CP, it raises the question: Could CP be one of the factors involved in the angiogenic switch? Figure 5.1 provides a summary of what is known of CP’s physiological role, specifically on angiogenesis and metastasis, *in vitro*.

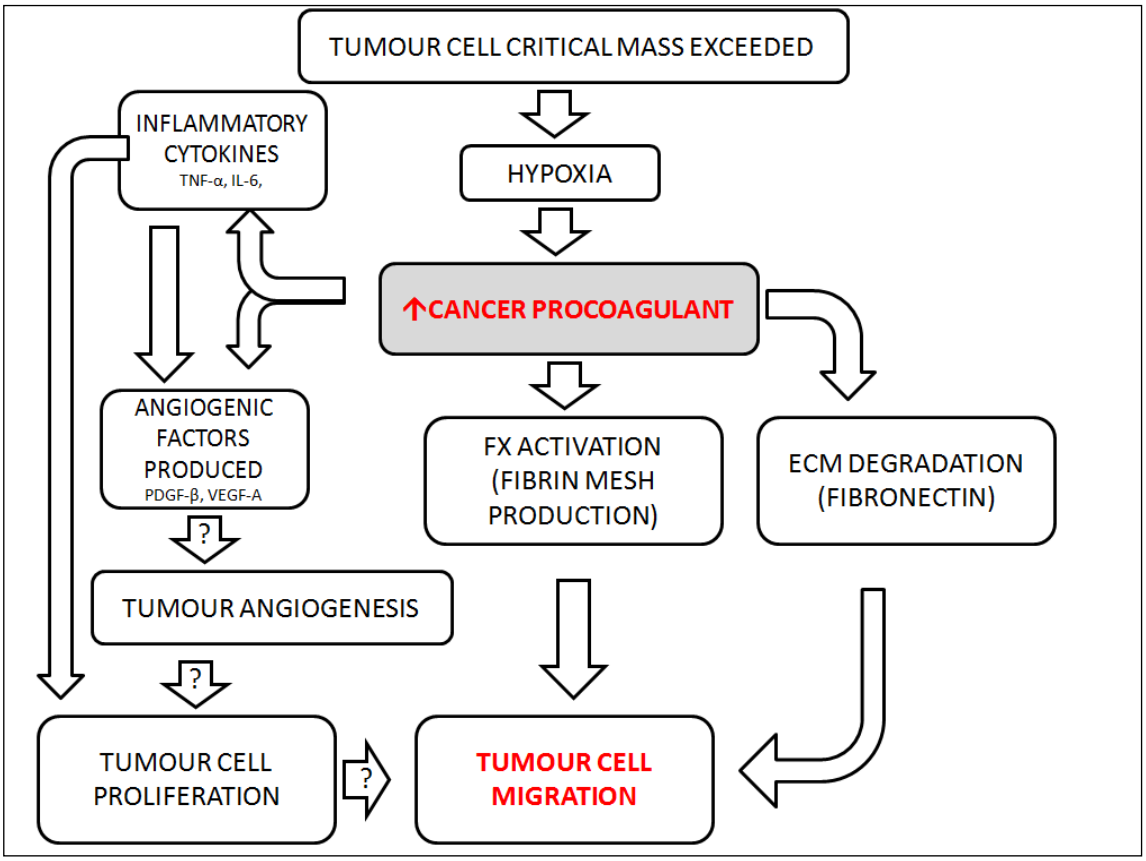


Figure 5.1 A summary of the effects of cancer procoagulant in angiogenesis and metastasis.

It was reported that CP degrades fibronectin (Low Ah Kee, 2011). In this study, substrates were designed for CP according to its cleavage sites on fibronectin. Synthetic substrates were designed by Low Ah Kee (2011) and the author identified Boc-QVR-AMC as the best substrate for CP, based on catalytic efficiency data. However, it was found that collagenase was capable of hydrolysing Boc-QVR-AMC, proving that Boc-QVR-AMC is a better substrate for collagenase than for CP. In an attempt to find a more specific substrate for CP that could not be cleaved by other proteases, we tested additional, novel substrates based on the cleavage site of CP on fibronectin as well as substrates designed by Low Ah Kee (2011) that, despite lower catalytic efficiencies than Boc-QVR-AMC, could potentially be more specific. It was found that AVSQSKP-AMC, which was designed according to the CP cleavage site on fibronectin, as well as PQVR-AMC (previously designed by Low Ah Kee, 2011) were the two best substrates for CP. This conclusion was based, not only on catalytic efficiency, but also on specificity, since none of the enzymes tested in this study, besides CP, could hydrolyse these substrates. Interestingly, it was found that CP hydrolysed a proline in the P1 position, which has not previously been known. It would be interesting to see whether CP can cleave a Pro-Pro bond. Based on the results of this study and those of Low Ah Kee (2011) it is evident that the hydrolysis of shorter substrates by CP with a bulky residue in the P4 position (Boc-QVR-AMC and PQVR-AMC) shows higher catalytic efficiencies than longer length substrates (AVSQSKP-AMC), although the latter does provide improved specificity. This study only begins to unravel the specificity of CP and future studies could improve on the design of the current substrates to improve the catalytic efficiency, whilst maintaining the specificity. Figure 3.22 (Chapter 3) provides a comprehensive summary of the possible substrates that could be designed for CP in future studies and also provides information on it's active site and mechanism of catalysis. Obtaining a better substrate that could also be used for detecting CP in a crude extract (such as serum) would be beneficial as a rapid means of detecting CP and, therefore, cancer.

In conclusion, this study has shown that CP has an effect on both endothelial and tumour cells, particularly affect the migration of these cells, through up-regulation of pro-inflammatory and pro-angiogenic cytokines. Furthermore, two novel substrates, PQVR-AMC and AVSQSKP-AMC were identified which may prove to be useful for a direct assay to determine CP activity as well as an early tumour marker detection kit.

The study portrays a role for CP in metastasis and angiogenesis (Figure 5.1) however; the exact mechanism of action of CP in these processes remains to be determined. The evidence in this study indicates that CP possibly promotes detachment of tumour cells from the primary tumour and also promotes migration of endothelial cells to initiate angiogensis. Further studies could look at the effect of CP on endothelial cell migration in matrigel to see if it would promote tube formation. This study provides a platform for many exciting future studies to unravel the role of this elusive protease in angiogenesis and metastasis.

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Appendix A – Ethical Clearance Letter

• PO Box 77000 • Nelson Mandela Metropolitan University
• Port Elizabeth • 6031 • South Africa • www.nmmu.ac.za

Nelson Mandela Metropolitan University

for tomorrow

Chairperson: Research Ethics Committee (Human)
Tel: +27 (0)41 504-2235

Ref: [H13-SCI-BIO-001/Approval]

RECH Secretariat: Mrs U Spies

23 April 2013

Prof C Frost
NMMU
Building 12
Biochemistry & Microbiology
Summerstrand South Campus

Dear Prof Frost

THE ISOLATION OF CANCER PROCOAGULANT FROM AMNION CHORION MEMBRANE TO INVESTIGATE ITS ROLE IN CANCER, COAGULATION AND ANGIOGENESIS

PRP: Prof C Frost
PI: Prof C Frost

Your above-entitled application for ethics approval served at the Research Ethics Committee (Human).

We take pleasure in informing you that the application was approved by the Committee.

The ethics clearance reference number is **H13-SCI-BIO-001**, and is valid for three years. Please inform the REC-H, via your faculty representative, if any changes (particularly in the methodology) occur during this time. An annual affirmation to the effect that the protocols in use are still those for which approval was granted, will be required from you. You will be reminded timeously of this responsibility, and will receive the necessary documentation well in advance of any deadline.

We wish you well with the project. Please inform your co-investigators of the outcome, and convey our best wishes.

Yours sincerely

CB Cilliers

Prof CB Cilliers
Chairperson: Research Ethics Committee (Human)

cc: Department of Research Capacity Development
Faculty Officer: Science

Appendix B – Consent Form

The isolation of CP from Placental Tissue

Nelson Mandela Metropolitan University
for tomorrow

Principal investigator: Prof C Frost
Nelson Mandela Metropolitan University
Department of Biochemistry and Microbiology
P.O. Box 77000
Port Elizabeth
6031, South Africa
+27 41 504 4123

INFORMATION AND INFORMED CONSENT FORM

A DECLARATION BY PARTICIPANT
I, (name and I.D. number) ……..

INFORMED CONSENT

I have been informed about the project that is being researched. The protein isolated from placental tissue for this project will provide biochemical information of this protease. This study hopes to contribute to a better understanding of this molecule's role in coagulation and would provide information which would contribute to the national and international knowledge base. The donation of this biological material (which otherwise would be incinerated) would have no harmful effects for the donor. All information of donors will remain confidential.

B I HEREBY VOLUNTARILY CONSENT TO PARTICIPATE IN THE ABOVE-MENTIONED PROJECT	
Signature or right thumb print of participant	Signature of witness:……..... Full name of witness……..

C STATEMENT BY INVESTIGATOR	
I have explained the information regarding the research to the participant named above. The participant was encouraged and given enough time to ask me questions. The conversation was conducted in:…… If a translator was used: Name of translator:……..	
Signed/confirmed by all parties at	On (date)
Signature of investigator (or nurse on behalf of investigator) ……..	Signature of witness:……... Same witness as above……..

This project has been approved by the Nelson Mandela Metropolitan University's Ethics Committee. Tissue donated will only be used for this research project.

Faculty of Science
NMMU
Tel: +27 (0)41 504-4123 Fax: +27 (0)41-504-2441
E-mail Faculty Chairperson: carminita.frost@nmmu.ac.za

August 2013

Ref: Donation of tissue samples for research purposes

Contact person: Prof. CL Frost

Dear Participant

You are being asked to participate in a research study. We will provide you with the necessary information to assist you to understand the study and explain what would be expected of you (participant). These guidelines would include the risks, benefits, and your rights as a study subject. Please feel free to ask the researcher to clarify anything that is not clear to you.

To participate, it will be required of you to provide a written consent that will include your signature, date and initials to verify that you understand and agree to the conditions.

You have the right to query concerns regarding the study at any time. Immediately report any new problems during the study, to the researcher. Telephone numbers of the researcher are provided. Please feel free to call these numbers (27 41 504 4123).

Furthermore, it is important that you are aware of the fact that the ethical integrity of the study has been approved by the Research Ethics Committee (Human) of the university. The REC-H consists of a group of independent experts that has the responsibility to ensure that the rights and welfare of participants in research are protected and that studies are conducted in an ethical manner. Studies cannot be conducted without REC-H's approval. Queries with regard to your rights as a research subject can be directed to the Research Ethics Committee (Human), Department of Research Capacity Development, PO Box 77000, Nelson Mandela Metropolitan University, Port Elizabeth, 6031.

If no one could assist you, you may write to: The Chairperson of the Research, Technology and Innovation Committee, PO Box 77000, Nelson Mandela Metropolitan University, Port Elizabeth, 6031.

Participation in research is completely voluntary. You are not obliged to take part in any research. If you choose not to participate in medically related research, your present and/or future medical care will not be affected in any way and you will incur no penalty and/or loss of benefits to which you may otherwise be entitled.

If you do partake, you have the right to withdraw at any given time, during the study without penalty or loss of benefits. However, if you do withdraw from the study, you should return for a final discussion or examination in order to terminate the research in an orderly manner.

If you fail to follow instructions, or if your medical condition changes in such a way that the researcher believes that it is not in your best interest to continue in this study, or for administrative reasons, your participation maybe discontinued. The study may be terminated at any time by the researcher, the sponsor or the Research Ethics Committee (Human).

Although your identity will at all times remain confidential, the results of the research study may be presented at scientific conferences or in specialist publications.

This informed consent statement has been prepared in compliance with current statutory guidelines.

Yours sincerely

Prof. CL Frost
RESEARCHER

Appendix C – Confirmation letter from Greenacres Hospital

Netcare Greenacres Hospital

Tel: +27 (0) 41 390 7000
Fax: +27 (0) 41 390 7089
Cnr Cape and Rochelle Roads, Greenacres, South Africa
PO Box 27488, Greenacres, 6057, South Africa
www.netcare.co.za

LETTER CONFIRMING KNOWLEDGE OF NON-TRIAL RESEARCH TO BE CONDUCTED IN THIS NETCARE FACILITY

Dear Jason Krause

Re: Isolation, purification and partial characterisation of cancer procoagulant from amnion-chorion membranes and its role in metastasis (Title of research)

We hereby confirm knowledge of the above named research application to be made to the Netcare Research Operational Committee and in principle agree to the research application for Netcare Greenacres Hospital/site/division, subject to the following:

1. That the data collection may not commence prior to receipt of FINAL APPROVAL from the Sustainability Committee of Netcare (Research Operational Committee).
2. A copy of the research report will be provided to Netcare Research Operational Committee once it is finally approved by the tertiary institution, or once complete.
3. Netcare has the right to implement any Best Practice recommendations from the research.
4. That the Hospital/Site/Division Management reserves the right to withdraw the approval for research at any time during the process, should the research prove to be detrimental to the subjects / Netcare or should the researcher not comply with the conditions of approval.

We wish you success in your research.

Yours faithfully

22 July 2013.

Signed by Hospital/Site/Division Management **Date**

Nursing Manager

(Specify designation)

Appendix D - SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophores (SDS-PAGE) was used to visualize the homogeneity of the isolated CP fractions and to determine its molecular mass. In electrophoresis, charged molecules are separated in an electric field. SDS, a detergent, disrupts non-covalent protein bonds by binding to the hydrophobic parts of proteins, coating the protein with a negative charge to allow the protein to migrate through the gel based on its molecular mass (Sheehan, 2009).

Samples were electrophoresed on 12% polyacrylamide gels for 1.5 hours under reducing and non-reducing conditions according to the method of Laemmli (1970) A vertical minigel system (Bio-Rad) with an 8 x 7cm cell format was used. The resolving gel (12%) was cast 0.75mm thick from the following mixture: 4.35ml distilled water, 2.5ml 1.5M Tris-HCl (pH 8.8), 100µl 10% (w/v) SDS, 3.25ml acrylamide mixture (30%, w/v, acrylamide, 1%, w/v, bis acrylamide), 5µl TEMED and 50µl 10% ammonium persulfate. The stacking gel mixture was prepared as follows: 3.213ml distilled water, 1.25ml 0.5M Tris-HCl (pH 6.8), 50µl 10% (w/v) SDS, 0.488ml acrylamide mixture, 5µl TEMED and 25µl 10% (w/v) ammonium persulfate. Both stacking and resolving gel solutions were degassed for 15 minutes prior to the addition of TEMED and casting. The sample buffer was prepared as follows: 4ml distilled water, 1ml 0.5M Tris-HCl (pH 6.8), 0.8ml glycerol, 1.6ml 10% (w/v) SDS and 1ml 0.05% (w/v) bromophenol blue. Mercaptoethanol was added to the sample buffer to a final concentration of 5% (v/v) when reducing conditions were employed. The sample was prepared by adding equal volumes of sample buffer and sample to an eppendorf microfuge tube to a final volume of 20µl. Under reducing conditions, the sample buffer contained mercaptoethanol and the sample was boiled with sample buffer for 5 minutes. Under non-reducing conditions, the sample buffer did not contain mercaptoethanol and no boiling was performed. The 5x electrode buffer (pH 8.3) was composed of Tris (15g/l), glycine (72g/l) and SDS (5g/l); 50ml of 10x electrode buffer was diluted with 450ml distilled water for one electrophoretic run. Electrophoresis was started at 100V and 64mA for 15 minutes at room temperature until the dye front migrated through the stacking gel. Electrophoresis was continued for 1 hour at 150V and 64mA until the dye front reached the end of the resolving gel.

The protein standard used was peqGOLD protein marker IV (170, 130, 95, **72**, 55, 43, 34, 26, 17 and **10** kDa). The calibration curve obtained from the preparation of this marker is shown in Figure 2.4. The gel staining solution contained 0.1% (w/v) CBB in 40% (v/v) methanol and 10% (v/v) acetic acid. The staining solution was filtered after the dye was dissolved. The gel was soaked in an excess of staining solution and gently shaken for an hour. It was destained with a large excess of destain solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. The gel was washed at 15 minute intervals with destain solution until a satisfactory clear background was obtained.