Towards the Development of Novel Cancer Therapies:

Selection and Characterisation of Compounds that Inhibit Tumour Cell Proliferation, Angiogenesis and Lymphangiogenesis

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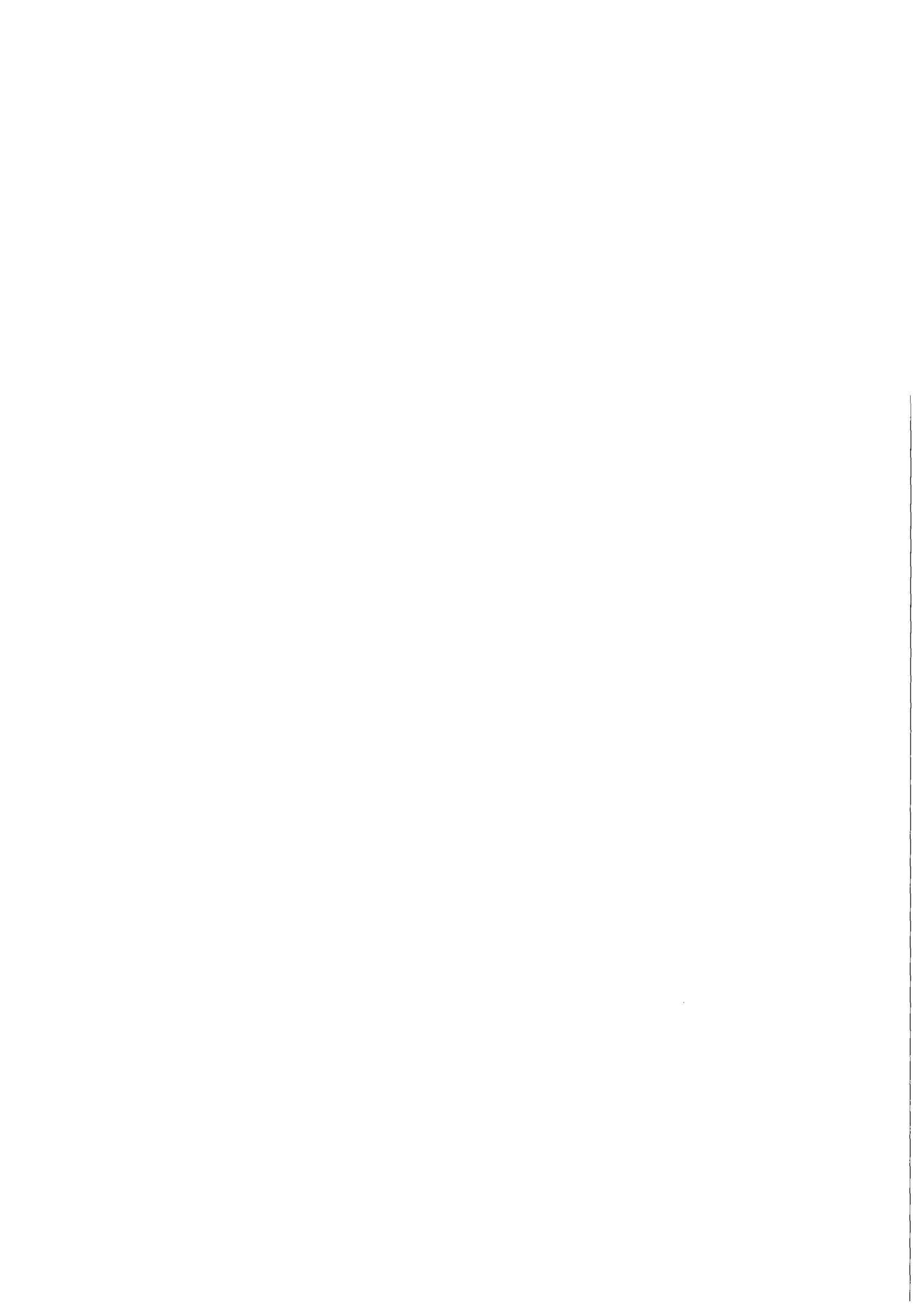
Towards the development of novel cancer therapies:

Selection and characterisation of compounds that inhibit tumour cell proliferation, angiogenesis and lymphangiogenesis

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Zur Entwicklung neuer Krebstherapien:

Selektion und Charakterisierung von Verbindungen, die die Tumorzell-Proliferation, Angiogenese und Lymphangiogenese hemmen.

Zusammenfasung

Aufgrund ihrer geringen Toxizität, des reduzierten Risikos einer Arzneimittelresistenz und der erhöhten Anti-Krebswirkung, besitzt die Anti-Angiogenese-Therapie das Potential zu einem wichtigen Ansatzpunkt in der Krebshandlung zu werden. Die Mitglieder der vaskulären endothelialen Wachstumsfaktoren (VEGF) Familie und ihre Tyrosinkinase-Rezeptoren (RTK) sind die am besten charakterisierten Vermittler der Angiogenese, die zur Zeit als potentielle Ziele in der Anti-Aniogenese-Strategie eingesetzt werden. Die Tumor-induzierte Lymphangiogenese ist ein anderer Prozess, dessen wichtige Rolle bei der Tumorprogression und bei der Metastasierung jetzt offensichtlich wird. Es wird angenommen, daß die Tumor-induzierte-Lymphangiogenese von Mitgliedern der VEGF-Familie, nämlich VEGF-C und VEGF-D, welche Signaltransduktion durch die RTK VEGFR-3 iniziieren, reguliert wird.

Das Ziel dieser Arbeit war es, neue Anti-Krebsagentien zu entwickeln, die das Tumorwachstum blockieren indem sie direkt in die Tumorzellproliferation, die Angiogenese und/oder Lymphangiogenese eingreifen, und so Tumorwachstum und Metastasierung verhindern. Zu diesem Zweck wurde ein *in vitro* Assay etabliert, der es ermöglicht, kleine synthetische Verbindungen, genannt Indolinone, hinsichtlich ihrer Fähigkeit mit der normalen Funktion verschiedener RTKs inklusive der VEGFRs, zu interferieren. Um das inhibitorische Potential der wirksamsten Indolinone bezüglich VEGFR zu bestimmen, wurden sie in einem zellulären Phosphorylierungstest unter Verwendung von Ratten-VEGF-Liganden zu Stimulierung von VEGFRs getestet. Die rekombinanten VEGF Proteine wurden in einem Drosophila-Expressionssystem hergestellt, über eine Nickel-säule aufgereinigt und ihre biologische Aktivität gezeigt. Sie wurden auch in einen Versuch zur Etablierung von lymphatischen Endothel in Kultur eingesetzt.

Durch den Screen wurden potentielle Inhibitoren von VEGFRs, namentlich AE87, AE106, MAZ51 und MAZ51-2 identifiziert. Alle 4 Indolinone zeigen starke antiangiogene Eigenschaften, die durch ihre Fähigkeit, die Endothelzell-Proliferation und deren Sprassung zu inhibieren, charakterisiert wurden. Weiterhin zeigten AE87, AE106, MAZ51 und MAZ51-2 die neue Fähigkeit, VEGFR-3 Phosphorylierung zu inhibieren. Da die selbe Inhibition nicht im VEGFR-2 zellulären Phosphorylierungstest beobachtet wurde, werden diese Inhibitoren nützlich sein für die Unterscheidung des Effekts von VEGF-C und VEGF-D, auf VEGFR-2 und VEGFR-3. MAZ51 und MAZ51-2 stellten sich als die wirksamsten untersuchten Indolinone heraus. MAZ51 z. B. induziert die Apoptose in Endothel- und Tumorzellen. Von besonderer Bedeutung ist die Unterdrückung des Tumorwachstums in Ratten, wenn MAZ51 intraperitoneal injiziert wird.

Eine einführende Charakterisierung der Antitumoreigenschaft des Pflanzenderivats-Acylphloroglucinol-Typs Hyperforin wurde ebenso durchgefürt. Hyperforin ist fähig, das Tumorwachstum zu reduzieren. Zum einen direkt durch die Induktion der Apoptose der Tumorzellen und zum anderen indirekt durch die Inhibierung der Proliferation der Endothelzellen.

Die Substanzen, die durch diese Arbeit identifiziert wurden, könnten von pharmazeutischem Wert sein und unterstützen die Entwicklung von besseren Krebstherapien. Die zukünftige Arbeit sollte sich darauf konzentrieren, die präzisen Reaktionmechanismen der Indolinone und von Hyperforin aufzuklären und mögliche Nebeneffekte der Substanzen zu identifizieren. Kleinere Modifikationen der Substanzstrukturen sind wahrscheinlich von Nöten, um ihre Löslichkeit und Spezifität zu steigern.

Abstract

Anti-angiogenic therapy, due to its low toxicity, reduced risk of drug resistance and increased anti-cancer efficacy, has the potential to become a major approach in treating cancer. Members of vascular endothelial growth factor (VEGF) family and their tyrosine kinase receptors (RTKs) are the best-characterised mediators of angiogenesis, which are being currently used as potential targets in the anti-angiogenic strategy. Tumour-induced lymphangiogenesis is another process that is now being recognised to play an important role in tumour progression and metastasis. It too is thought to be regulated by members of the VEGF family, namely VEGF-C and VEGF-D, which signal through the RTK VEGFR-3.

The goal of this work was to develop novel anti-cancer agents that would block tumour growth by targeting tumour cell proliferation directly, angiogenesis and/or lymphangiogenesis, thereby preventing its growth and metastasis. To this end, an *in vitro* assay was established which permitted screening of small synthetic compounds called indolinones for their ability to interfere with normal functioning of different RTKs, including the VEGFRs. To further assess the VEGFR inhibitory potential of the most potent indolinones, they were tested in a cellular phosphorylation assay using rat VEGF ligands to stimulate VEGFRs. The recombinant VEGF proteins were produced in a *Drosophila* expression system, purified via a histidine tag and shown to be biologically active. They were also used in an attempt to establish lymphatic endothelium in culture.

The screen identified four potential inhibitors of VEGFRs, named AE87, AE106, MAZ51 and MAZ51-2. All the four indolinones showed potent anti-angiogenic properties that were characterised by the ability to inhibit endothelial cell proliferation and sprouting *in vitro*. Furthermore, AE87, AE106, MAZ51 and MAZ51-2 showed the novel ability to inhibit VEGFR-3 phosphorylation. Since the same inhibition was not observed in VEGFR-2 cellular phosphorylation assays, these inhibitors will be useful for dissecting the effects of VEGF-C and VEGF-D exerted via VEGFR-2. MAZ51 and MAZ51-2 proved to be the most potent of the screened indolinones. MAZ51, for instance, induced apoptosis in endothelial and tumour cells. Importantly, when injected intraperitoneally, it could suppress tumour growth in rats.

Initial characterisation of the anti-tumour properties of the plant-derived acylphloroglucinol-type compound hyperforin was also performed. It was found to be able to reduce tumour growth directly by inducing tumour cells to apoptose and indirectly via inhibition of endothelial cell proliferation.

The substances identified in the course of this work may be of pharmaceutical value and should assist development of better anti-cancer protocols. Future work should concentrate on the elucidation of the precise mechanism of action of the indolinones and hyperforin and identification of possible adverse effects of the substances. Minor modifications in the substance structure may be necessary to improve their solubility and specificity.

Acknowledgements

Table of contents

List of figures

List of tables

Abbreviations

A	Adenosine
aa	aminoacids
aFGF	acidic fibroblast growth factor
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp(s)	base pair(s)
BSA	Bovine serum albumin
C	Cytidine
°C	Degrees celsius
cDNA	Complementary DNA
Ci	Curie
cm	centimetre
CTP	Cytidine triphosphate
ddNTP	Di-deoxynucleotide triphosphate
DES	*Drosophila* expression system
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-N,N-tetracetate
e.g.	example given
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunoabsorbant assay
et al.	and others (Lat. *et ali*)
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
bFGF	Basic fibroblast growth factor

FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Flk	Fetal liver kinase
Flt	fms-like kinase
g	gram
G	Guanosine
GF	Growth factor
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
^{3}**H**	Tritium
HBS	HEPES buffered saline
HDMEC	Human dermal microvascular endothelial cells
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hr(s)	hour(s)
HRP	Horseradish peroxidase
HSPGs	Heparan sulphate proteoglycans
HUVEC	Human umbilical vascular endothelial cells
hVEGF	Human VEGF
IC$_{50}$	Inhibitory concentration 50%
Ig	Immunoglobulin
IGFR	Insulin-like growth factor receptor
IHC	Immunohistochemistry
IP	Immunoprecipitation
i.p.	Intraperitoneal
kDa	Kilodalton (10^{3} daltons)
KDR	Kinase insert domain receptor
l	litre
M	Molar
MHC	Major histocompatibility complex
m	milli- (10^{-3})
mA	milliamper
mab	Monoclonal antibody
mg	milligram

min	minute (s)
ml	millilitre
mM	millimolar
mRNA	Messenger RNA
μ	micro- (10^{-6})
μCi	microcurie
μg	microgram
μl	microlitre
μM	micromolar
μm	micrometer
n	nano- (10^{-9})
ng	nanogram
Ni-NTA	Nickel nitrilotriacetic acid
nm	nanometer
OD	Optical density
o/n	overnight
p	pico- (10^{-12})
PAE	Porcine aortic endothelial (cells)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PECAM	Platelet-endothelial cell adhesion molecule-1
PKA	Protein kinase A
PKB	Protein kinase B
pmol	picomols
PVDF	Polyvinylidenedifluoride
PY	Phosphotyrosine
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse transcription PCR
RTK	Receptor tyrosine kinase

s.c.	subcutaneous
SDS	Sodium-lauryl-sulfate
SE	Standard error
sec	second(s)
SF/HGF	Scatter factor/Hepatocyte growth factor
T	Thymidine
TBE	Tris-borate-EDTA
TEMED	N, N, N', N' tetramethylene-diamine
Tie	Tirosine kinase with immunoglobulin and epidermal growth factor homology domains
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Tris	Tris-(hydroxymethyl)-aminomethane
TTP	Thymidine triphosphate
U	Unit(s)
V	Volt
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHD	VEGF-homology domain
v/v	Volume per volume
W	Watt
w/v	Weight per volume

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Chapter 1

Introduction

1.1 Cancer

"Cancer". Who of us would not shudder at hearing such a diagnosis? Indeed, this disease, accounting for one of five fatalities in the developed countries, in many cases still remains largely incurable. Obviously enough, conquering cancer has long been a major challenge for researchers throughout the world. Cancer research, however, is not only of clinical significance. It also promises to cast light on those fundamental mechanisms that determine behaviour of the cells in a multicellular organism.

1.1.1 Tumour generation

The term "cancer" refers not to a single illness but rather describes a family of diseases having common key features. These include uncontrolled cell reproduction (*i.e.* tumour formation), loss of differentiation, aneuploidy, and resistance to apoptosis. The process of tumorigenesis is thought to be initiated by distinct genetic alterations which can be either inherited or acquired upon exposure of DNA to different mutagenic factors (Kinzler and Vogelstein, 1998). Such a genetic insult typically results in the mutation, misexpression, or deletion of regulatory genes that play essential roles in key cellular processes such as proliferation, differentiation, cell cycle progression and apoptosis (Yokota, 2000). With respect to tumorigenesis, such affected genes are usually divided into two groups, referred to as proto-oncogenes (which usually exert their effect via gain of function) or tumour-suppressor genes (whose role is typically associated with loss of function) (Weinberg, 1995). The activity of oncogenes and impaired function of tumour suppressor proteins leads to uncontrolled cell proliferation and escape from apoptotic signals, causing expansion of the transformed cell population (*e.g.* Bishop, 1991). Such tumours may remain benign until further genetic alterations take place. These secondary insults to key genes are thought to happen in steps and further contribute to the malignant transformation of neoplastic cells (Foulds, 1975; Fearon and Vogelstein, 1990).

1.1.2 Apoptosis and tumorigenesis

As can be appreciated from the preceding section, DNA damage is a driving force behind the development of tumours. Cells possess many properties that allow them to detect damaged DNA and respond appropriately. One response to DNA damage is apoptosis. Thus, in order to survive with damaged DNA, tumour cells need to suppress apoptosis inducing signals (Sigal and Rotter, 2000). Apoptosis, also referred

to as programmed cell death, is the most common form of eukaryotic cell death. In contrast to necrosis, it is a regulated physiological cell suicide mechanism that maintains tissue homeostasis (Wyllie, 1997; Hengartner, 2000). The activation of the apoptosis programme occurs by the two major pathways: a) the 'extrinsic' cell death pathway initiated by external factors through the specialised TNF-family death receptors such as the TNF-R1, CD95(Fas) and TRAIL (Ashkenazi and Dixit, 1998) and b) the 'intrinsic' cell death pathway activated by the release of pro-apoptotic factors from mitochondria which include cytochrome c and Apaf-1 (Green and Reed, 1998). The gross effect of either pathway is characterised by the activation of specific proteases called caspases and endogenous endonucleases, resulting in the DNA fragmentation, nuclear and cytoplasm condensation, membrane blebbing referred to as zeiosis and disintegration of the cell into apoptotic bodies (Hengartner, 2000). An important feature of apoptosis is the lack of the necrosis-associated inflammation as the apoptotic bodies are efficiently fagocytised by other cells (Hengartner, 2000).

In order to grow progressively, tumour cells have to circumvent apoptosis, which is one of the cellular responces to DNA damage (Pucci et al., 2000). Consequently, mutations in proteins such as p53 which regulate the expression of genes involved in apoptosis have been identified in the majority of cancers (Sigal and Rotter, 2000). It is therefore thought that the ability to restore the normal apoptosis mechanisms in tumour cells might potentially lead to the elimination of the cancer (Sigal and Rotter, 2000).

1.1.3 Tumour progression and metastasis

It is the ability of cancer to metastasise that makes it so hard to eliminate, as some tumours, for example melanoma, start to metastasise while measuring only several millimetres in diameter and thereby are very difficult to detect before they have begun to disseminate. The onset of metastasis is associated with poor prognosis since many vital organs become targeted and subsequently destroyed by the disease.

In order to become metastatically competent, a tumour cell must acquire several properties (reviewed in Sleeman, 2000). Tumour cells metastasise via three main routes: by direct extension, through the blood circulation and through the lymphatic system. Therefore, the major three properties that are thought to be necessary for a tumour cell to leave the primary tumour and enter a cavity or a capillary are adhesiveness, ability to produce and/or activate proteases, and motility. Thus, changes

in adhesive properties permit detachment of metastatic cells from neighbouring cells and establishment of new contacts with the extracellular matrix (ECM) and host cells they encounter on their way of invasion. The production and activation of proteases are required to degrade the ECM and thereby allow the metastatic cells to invade surrounding tissues. Finally, tumour cells need to become actively motile in order to migrate away and enter vessels of the circulatory system. Further metastatic dissemination occurs in steps which include intravasation (enter into a lymphatic or blood vessel), transport in the blood or lymphatic stream, extravasation (exit from the blood stream into the surrounding tissues) and formation of secondary tumours (**Fig. 1.1**). Therefore, during their voyage tumour cells need further cellular properties if they are to survive and reach their target organ(s) (Sleeman, 2000).

How do tumour cells acquire the properties they require if they are to successfully metastasise? During tumour progression from a benign lesion to malignancy, mutations in the regulatory genes such as p53 (Hollstein *et al.*, 1991) can lead to disruption of those control mechanisms that ensure the fidelity of DNA replication and repair. The lack of apoptosis, which is normally triggered when DNA is seriously damaged, ensures that the tumour cell will continue its passage through the cell cycle and undergo mitosis. This leads to genetic instability of tumour cells, which is presumably reflected in loss of differentiation, high mitotic index, aneuploidy and gross chromosomal aberrations often associated with malignancies (Sleeman, 2000). Together with the concomitant increase in the mutation rate, the genomic abnormalities ensure that the population of the tumour becomes largely heterogeneous (Fidler, 1978). Thus, tumours then contain a range of tumour cells with different assortments of molecular and cellular properties. It is thought that only those tumour cells possessing the properties necessary to overcome the selective pressures they encounter as they try to metastasise will be able to successfully form secondary tumours.

1.1.4 Tumour-host interactions

In both primary tumours and their metastases, cancer cells constantly interact with each other as well as with host cells. Thus, an important prerequisite for tumour growth and metastasis formation is the development of stroma, which is composed of fibroblasts, blood vessels, immune cells and components of the ECM (Dvorak, 1986; Wernert, 1997). Stromal fibroblasts play an important role in tumour invasion as they

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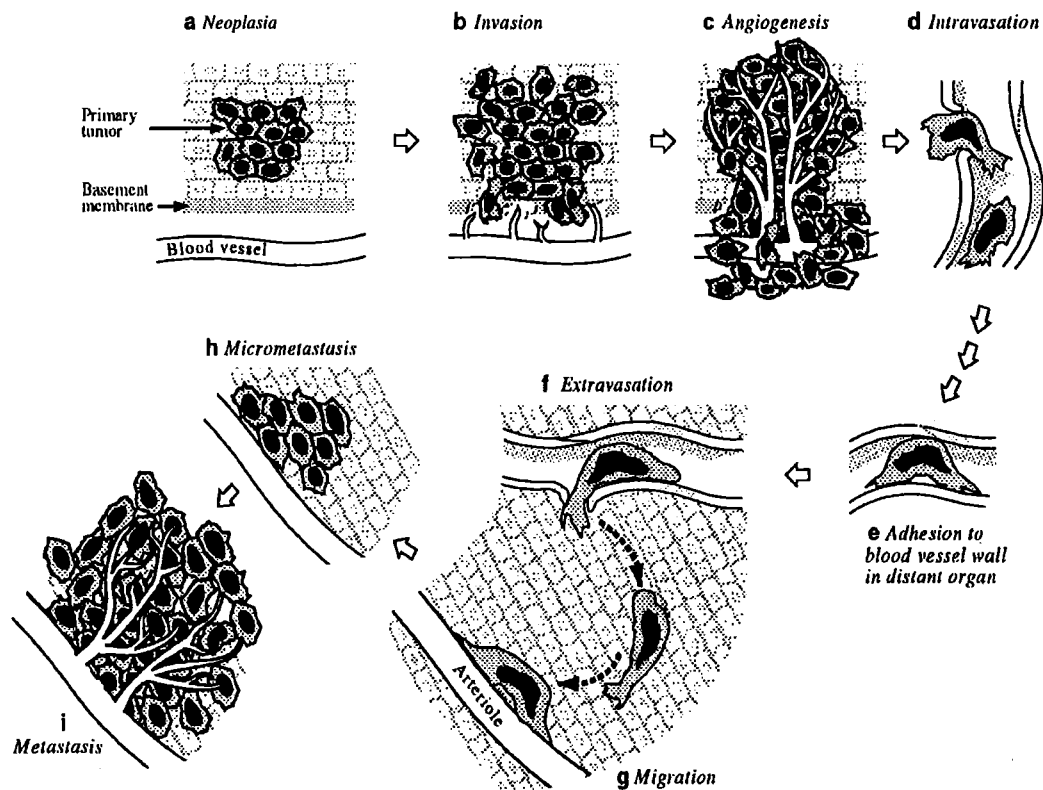
Figure 1.1 *Overview of tumour progression and metastasis.* **a)** As long as neoplastic cells are clustered together, tumour remains benign; **b)** tumour turns into malignant state when it acquires an invasive phenotype (*i.e.* ECM degradation and migration); **c)** tumour induces angiogenesis to secure its own blood supply; **d)** capillaries are entry points for metastatic cells; **e)** to exit circulation metastatic cells have to attach specifically to vascular endothelial cells and **f)** extravasate through the vessel wall; **g)** to grow successfully, metastatic cells migrate to sites proximal to arterioles; **h)** micrometastasis can remain dormant for prolonged time during which angiogenesis is subdued; **i)** secondary tumour site angiogenesis releases the metastatic colony from the dormancy and allows its rapid growth. *For simplicity, only hematogenous spread of metastatic cells is shown (adopted from Zetter, 1998).*

participate in the degradation of the ECM by secreting matrix degrading proteases as well as their downstream-activators. Moreover, factors derived from the stromal cells and ECM such as scatter factor/hepatocyte growth factor (SF/HGF) as well as interactions between neoplastic cells and the ECM can play a role in both tumour cell migration and proliferation. Another important aspect of tumour host interactions is reflected in the evasion of tumour cells from the surveillance of the immune system. To escape from the immunological destruction, tumours need to further change their properties. These include downregulating or shedding tumour-associated antigens, decreasing or losing expression of MHC antigens (Pawelec *et al.*, 1997), and expression of molecules inducing apoptosis in anti-tumour T cells (Walker *et al.*, 1997).

1.1.5 Tumour angiogenesis and lymphangiogenesis

Perhaps the most important tumour-host interaction is the induction of angiogenesis. As illustrated by **Figure 1.1**, for a solid tumour to grow successfully, it also must secure its own blood system to supply tumour cells with nutrition as well as to relieve the growing mass from hypoxia (Folkman *et al.*, 1963; Folkman, 1972). This process, referred to as tumour-induced angiogenesis, is mediated via soluble growth factors either secreted by tumour cells or derived from the host tissue. The established capillary network also permits tumour cells endowed with an "invasive" phenotype to enter capillary beds and subsequently be transported in the circulation.

Since many human tumours metastasise at least initially via the lymphatic system the induction of new lymphatics is thought to be an important step in tumour progression, too (Sleeman, 2000). Lymphangiogenesis may be invoked to help tumour cope with rising interstitial pressure (Jain, 1987). Recent studies demonstrate that the tumour-induced lymphangiogenesis can promote metastasis formation (Mandriota *et al.*, 2000; Skobe *et al.*, 2001; Stacker *et al.*, 2001).

1.1.6 Approaches to anti-cancer therapy

Better understanding of the processes occurring in the tumour cell may make it possible to design various therapies aimed at altering or inactivating key tumour cell-specific genes, which would cause tumour cells to apoptose or revert from their malignant phenotype. Another potential line of anti-cancer research is to inhibit tumour-host interactions, which would indirectly interfere with tumour growth. For

instance, anti-angiogenesis therapies are being developed to deprive the tumour of the blood supply, causing its regression and, most optimistically, complete ablation (Kerbel, 2000). Furthermore, since the vast majority of human carcinomas tend to metastasise via the lymphatics (Beitz and Calabresi, 1993), the development of anti-lymphangiogenesis approaches might block metastatic spread via the lymphatics.

In this thesis I have characterised and investigated the potential anti-tumour effects of a number of chemical compounds that may act directly on tumour cells and/or inhibit important tumour-host interactions. Since the work in this thesis is partly concerned with the development of anti-angiogenic and anti-lymphangiogenic therapies, the following sections will review the existing literature on the processes of tumour-induced angiogenesis and lymphangiogenesis. The current state of the clinical utility of the knowledge on tumour-induced angiogenesis will also be addressed here.

1.2 Angiogenesis

1.2.1 Vasculogenesis vs. angiogenesis

The circulatory system appeared early in the evolution of multicellular organisms. Gradually it evolved into a complex and highly specialised organ system playing a central role in the maintenance of organism homeostasis. Thus in vertebrates, the circulatory system is not restricted to transporting oxygen, nutrients, carbon dioxide and metabolic wastes but also serves as a means of communication between tissues and organs ensuring an adequate response of the organism to the environment.

The circulatory system is one of the earliest organ systems to develop and become functional during embryogenesis. Embryonic vasculature develops in two successive steps (**Fig. 1.2**). In the first step, termed vasculogenesis, multipotent mesodermally-derived precursors called hemangioblasts differentiate *in situ* to become endothelial cells (Wanger, 1980; Risau, 1997) that later organise into a primary capillary plexus (Risau and Flamme, 1995). During the second step, called angiogenesis, the sprouting and remodelling of the primary plexus as well as the recruitment of periendothelial cells (such as smooth muscle cells and pericytes) occurs to create a mature arteriovenous vascular system (Risau, 1997; Folkman, 1995a).
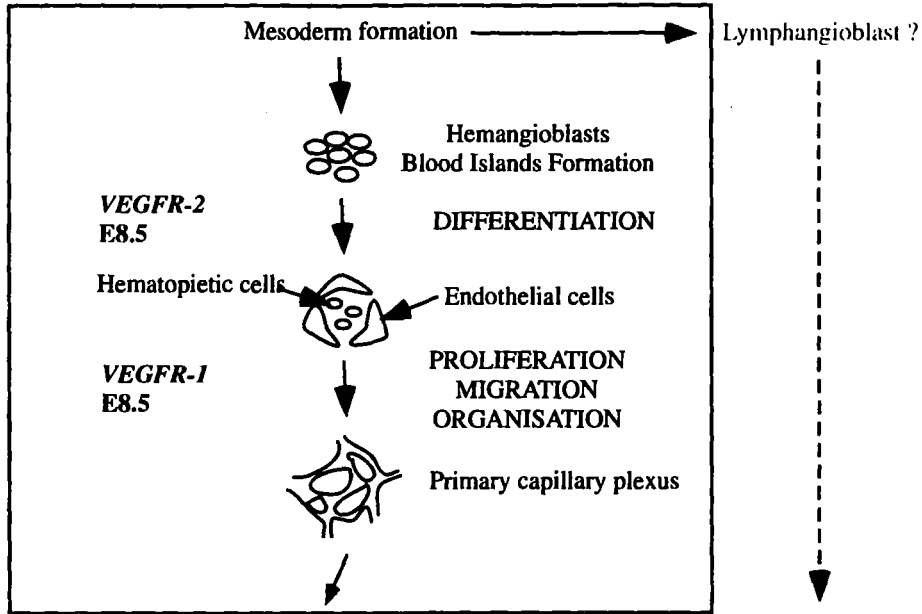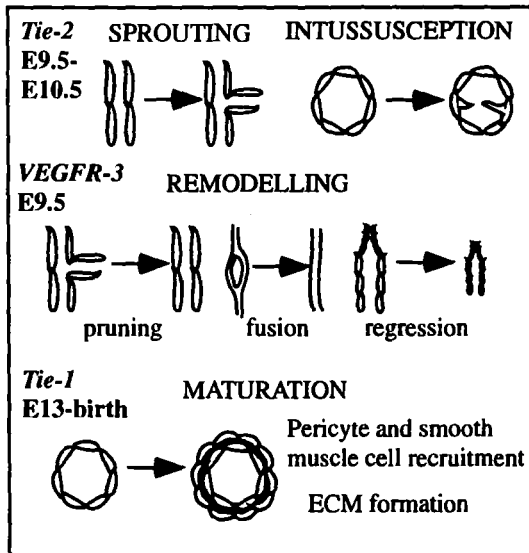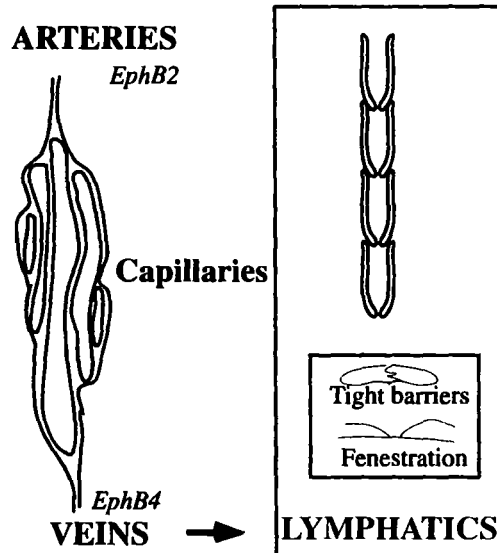
Figure 1.2 *Schematic illustration of the relationship among the processes of vasculogenesis, angiogenesis and lymphangiogenesis.* The stages at which inactivation of the indicated endothelial specific receptor tyrosine kinases is lethal on the basis of gene targeting studies are marked on the left *(adopted from Saaristo et al., 2000).*

1.2.2 Molecular mechanisms of angiogenesis regulation

Whereas vasculogenesis is restricted to early stages of embryogenesis, angiogenesis can be found in both embryonic development and postnatal life. Adult angiogenesis is, however, kept to a minimum. It is triggered during physiological processes such as wound healing and the female reproductive cycle, but is turned off after these processes are completed (Folkman, 1995). It therefore appears that physiological angiogenesis is constantly being tuned via a dynamic balance of positive and negative factors that operate within the vascular microenvironment (Pepper, 1997; Iruela-Arispe and Dvorak, 1997). In certain pathological conditions, however, this fine balance is disturbed leading to excessive proliferation of endothelial cells. Such abnormal angiogenesis is a characteristic feature of major diseases such as diabetic retinopathy, arthritis, psoriasis, ischimia, atherosclerotic plaques, and cancer (Garner, 1994; Folkman, 1995).

1.2.2.1 Positive regulators of angiogenesis

The development of various *in vitro* and *in vivo* angiogenesis assays as well as genetic manipulation of the mouse genome has allowed a rather large panel of positive regulators of angiogenesis to be identified during the past several years (**Table 1.1**). Typically, angiogenic factors secreted in a paracrine or autocrine fashion bind to and activate their corresponding receptors expressed on the surface of endothelial cells. Thereby activated endothelial cells become capable of proliferation, migration, and differentiation resulting in the formation of new capillaries (Klagsbrun and D'Amore, 1996). Besides direct endothelial cell stimulation, however, angiogenesis can be promoted by activation of other cell types such as smooth muscle cells (SMC) and pericytes that further contribute to vessel formation and maturation (Rissau, 1997). In the following section I will describe some of the best-characterised regulators of angiogenesis.

a) Vascular endothelial growth factor (VEGF) family and VEGFR receptors

VEGF family members (**Fig. 1.3**) are important regulators of angiogenesis and lymphangiogenesis. The VEGF family consists of six known members: VEGF, PlGF, VEGF-B, VEGF-C, VEGF-D and VEGF-E (orf virus VEGF; Ogawa *et al.*, 1998). All of them share a common feature, namely the presence of regularly spaced eight cysteine residues, which constitute the so-called cysteine knot motif. Their receptors,

called VEGFRs, belong to the PDGF receptor class, within which they form a distinct receptor subfamily characterised by seven Ig-homology domains, a transmembrane sequence and an intracellular portion containing a split kinase domain (Shibuya *et al.*, 1990).

Vascular endothelial growth factor (VEGF, or VEGF-A), previously known as vascular permeability factor (VPF) and vasculotropin (VAS), is by far the best-characterised endothelial-specific mitogen, playing a pivotal role in the positive regulation of angiogenesis (Dvorak *et al.*, 1995; Klagsbrun and D'Amore, 1996). VEGF is a 34-45 kDa cysteine-linked homodimeric glycoprotein represented by five isoforms derived by alternative exon splicing of a single VEGF gene: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (Park *et al.*, 1993; Poltorak *et al.*, 1997). The five isomorphic proteins vary in their heparin-binding ability resulting in the different extracellular matrix (ECM) immobilisation, and hence bioavailability, of the VEGF molecules. Thus $VEGF_{121}$ is a freely diffusible protein. $VEGF_{145}$ and $VEGF_{165}$, the major isoform, are partially bound to the cell surface and the ECM. $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM. All the forms of VEGF are mitogenic towards vascular endothelial cells in a paracrine manner both *in vitro* (*e.g.* Pepper *et al.*, 1992; Nicosia *et al.*, 1994) and *in vivo* (*e.g.* Leung *et al.*, 1989; Tolentino *et al.*, 1996). VEGF is also known to induce vascular permeability of blood vessels (Dvorak *et al.*, 1995) and expression of different proteases by vascular endothelial cells (Pepper *et al.*, 1991; Unemori *et al.*, 1992). The latter two VEGF-mediated processes ultimately lead to profound alterations in the ECM that facilitate endothelial cell migration and therefore promote angiogenesis.

VEGF exerts its angiogenic effect in a dose-dependent fashion as was demonstrated by heterozygous mutations inactivating the VEGF gene (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996), where the lack of only a single VEGF allele resulted in embryonic lethality in day 11.5-12.5 mice due to abnormal angiogenesis and hematopoiesis. Conversely, overexpression of VEGF in developing avian embryos led to the formation of a hyperfused vascular network and vascularisation of normally avascular embryonic areas (Drake and Little, 1995). It seems, therefore, that the maintenance of appropriate VEGF levels is of vital importance for both the embryo and adult. Thus, diverse molecular mechanisms are responsible for the regulation of VEGF expression (Breier *et al.*, 1997). VEGF expression is triggered by different

pathophysiological factors ranging from hypoxia (Shweiki *et al.*, 1992; Forsythe *et al.*, 1996), hypoglycaemia (Klagsbrun, 1999) and certain cytokines such as EGF and TGF-β (Frank *et al.*, 1995) to oncogenes such as *ras* (Rak *et al.*, 1995) and mutated tumour-suppressor genes such as p53 (Kieser *et al.*, 1994) and von Hippel-Landau gene (Wizigmann-Voos *et al.*, 1995).

Ligand	Receptor(s)
VEGF family	
VEGF	VEGFR-1, VEGFR-2
PIGF	VEGFR-1
VEGF-B	VEGFR-1
VEGF-C	VEGFR-2, VEGFR-3
VEGF-D	VEGFR-2, VEGFR-3
FGF family	
FGF-1	FGFR-1, FGFR-2, FGFR-3, FGFR-4 and HSPGs
FGF-2	FGFR-1, FGFR-2 and HSPGs
Angiopoietins	
Ang1	Tie-2/Tek
Ang2	Tie-2/Tek
?	Tie-1
PDGF family	
PDGF-AA	PDGFR-αα
PDGF-BB	PDGFR-αα, PDGFR-αβ, PDGFR-ββ
PDGF-AB	PDGFR-αα, PDGFR-αβ
TGF-β1	TGFR I, TGFR II, endoglin
TNF-α	p55, p75
SF/HGF	c-met receptor
EGF	EGFR

Table 1.1 *Positively acting angiogenic factors (adopted from Ahrendt et al., 1998).*

VEGF activates endothelial cells by binding to its cognate VEGF receptors (VEGFRs). They are subclass-III receptor tyrosine kinases (RTKs), homologous to the PDGF-receptor family. Two high-affinity VEGF RTKs, almost exclusively expressed on the cells of endothelial lineage (Hanahan, 1997), have been described so far: VEGFR-1 (also denoted fms-like kinase, Flt-1) (de Vries *et al.*, 1992) and VEGFR-2 (also called KDR (human) and Flk-1, foetal liver kinase-1 (murine)) (Terman *et al.*, 1992). Both VEGFR-1 and VEGFR-2 have seven Ig-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine
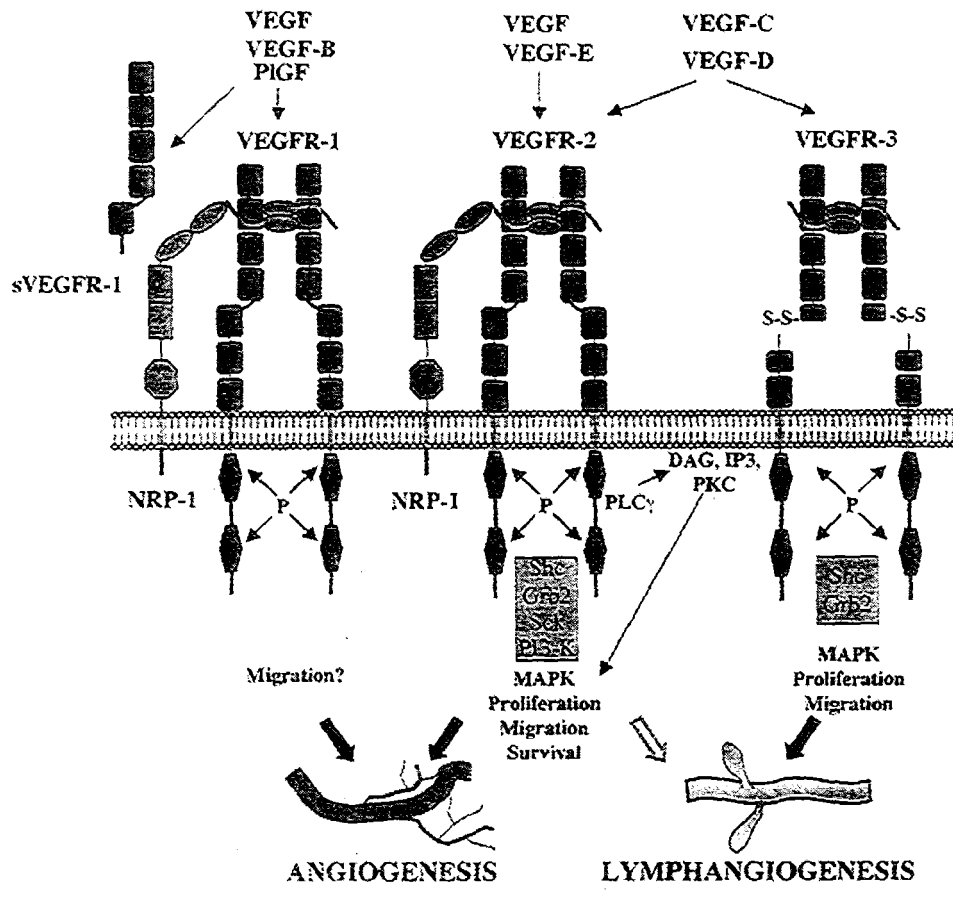
Figure 1.3 *Schematic representation of VEGF receptor family and their ligands.* VEGFs are showen in red, VEGFR-1, VEGFR-2, and VEGFR-3 in blue, and neuropilin in green. The different structural elements of the receptors are illustrated as follows: blue lozenge, Ig domain; blue hexagon, tyrosine kinase domain; green oval, CUB domain; green rectangle, domain homologous to coagulation factors V and VIII; green octagon, MAM domain *(taken from Petrova et al., 1999).*

kinase sequence interrupted by a kinase-insert domain (Shibuya *et al.,* 1990; Matthews *et al.,* 1991; Terman *et al.,* 1991). This structural similarity defines the subfamily of vascular endothelial growth factor receptors that contains an additional member called VEGFR-3 (Flt-4), which, however, is not a receptor for VEGF (Mustonen and Alitalo, 1995). In fact, another VEGF-binding receptor, neuropilin-1 (NP-1), which is a cell surface glycoprotein that binds collapsins/semaphorins and mediates axonal guidance during embryonic development and does not belong to the VEGFR family, was described more recently (Soker *et al.,* 1998). NP-1 appears to modulate VEGF binding to VEGFR-1 and VEGFR-2 acting as an isoform-specific $VEGF_{165}$ receptor (Soker *et al.,* 1998).

Gene knockout studies in mice have clearly shown the importance of both VEGFR-1 and VEGFR-2 for vasculogenesis. However, temporal signalling through these two receptors seems to be quite different. Thus, VEGFR-2 knockout mice die *in utero* between embryonic day 8.5 and 9.5, displaying defects in both angioblastic and hematopoietic lineages (Shalaby *et al.,* 1995). On the other hand, in VEGFR-1-null mice, which also die between E8.5 and 9.5, it is still possible to detect migrating and proliferating angioblasts, although they fail to assemble into functional vessels (Fong *et al.,* 1995). Interestingly, although VEGFR-1 binds VEGF with a 10-fold higher affinity than VEGFR-2 (Terman *et al.,* 1992; de Vries *et al.,* 1992), it does not seem to transduce the VEGF signal efficiently (Seetharam *et al.,* 1995; Kroll and Waltenberger, 1997, Gerber *et al.,* 1998). This suggests that VEGFR-1 may serve as a negative regulator of angiogenesis by sequestering VEGF. Consistent with this hypothesis, and in contrast to the aforementioned VEGFR-1-null mice, transgenic mice expressing a truncated form of VEGFR-1, which lacks the tyrosine kinase domain, develop normally (Hiratsuka *et al.,* 1998).

Placenta growth factor, another member of the VEGF family abundantly expressed in placenta, has two isoforms ($PlGF_{131}$ and $PlGF_{152}$), which differ only by the insertion of a heparin-binding 21-aa stretch resulting in different ECM binding properties (Maglione *et al.,* 1993; Hauser and Weich, 1993). Unlike the VEGF homodimer, which can bind to both VEGFR-1 and VEGFR-2, the PlGF homodimer binds only to VEGFR-1 (Cao *et al.,* 1996). PlGF can form, however, heterodimers with VEGF thereby acquiring VEGFR-2-binding properties (Cao *et al.,* 1996).

Vascular endothelial growth factor-B (VEGF-B), also known as VEGF-related factor (VRF, Grimmond *et al.*, 1996), like PlGF, is a selective ligand for VEGFR-1. Its isoforms (VEGF-B$_{167}$ and VEGF-B$_{186}$) are produced as disulphide-linked homodimers with high affinity for VEGFR-1 (Olofsson *et al.*, 1996; Olofsson *et al.*, 1996a). Although experimental data suggest it has a mitogenic activity towards endothelial cells *in vitro* (Olofsson *et al.*, 1996), its exact *in vivo* function remains obscure.

Vascular endothelial growth factor-C (Joukov *et al.*, 1996) (also known as VEGF-related protein, VRP; Lee *et al.*, 1996), and *vascular endothelial growth factor-D* (originally called *Fos*-induced growth factor, FIGF; Orlandini *et al.*, 1996) are two VEGF-related proteins that appear to play a role in both angiogenesis and lymphangiogenesis. Their signalling specificity is regulated proteolytically. Unprocessed VEGF-C and VEGF-D are initially able to bind only their lymphangiogenesis-specific receptor VEGFR-3 (Joukov *et al.*, 1997; Stacker *et al.*, 1999). After proteolytic maturation they acquire the ability to bind to and activate VEGFR-2, which can lead to angiogenesis (Joukov *et al.*, 1997; Stacker *et al.*, 1999) (**Fig. 1.4**). Since VEGF-C and VEGF-D can bind to and activate VEGFR-2, they may also be involved in the regulation of VEGF-mediated signalling.

b) Fibroblast growth factor (FGF) family and receptors

Fibroblast growth factor 1 (FGF-1, acidic FGF, aFGF) and *fibroblast growth factor 2* (FGF-2, basic FGF, bFGF) (Folkman and Shing, 1992; Fernig and Gallagher, 1994) are another example of proteins involved in the positive regulation of angiogenesis. Both FGF-1 and FGF-2 are members of a family of 17 structurally related proteins (Botta *et al.*, 2000; Powers *et al.*, 2000). Interestingly, unlike the other family members, they lack signal sequences, which poses the question of how they are secreted. Different isoforms of FGF-2 (18, 22, 22.5, and 24 kDa) that result from alternative translation initiation have been described (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). FGF-1 is also represented by 3 isoforms: 154 aa and truncated forms of 140 and 134 aa (Esch *et al.*, 1985; Gimenez-Gallego *et al.*, 1985; Burgess *et al.*, 1986; Harper *et al.*, 1986). The different isoforms (which are the result of differential regulation of translation) may be important in the fine modulation of angiogenic signalling by these molecules.

In order to be able to transduce their signals, FGF-1 and FGF-2 must bind to their cognate receptors. FGF-1 binds with high affinity to all four FGF tyrosine kinase receptors (FGFR-1, FGFR-2, FGFR-3, and FGFR-4), whereas FGF-2 binds with higher affinity to FGFR-1 and FGFR-2. The FGFRs have a common structure (Lee *et al.*, 1989; Dionne *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991): their extracellular portion consists of two or three Ig-like loops, with an acidic region between the first and second Ig loops. The intracellular domain includes the catalytic tyrosine kinase domain split by a short kinase insert. As another level of signalling regulation, multiple isoforms of the FGFRs are generated via alternative splicing, which include soluble and truncated receptors having impaired signalling functions (Johnson and Williams, 1993).

FGF-1 and FGF-2, like other family members, can also bind to another class of low-affinity receptors called heparan sulphate proteoglycans (HSPGs), which are present in abundance on the cell surface and in the ECM (Saksela *et al.*, 1988; Vigny *et al.*, 1988; Bashkin *et al.*, 1989; Kiefer *et al.*, 1990). Interaction with the HSPGs is thought to stabilise and protect FGF-1 and FGF-2 from proteases (Saksela *et al.*, 1988; Sommer and Rifkin, 1986) as well as to provide a reservoir for the growth factors ensuring their long-term action (Flaumenhaft *et al.*, 1989; Presta *et al.*, 1989). The importance of low affinity, heparin-like binding sites for binding of FGF-2 to its high affinity receptors has further been studied in heparan sulfate-deficient cells. When transfected with a cloned FGF receptor cDNA, these mutants failed to bind FGF-2 unless free heparin or heparan sulfate were present in cell medium. It was therefore proposed that an obligatory interaction of low and high affinity receptors constitutes a novel mechanism for the regulation of growth factor-receptor interactions (Yayon *et al.*, 1991).

Similar to other growth factor tyrosine kinase receptors, FGFRs are activated by dimerisation. Signals mediated by FGF-1 and FGF-2 via the FGFRs and transduced farther via signal transduction pathways (for review, see Schlessinger and Ullrich, 1992) ultimately lead to endothelial cell mitogenesis and migration as well as to the production of proteases capable of digesting components of the basement membrane (Friesel and Maciag, 1995). Flamme and Risau (1992), in an avian model system, also demonstrated that FGF-1 and FGF-2 are able to induce differentiation of ventral mesodermal cells into hemangioblasts, substantiating the importance of FGF-1 and FGF-2 in vasculogenesis. Furthermore, both FGF-1 and FGF-2 have been shown to be

mitogenic for smooth muscle cells (Isner, 1996). This suggests they play a role in periendothelial cell recruitment.

c) Indirectly acting angiogenic factors

Unlike the direct-acting angiogenic factors (*i.e.* the members of the VEGF and FGF growth factor families) some other angiogenic mediators are non-mitogenic for endothelial cells and even inhibitory for their proliferation *in vitro* (Folkman and D'Amore, 1996). This action is rather indirect, resulting in the up-regulation of the expression of direct-acting factors from endothelial, stromal or other cells. For example, TGF-βs, a large family of homodimeric peptides, play a role in embryonic angiogenesis by inducing the differentiation of mesenchymal cells into the smooth muscle cell/pericyte lineage and promoting matrix deposition (Sato *et al.*, 1990; Folkman and D'Amore, 1996). Although TGF-β1 inhibits endothelial cell growth *in vitro*, it can indirectly stimulate angiogenesis by inducing the expression of several angiogenic growth factors such as VEGF and FGF-2 by smooth muscle cells (Flaumenhaft *et al.*, 1992; Isner, 1996; Cockerill *et al.*, 1995).

PDGF, another indirectly-acting angiogenic factor expressed by endothelial cells, serves as a mitogen for underlying smooth muscle cells (Banai *et al.*, 1994) subsequently promoting smooth muscle cell expression of VEGF (Goad *et al.*, 1996). PDGF is also thought to be involved in the recruitment of pericytes and in the differentiation of endothelial cell precursors (Hirschi and D'Amore, 1996).

d) Tie receptors and angiopoietins

Signalling of the aforementioned growth factors and cytokines results in the activation of endothelial cells manifested by their increased proliferation, release of proteases degrading vascular basement membrane, migration towards the angiogenic stimuli, and coalescence into new vessel structures (**Fig. 1.2**). Once the early vascular network is established, it undergoes several maturation steps. One of these steps is thought to involve attraction of smooth muscle cells and pericytes around the endothelium. Interaction of endothelial and perivascular cells, in turn, results in inhibition of endothelial cell proliferation and subsequent vessel maturation (Beck and D'Amore, 1997). A novel family of RTKs, the Tie (Tyrosine kinase with

Immunoglobulin and Epidermal growth factor homology domains), and its angiopoietin ligands have been implicated in this process.

To date, two members of the Tie family, Tie-1 and Tie-2 (also known as Tek), have been identified (Dumont *et al.*, 1992; Sato *et al.*, 1993). Like the members of VEGF tyrosine kinase receptor family, expression of the Tie receptors is almost exclusively restricted to the endothelial cell lineage (Hashiyama *et al.*, 1996). The gene knockout strategy helped to unveil the critical role the Tie receptors play during embryonic angiogenesis. Thus, mice lacking Tie-1 die perinatally displaying severe oedema and subsequent haemorrhage caused by hyperactivity of endothelial cells failing to form mature blood vessels (Puri *et al.*, 1995; Sato et al., 1995). Unfortunately, due to the lack of identified ligands for Tie-1 its precise function in angiogenesis signalling remains rather vague.

Tie-2/Tek seems to play even more crucial role as demonstrated by the finding that transgenic mice deficient in Tie-2 alleles die earlier (between embryonic day 9.5 and 10.5) due to defects in blood vessel architecture. These defects (more severe than in the Tie-1-deficient mice) are characterised by malformations in cardial endothelial cell lining, reduction in total endothelial cell number, failure to recruit pericytes, lack of a branching network, and an inability to remodel the primary capillary plexus into large and small vessels (Dumont *et al.*, 1994; Sato *et al.*, 1995). Thus, Tie-2 is thought to be important in mediating interactions between endothelial cells and the ECM as well as peripheral stromal cells – necessary prerequisites for stabilisation and maturation of blood vessels.

Two ligands for the Tie-2 receptor (referred to as *angiopoietins*) have been identified to date. *Angiopoietin-1* (Ang1), a secreted 75 kDa glycoprotein, can induce autophosphorylation of Tie-2 *in vitro*. However, this does not lead to either endothelial cell mitogenesis or tube formation (Davis *et al.*, 1996). Ang1 was rather shown to play a role in regulating the assembly of non-endothelial vessel wall components during embryonic angiogenesis, consistent with findings in the Tie-2 knockout mice (Suri *et al.*, 1996). In contrast to Ang1, *angiopoietin-2* (Ang2) has been demonstrated to suppress the kinase activity of Tie-2 at low concentrations, acting as an Ang1 antagonist, which does not block endothelial cell proliferation (Maisonpierre *et al.*, 1997). Kim *et al.* (2000) were able to demonstrate, however, that at high concentrations Ang2 does induce phosphorylation of Tie-2, acting as an

apoptosis survival factor for endothelial cells during serum-deprivation-induced apoptosis. Thus the role of Ang2 in signalling angiogenesis is still controversial.

e) Integrin receptor family

Angiogenesis is a complex multistep process that involves endothelial cell survival, proliferation, migration as well as the functional maturation of newly formed blood vessels. All these steps rely upon the intimate interaction between endothelial cells and between endothelium and the surrounding ECM. These interactions are mediated by various cell adhesion molecules, of which integrins seem to be the most prominent ones (Brooks, 1996; Brooks et al., 1994; Bischoff, 1997).

Integrins are heterodimeric cell-surface receptors constituted by two non-covalently bound transmembrane glycoproteins (α and β) that connect components of the cytoskeleton to the ECM or immunoglobulin family molecules expressed on the surface of other cells through short peptide sequences such as RGD (Arg-Gly-Asp) present in their ligands (Ruoslahti and Pierschbacher, 1986). While integrins are well known to mediate cellular adhesion and migration (Hynes, 1992), they also participate in regulating the cell cycle (Guadango et al., 1993; Varner et al., 1995). One member of the integrin family, namely $\alpha_v\beta_3$, has been demonstrated to be almost exclusively expressed on the surface of endothelial cells and smooth muscle cells in newly-formed blood vessels, but is absent in mature or quiescent vessels (Brooks et al., 1994; Clark et al., 1996). A requirement for $\alpha_v\beta_3$ during the development of new capillaries has been shown experimentally. Interference with the interaction of the integrin $\alpha_v\beta_3$ with its ECM ligand by anti-$\alpha_v\beta_3$ antibodies or RGD-containing peptide antagonists induced apoptosis in proliferating endothelial cells of nascent vessels, leading to the inhibition of angiogenesis (Brooks et al., 1994a). This phenomenon is currently being utilised in the development of anti-angiogenesis therapies.

1.2.2.2 Negative regulators of angiogenesis

ECM is known to participate in the negative regulation of angiogenesis. Thus extracellular matrix components and in particular certain proteoglycans bind and store endothelial cell growth factors, thereby making them inaccessible to endothelial cells (Vlodavsky et al., 1991). For example, both bFGF and VEGF bind to heparan sulphate proteoglycan. Furthermore, a number of endogenous negative regulators of

endothelial cell proliferation and angiogenesis represent fragments of different ECM proteins, such as fibronectin (Homandelberg *et al.*, 1985), thrombospondin-1 (Tolsma *et al.*, 1993; Weinstat-Saslow *et al.*, 1994), and collagen XVIII (*i.e.* endostatin, O'Reilly *et al.*, 1997).

Interestingly, some other non-ECM anti-angiogenic factors have been described that are fragments of larger proteins which themselves are devoid of inhibitory activity. They include angiostatin (38 kDa fragment of plasminogen, O'Reilly *et al.*, 1994), vasostatin (calreticulin fragment, Pike *et al.*, 1998), 16 kDa fragment of prolactin (Clapp *et al.*, 1993), 7.8 kDa fragment of platelet factor 4 (Gupta *et al.*, 1995) and a fragment of murine EGF (Nelson *et al.*, 1995). These data suggest that there may be a general mechanism by which inhibitors of endothelial growth can be produced locally by specific proteolytic cleavage of a larger protein.

Several other negative angiogenesis modulators have been identified to date. They include thrombospondin-2 (Streit *et al.*, 1999), α-interferon (Zetter, 1998; Sidky and Borden, 1987), tissue inhibitors of metalloproteinases (Johnson, 1994), and epigallocatechin-3-gallate (Cao and Cao, 1999).

Fragments of other proteins
ECM components
29 kDa fragment of fibronectin
thrombospondin-1 fragment
endostatin (fragment of collagen XVIII)
Non-ECM factors
angiostatin (38 kDa fragment of plasminogen)
vasostatin (fragment of calreticulin)
16 kDa fragment of prolactin
7.8 kDa fragment of platelet factor 4
Thrombospondin-1 and 2
α-interferon
tissue inhibitors of metalloproteinases
epigallocatechin-3-gallate

Table 1.2 *Negatively acting angiogenic factors.*

19

1.2.3 Tumour angiogenesis

In the early 1970s Judah Folkman, a pioneer in the field of angiogenesis, postulated that the continued growth of solid tumours and the process of metastasis are dependent on the formation of new blood vessels (Folkman, 1971). This hypothesis was based on the observations that tumours fail to grow beyond the size of 1-3 mm in diameter without inducing angiogenesis (Algire and Chalkley, 1945; Folkman *et al.*, 1963). Therefore, during the prevascular phase, many tumours may remain dormant and clinically undetectable, as the cell proliferation rate in the avascular tumour is counterbalanced by the rate of cell apoptosis (Holmagern *et al.*, 1995; O'Reilly *et al.*, 1996). However, continued genetic change in tumour cells can eventually result in the so-called "angiogenic switch", which triggers the growth of new capillaries towards the tumour mass (Hanahan and Folkman, 1996). This switch is characterised by induction of the proteolytic process, which disrupts the local host microvasculature, the release of diffusible angiogenic factors (*e.g.* VEGF, FGF-2, etc.) and/or the down-regulation of angiogenesis inhibitors (*e.g.* thrombospondin and angiostatin).

Upon angiogenic stimulation, blood vessels growing into tumour form by sprouting or intussusception[*] from pre-existing vasculature. Tumour cells, however, can also recruit angioblasts, circulating endothelial precursors shed from the vessel wall or mobilised from the bone marrow, thus mimicking the process of vasculogenesis (Takashi *et al.*, 1999; Peichev *et al.*, 2000; Rafii, 2000; Asahara *et al.*, 2000). In addition to angiogenesis, tumour cells have been shown to be able to coopt host vessels especially during metastasis (Holash *et al.*, 1999).

VEGF is one of the most prominent angiogenic factors up-regulated in tumours. Indeed, numerous tumour cell lines have been shown to express the VEGF mRNA and secrete the VEGF protein *in vitro* (Senger *et al.*, 1986; Rosenthal *et al.*, 1990). Also, most human tumours show up-regulation of VEGF at the mRNA level (*e.g.* Olson *et al.*, 1994; Brown *et al.*, 1993; Brown *et al.*, 1995). It is thought that during tumour growth concomitant oxygen deprivation (hypoxia) plays one of the leading roles in the induction of VEGF expression (Shweiki *et al.*, 1992; Plate *et al.*, 1992).

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[*] *Intussusception* (L. *intus* within + susception), as opposed to sprouting, is another mechanism of angiogenesis in which interstitial tissue columns get inserted into the lumen of pre-existing vessels and partition the vessel lumen (Patan *et al.*, 1996).

The hypoxia-induced rise in VEGF expression is due both to increased transcription mediated by HIF-1 (hypoxia-inducible factor-1) and an increase in VEGF mRNA stability (Semenza, 1996). Interestingly, expression of oncogenic *ras* potentiated the induction of VEGF by hypoxia (Mazure *et al.*, 1996). Moreover, loss of tumour-suppressor genes such as the von Hippel-Lindau gene has been shown to result in stabilisation of VEGF mRNA in the absence of hypoxia (Gnarra *et al.*, 1996). Hypoglycaemia seems to be another environmental factor playing a role in the induction of VEGF expression in tumours as it regulates gene expression in an overlapping pattern with hypoxia (Stein *et al.*, 1995).

The role of VEGF in tumorigenesis was further substantiated by experiments in which the growth of human tumour cell lines injected subcutaneously into nude mice was dramatically reduced by treating the animals with VEGF-specific monoclonal antibodies (Kim *et al.*, 1993; Warren *et al.*, 1995). Since VEGF is a potent vascular permeability factor, many vessels found in tumours are hyperpermeable to plasma proteins and to other circulating macromolecules (Dvorak, 1986). Therefore, in addition to its direct endothelial cell stimulation effect, VEGF may promote tumour angiogenesis by causing pre-existing blood vessels to become leaky leading to the formation of an extravascular fibrin gel, a substrate for endothelial and tumour cell growth (Dvorak *et al.*, 1987).

FGF-2 is another angiogenic factor described in tumours (Folkman and Klagsbrun, 1987). It is secreted by tumour cells as well as tumour-associated macrophages (Lewis *et al.*, 1995), or mobilised from the ECM by proteases. Thus, its release has been detected in various human tumours, which was correlated with the extent of their vascularisation (Kandel *et al.*, 1991; Li *et al.*, 1994; Nanus *et al.*, 1993). Abnormally elevated levels of FGF-2 were also detected in the serum and urine of patients with a variety of solid tumours, leukaemias, and lymphomas (Nguyen *et al.*, 1994). Furthermore, a number of other angiogenic factors have been described as being overexpressed in different tumours including scatter factor (SF/HGF) (Rosen *et al.*, 1994; Yamashita *et al.*, 1994; Joseph *et al.*, 1995), EGF (Ebert *et al.*, 2000), and PDGF (Hermanson *et al.*, 1992).

Consistent with the hypothesis that tumours may down-regulate endogenous inhibitors of angiogenesis, down-regulation of thrombospondin 1 (TSP1) has been documented in various tumour systems (Good *et al.*, 1990; Dameron *et al.*, 1994;

Zabrenetzsky *et al.,* 1994). Decreased TSP1 production has been linked to the loss of a tumour suppressor gene in several tumour cell lines (Dameron *et al.,* 1994).

The newly formed microvessels generally resemble normal vessels but have several important differences (D'Amore and Thompson, 1987): blood vessels of the tumour usually lack pericytes, their base membrane composition is altered and the endothelial cells are larger and irregular. As a consequence, these vessels have increased permeability, which may contribute to tumour metastasis. Thus, entering the vascular phase, tumours acquire the potential for rapid growth, invasion into host tissue, and metastasis (Weidner *et al.,* 1991; Zetter, 1998). Indeed, multiple studies have confirmed a direct correlation between the degree of tumour vascularisation and metastasis (Weidner *et al.,* 1991; Weidner *et al.,* 1993; Brawer, 1996; Yamakazi *et al.,* 1994; Angeletti *et al.,* 1996; Maeda *et al.,* 1995; Wiggins *et al.,* 1995; Gasparini *et al.,* 1993; Hollingsworth *et al.,* 1995).

1.3 Lymphangiogenesis

1.3.1 Lymphatic system and its function

The circulatory system of higher vertebrates consists of blood and lymphatic vessels that differ in their structural and functional features. The primary structural characteristics of the lymphatics, which distinguish them from the blood vessels, are the discontinuous or absent basal lamina, intricate overlapping intercellular junctional complexes, and specialised anchoring filaments which hold the vessel open as tissue pressure rises (Casley-Smith and Florey, 1961; Leak, 1970; Leak, 1972). These attributes allow the lymphatics to play their physiological role by re-absorbing large biological macromolecules, cellular particles and interstitial fluid, thereby maintaining homeostasis and a constant colloid osmotic pressure within the interstitial tissue (Ryan, 1987; Guyton and Hall, 1996). The lymphatic system is also important in the absorption of lipids from the small intestine. Furthermore, it serves as a filter for the entry of pathogens into the circulation and as a site where T-cell clonal expansion occurs in response to activated antigen-presenting cells (Guyton and Hall, 1996).

The lymphatics have also been implicated in a variety of pathological events including lymphoedema, inflammation, infectious and immune diseases, fibrosis and tumours such as Kaposi's sarcoma and lymphangioma (Witte *et al.,* 1997). In addition, due to their highly permeable nature and poorly developed basement

membranes, the lymphatics have been proposed to be the main routes for tumour dissemination (Cann *et al.*, 1995). Indeed, the majority of human carcinomas metastasise at least initially via the lymphatic vessels (Beitz and Calabresi, 1993). Moreover, active interactions of tumour cells with the lymphatics *in vivo* could be demonstrated (Papoutsi *et al.*, 2000; Mandriota *et al.*, 2000; Skobe *et al.*, 2001; Stacker *et al.*, 2001).

1.3.2 Lymphangiogenesis in embryo and adult

The growth of lymphatic vessels from a pre-existing lymphatic network is termed *lymphangiogenesis*. In the human embryo, lymphatics appear later than blood vessels (*i.e.* 6- to 7-week-old embryos) taking their origin from endothelial-lined lymph sacs, which are located immediately adjacent to veins (van der Putte, 1975). However, due to the lack of specific markers, the origin of the lymph sacs still remains controversial (Yoffey and Courtice, 1970; van der Putte, 1975). Two alternative hypotheses have been proposed. One of them suggests the existence of lymphangioblast precursors of lymphatic endothelium originating from the early mesenchyme (Huntington, 1908; Kampmeier, 1912). The other maintains that embryonic lymphatics derive from central veins by sprouting (Ranvier, 1895; Sabin, 1909). The latest report from Wigle and Oliver (1999) strongly supports the second hypothesis (**Fig. 1.2**).

In the adult organism, lymphangiogenesis occurs after tissue injury (Strange *et al.*, 1989; Junghans and Collins, 1989) and obstruction or damage of the lymphatic vessels (Battezzati and Donini, 1972), where it serves to reduce the increased interstitial fluid pressure associated with oedema and inflammation (Anthony *et al.*, 1997). An increasing amount of evidence suggests the existence of the tumour-induced lymphangiogenesis, mediated by soluble lymphangiogenic factors, VEGF-C and VEGF-D, and their receptor VEGFR-3 (Salven *et al.*, 1998; Stacker *et al.*, 2001; Skobe *et al.*, 2001; Mandriota *et al.*, 2001).

1.3.3 Signalling lymphangiogenesis

Until recently, little was known about the molecular mechanism by which lymphangiogenesis is induced and maintained. However, following the identification of VEGFR-3, a receptor tyrosine kinase whose expression has been shown to be largely restricted to the lymphatic endothelium in the adult (Finnerty *et al.*, 1993; Kaipainen *et al.*, 1995), and its corresponding ligands VEGF-C and VEGF-D, the

mechanisms underlying the growth and development of new lymphatic vessels are beginning to be deciphered.

1.3.3.1 Role of VEGFR-3 in lymphangiogenesis

VEGFR-3 (also known as foetal liver kinase-4, Flt4), a member of the VEGFR family of type III receptor tyrosine kinases (**Fig. 1.3**), was cloned from human placental and erythroleukimia cell cDNA libraries (Aprelikova *et al.*, 1992; Pajusola *et al.*, 1992; Galland *et al.*, 1992; Galland *et al.*, 1993). It shows 31% and 36% homology with VEGFR-1 and VEGFR-2, respectively. Like the other VEGFR members, VEGFR-3 has an extracellular portion consisting of seven immunoglobulin-like domains and the intracellular portion containing a split kinase domain, characteristic of type III receptor tyrosine kinases (Pajusola *et al.*, 1992). In humans VEGFR-3 is present in 2 forms due to alternative splicing giving rise to 5.8 and 4.5 kb species of mRNA (Pajusola et al., 1993; Borg *et al.*, 1995). The unique feature of VEGFR-3 among the other VEGFRs, however, is that the translated protein is further processed via cleavage of the first immunoglobulin loop which remains re-associated to the remaining molecule via a disulphide linkage (**Fig. 1.3**).

During mouse embryogenesis, VEGFR-3 mRNA is localised at E8.5 in the developing blood vessels and in the angioblasts of the head mesenchyme, becoming confined to the lymphatic endothelium during E14.5 (Kaipainen *et al.*, 1995). Prior to the emergence of the lymphatic vessels, VEGFR-3 is thought to play an essential role in the development of the cardiovascular system. Thus, mice lacking functional VEGFR-3 die *in utero* after embryonic day 10 displaying defects in vascular re-modelling and maturation of large vessels and cardiovascular failure (Dumont *et al.*, 1998). Although VEGFR-3 plays an apparent role in angiogenesis, its importance for the development of the lymphatics has also been shown, when a congenital hereditary lymphoedema, known as "Milroy disease", was traced to certain missense mutations in the VEGFR-3 tyrosine kinase domain (Karkkainen *et al.*, 2000; Irrthum *et al.*, 2000). Consistent with these findings, the expression of a soluble form of VEGFR-3 in the skin of transgenic mice inhibited foetal lymphangiogenesis and induced a regression of already formed lymphatic vessels, which resulted in swelling of feet, oedema and dermal fibrosis. Interestingly, development of blood vasculature was not affected.

The latest studies emphasise the role of VEGFR-3 signalling in lymphangiogenesis. Thus, the ectopic expression of either of VEGFR-3 ligands as well as a VEGFR-3-specific mutant under the keratin 14 promoter in the skin resulted in the specific induction of lymphangiogenesis but did not cause angiogenesis (Jeltsch *et al.*, 1997; Veikkola *et al.*, 2001). These results indicate that VEGFR-3 activation is sufficient to induce specifically lymphangiogenesis *in vivo*, which however may depend on the context of VEGFR-3 expression (Jeltsch *et al.*, 1997; Veikkola *et al.*, 2001). Thus, consistent with the apparent role of VEGFR-3 in lymphangiogenesis, in the adult, physiological VEGFR-3 expression is restricted to lymphatic endothelial cells, some high endothelial venules and hematopoietic cells primarily of the megakaryoblastoid and erythroid lineage (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). However, VEGFR-3 has also been shown to be expressed in some fenestrated endothelia (Partanen *et al.*, 2000) as well as in blood vessels of chronic wounds (Paavonen *et al.*, 2000) and tumours (Valtola *et al.*, 1999). Importantly though, the overall pattern of VEGFR-3 expression suggests that VEGFR-3 may potentially serve as a useful marker of lymphatic vessels under normal physiological conditions.

1.3.3.2 VEGF-C and VEGF-D in lymphangiogenesis

VEGF-C and VEGF-D, the two known ligands for VEGFR-3, share 48% amino acid sequence identity (Achen *et al.*, 1998). They belong to the VEGF/PDGF family of growth factors with which they share a high degree of homology (*i.e.* they possess a VEGF-homology domain belonging to the cysteine knot family and containing six distinctly spaced cysteine residues). In addition, however, VEGF-C and VEGF-D also have long N- and C-terminal extensions, which are unique for the two VEGFR-3 ligands (Joukov *et al.*, 1996; Orlandini *et al.*, 1996; Achen *et al.*, 1998). The significance of these unique regions is unclear, though it is possible that the respective VEGF-C and VEGF-D unique peptide sequences may be involved in sequestration of the protein to components of the ECM (Joukov *et al.*, 1997; Marconcini *et al.*, 1999). Upon translation, VEGF-C and VEGF-D produce a protein of 58kD and 60kD, respectively. This protein undergoes step-wise proteolysis (**Fig. 1.4**) via cleavage of the N- and C -terminal regions of the translated protein. During this maturation process several forms with increased activity towards VEGFR-3 are generated to finally give rise to a protein of 21kD (Joukov *et al.*, 1997, Stacker *et al.*, 1999). The fully processed mature protein, termed ΔNΔC/VEGF-C (ΔNΔC/VEGF-D) acquires
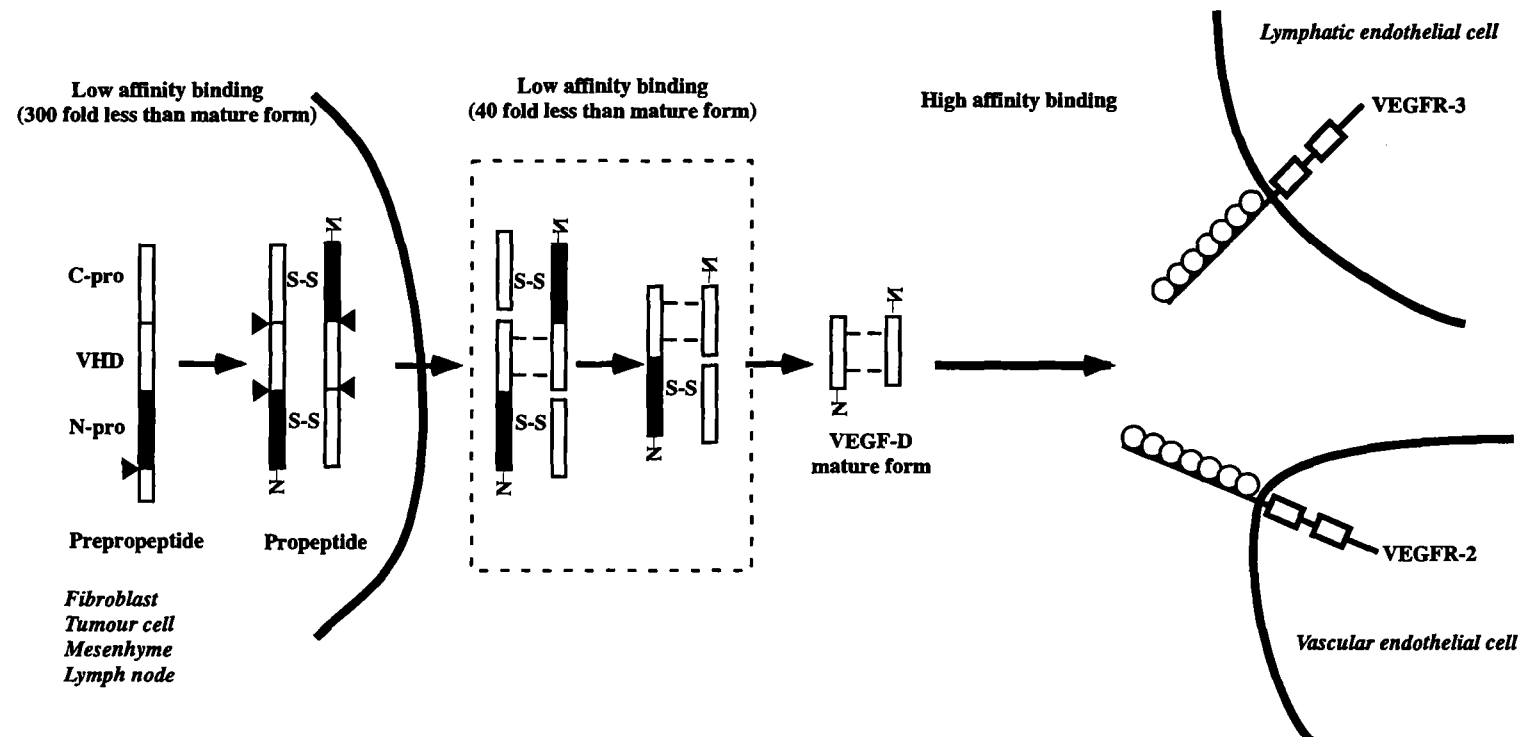
Figure 1.4 *Schematic representation of the proteolytic processing of the VEGF-C/VEGF-D proteins.* Initially synthesised as preproproteins containing N- and C-terminal propeptides (N-pro and C-pro) and a VEGF-homology domain (VHD), VEGF-C and VEGF-D undergo stepwise proteolytic processing. Several forms of the proteins with increased binding and activity towards VEGFR-3 and VEGFR-2 are generated among which the mature forms, coinsisting of dimers of the VHD, bind the receptors with highest activity. The processing shown is that for VEGF-D (Stacker *et al.,* 1999) and is similar to that for VEGF-C (Joukov *et al.,* 1997). VEGFR-2 is also expressed by at least some lymphatic vessels (Partanen *et al.,* 1999), whereas VEGFR-3 can be detected in angiogenic blood vessels in tumours (Valtola *et al.,*1999a), *taken from Lymboussaki et al. (2000).*

properties which permit its binding to VEGFR-2. Although mature VEGF-C and VEGF-D bind VEGFR-3 with a 3-fold higher affinity, they become capable of inducing VEGFR-2 signalling (Joukov *et al.*, 1997, Stacker *et al.*, 1999). Therefore, as already mentioned, both VEGF-C (Witzenbichler *et al.*, 1998; Cao *et al.*, 1998; Pepper *et al.*, 1998) and VEGF-D (Achen *et al.*, 1998; Marconcini *et al.*, 1999) also play a role in modulating angiogenesis. A ΔNΔC/VEGF-C mutant lacking VEGFR-2 activating property but still able to bind to and activate VEGFR-3 has been described (Joukov *et al.*, 1998).

VEGF-C was the first protein to be identified as lymphangiogenic (Oh *et al.*, 1997). When overexpressed in the skin of transgenic mice, VEGF-C resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement (Jeltsch *et al.*, 1997). The expression pattern of *VEGF-C* gene during embryogenesis also suggests that VEGF-C plays a role in the development of lymphatics. Thus, *VEGF-C* is expressed in the mesenchymal cells of post-implantation embryos around large sac-like structures in the jugular area, whereas VEGFR-3 is found lining the borders of these sacks (Kukk *et al.*, 1996). Exactly such venous sac-like structures, according to Sabin (1909), are thought to be the origin of the lymphatics.

Similarly to VEGF-C, VEGF-D has also been anticipated to be lymphangiogenic, given its equal receptor specificity (Lymboussaki *et al.*, 2000). A direct evidence came recently from the VEGF-D transgenic mice, where overexpression of VEGF-D in the skin under the keratin 14 promoter also resulted in the hyperproliferation of lymphatic but not blood vessels (Veikkola *et al.*, 2001).

The expression pattern for *VEGF-D* in mouse embryos overlaps with *VEGF-C* in many tissues in a manner complementary to VEGFR-3 (Kaipainen *et al.*, 1995). However, slight differences in the expression of *VEGF-C* and *VEGF-D* are reported to exist (Lymboussaki *et al.*, 2000). Interestingly, VEGF-C and VEGF-D have different properties with respect to induction of vascular permeability. While VEGF-C is able to increase the vascular permeability (Joukov *et al.*, 1997), VEGF-D is void of such an activity (Stacker *et al.*, 1999a).

1.3.4 Tumour-induced lymphangiogenesis

Lymphatic (lymphangenous) spread of cancer cells by direct invasion or tumour embolisation is a crucial prognostic indicator of tumour aggressiveness used for histological staging of many solid organ cancers (Willis, 1952). Furthermore,

auxiliary node (so called "sentinel lymph node") status is the most important prognostic information regarding treatment and prognosis for breast cancer patients (Cabanas, 2000). One can imagine that the ability of tumours to attract lymphatic vessel growth would be of great advantage, since it would not just facilitate their metastasis but would also relieve the interstitial pressure which builds up within the tumours (Jain, 1987). However, the existence of lymphatics within tumours has long been questioned (Zeidman *et al.*, 1955; Jain, 1989; Folkman, 1995a).

This situation is due to the difficulties in studying the lymphatics: inadequate methods and the lack of reliable markers for the lymphatic endothelium. Thus, lymphangiographic studies in which tracer dyes are injected into the lymphatic system or the interstitial space in order to evaluate the uptake of the dye by the tumour lymphatics generally indicate the absence of lymphatics within tumours (Butler *et al.*, 1975; Leu *et al.*, 2000). However, the high intratumoral interstitial fluid pressure (which is likely to push the dyes away from the tumour) may well influence the results of such a lymphangiographic study. More importantly, the absence of reliable markers specific for the lymphatic endothelium (Sleeman *et al.*, 2001) have made histological data on tumour lymphatics difficult to interpret. Very recently, however, using novel lymphatic endothelial markers VEGFR-3 and LYVE-1, a hyalouronan receptor found to be expressed almost exclusively on lymphatic endothelium (Banerji *et al.*, 1999), functional lymphatics and lymphangiogenesis were documented within tumours (Skobe *et al.*, 2001; Karpanen *et al.*, 2001; Stacker *et al.*, 2001).

In these studies, tumour-induced lymphangiogenesis was linked to VEGF-C and VEGF-D signalling. Thus, when VEGF-C-overexpressing breast cancer cells were orthotopically transplanted onto nude mice, tumours demonstrated increased intratumoral lymphangiogenesis and significantly enhanced metastasis to regional lymph nodes and to lungs (Skobe *et al.*, 2001; Karpanen *et al.*, 2001). Since these effects of VEGF-C could be inhibited by a soluble VEGFR-3 fusion protein, the role of VEGFR-3 in tumour-induced lymphangiogenesis and lymphatic metastasis was substantiated too (Karpanen *et al.*, 2001). Similar results were obtained in experiments with VEGF-D-overexpressing 293EBNA tumours implanted into immunodeficient mice. VEGF-D expression in these normally avascular and non-metastatic tumours resulted in the formation of intratumoral lymphatics and lymph node metastasis. Interestingly, VEGF-D expressed by tumour cells also promoted tumour vascularisation, which suggests the involvement of VEGF-D-mediated

VEGFR-2 signalling in this tumour model. These effects could be blocked, however, with a VEGF-D-specific antibody (Stacker *et al.*, 2001).

Experiments with Rip1Tag2 mice, which are characterised by spontaneous development of pancreatic β-cell tumours that are neither lymphangiogenic nor metastatic, further support the notion that lymphangiogenic factors, when expressed by tumours, may lead to lymphangiogenesis and promote metastasis. Thus, crossing Rip1Tag2 mice with other transgenics, in which VEGF-C expression is driven by the rat insulin promoter (Rip), gives rise to a mouse line, which tend to form tumours surrounded by lymphatics and develop pancreatic lymph node metastases. The surrounding lymphatic vessels were also shown to frequently contain tumour cell masses of β-cell origin (Mandriota *et al.*, 2001).

The role of VEGF-C in tumour progression and metastasis has also been studied clinically. Its expression by tumour and stromal cells in different types of cancer was usually found to correlate with lymphogenous metastasis and significantly poorer prognosis for patients (Ohta *et al.*, 1999; Yonemura *et al.*, 1999; Fellmer *et al.*, 1999; Tsrusaki *et al.*, 1999; Bunone *et al.*, 1999; Akagi *et al.*, 2000; Kabashima *et al.*, 2001). Consistent with the hypothesis that VEGF-D, like VEGF-C, secreted by tumour cells, activates endothelial cell receptors and thereby contributes to the regulation of tumour angiogenesis and lymphangiogenesis, VEGF-D positive tumour cells were identified in malignant melanoma (Achen *et al.*, 2001). Controversially, however, a report on the clinical significance of VEGF-D in lung adenocarcinoma indicates that VEGF-D expression may inversely correlate with the metastatic potential of the tumours (Niki *et al.*, 2000).

Consistent with the proposed role of VEGF-C and VEGF-D in tumour-induced lymphangiogenesis, the expression of VEGFR-3 has also been found to be up-regulated in different tumours. Thus, VEGFR-3 is highly expressed in the putative lymphatic endothelium of cutaneous lymphangiomatosis (Lymboussaki *et al.*, 1998), in vessels surrounding lymphomas (Jussila *et al.*, 1998) and in the vessels of external invasive margin of several human breast and prostate carcinoma specimens (Jussila *et al.*, 1998; Valtola *et al.*, 1999; Tsurusaki *et al.*, 1999). In oral squamous cell carcinoma, VEGFR-3 expression was found to be in correlation with the propensity for lymph node metastasis (Moriyama *et al.*, 1997). In addition, VEGFR-3 is expressed in tumours with presumed lymphatic origin including Kaposi's sarcoma

(Folpe *et al.*, 2000). Interestingly however, while not being expressed in adult blood vessels, VEGFR-3 is induced in some vascular endothelial cells of tumour-bearing tissues (Valtola *et al.*, 1999; Partanen *et al.*, 1999). VEGFR-3, therefore, also appears to be involved in tumour-induced angiogenesis. Consequently, cancer cells that produce VEGF-C or VEGF-D may have a growth advantage because of their ability to stimulate both blood vascular and lymphatic endothelia, triggering both lymph- and angiogenesis.

1.4 Inhibition of angiogenesis: a novel anticancer therapy

1.4.1 Role of anti-angiogenic strategy in fighting cancer

Historically, surgery has been the first treatment for cancer. However, it may not bring complete remission for the cancer patient as metastasis often ensues prior to the removal of the primary tumour. Therefore, at present, following the surgery, radiation and chemotherapy, separately or in combination, are used to target the remaining cancerous cells in the body. The major drawback of the latter two modalities, however, is the lack of specificity, which jeopardises normal cells of the organism (Boehm, 1998). So-called "biological therapy" was the fourth modality to be developed in the late 1970s, whose principle is cancer treatment by modification and exploitation of the cellular and molecular mechanisms of the natural host defence and of the regulation of tissue proliferation, differentiation, and survival (Hersh and Stopeck, 1997). The commonly used biological therapies include use of different cytokines, monoclonal antibodies, bone marrow growth factors, host defence cells, tumour vaccines, immunomodulators, anti-growth factors and gene therapy (Hersh and Stopeck, 1997). Thus, biological therapy, besides taking direct anti-tumour approaches (*e.g.* intratumoral delivery of the gene of interest to change its immunogenecity), resorts to another important strategy in the anti-cancer battle: namely, alteration of the tumour/host interactions. To this end, inhibition of tumour angiogenesis (combining principles of both chemo- and biological therapies) has the potential to be a powerful anti-cancer strategy aimed at preventing tumour cells from gaining access to vital nutrients and oxygen, thus stopping tumour growth.

For several reasons anti-angiogenic therapy has the potential of being a better treatment for cancer (Folkman, 1972; Folkman, 1996; Sato, 1998). Thus, due to the specific targeting of the dividing endothelial cells within angiogenic tumours,

inhibition of angiogenesis is likely to cause considerably less side effects (*e.g.* bone marrow suppression, gastrointestinal symptoms, hair loss, etc.) than the standard radio- and chemotherapies (Folkman, 1995a). Also, since growth of many tumour cells depends on just a few endothelial cells, the efficacy of the anti-angiogenic therapy may be considerably higher. Another advantage of the anti-angiogenic strategy is to be gained from the fact that therapeutic agents are not required to enter the tumour cells or cross blood-brain barrier. It is therefore anticipated that drug delivery should be much easier than in the conventional chemotherapy. Furthermore, targeting physiological cells of the organism (*i.e.* vascular endothelial cells) avoids the problem of genetic instability in cancer cells with the ensuing phenomenon of acquired drug resistance (Lengauer *et al.*, 1998). Finally, since endothelial cells are important for the growth of all solid tumours, anti-angiogenic therapy may have a broader anticancer spectrum than the other more conventional approaches.

Although the anti-angiogenic strategy has many advantages in comparison to the other currently used therapies, it also has potential drawbacks (Cohen, 1999). For example, it may interfere with physiological angiogenesis, such as the female reproductive cycle and wound healing. Some anti-angiogenic agents are suspected to have teratogenic effects (Phillips, 1998). More importantly, even though inhibition of angiogenesis can be employed to render tumours avascular and dormant, it alone is unlikely to completely eradicate tumours (Folkman, 1972). Thus, an emerging concept is that anti-angiogenesis therapy should be used in combination with the other established cancer treatment protocols such as surgery, radiation, chemotherapy, and immunotherapy (Kerbel, 2000).

1.4.2 Inhibition of angiogenic ligand signalling

Following the angiogenic switch, a tumour acquires the ability to express factors that are capable of stimulating angiogenesis. To date, members of the VEGF, bFGF and angiopoietin families have been demonstrated to play a predominant role in this process (Yancopoulos *et al.*, 2000). Consequently, one of the first steps in the development of anti-angiogenic approaches has been the search for antagonists of the aforementioned positive regulators of angiogenesis. To this end, naturally occurring or synthetic inhibitors, monoclonal neutralising antibodies, soluble receptors and antisense cDNA for the endothelial growth factors have been applied to suppress tumour growth *in vivo* (Witte *et al.*, 1998; Kerbel, 1998).

Although inhibition of bFGF (Ensoli *et al.*, 1994) or angiopoietin/Tie2 (Lin *et al.*, 1997; Lin *et al.*, 1998) have been shown to suppress tumour growth, interference with VEGF signalling via its cognate receptors has been by far the most popular line of anti-angiogenic research for the last decade. Thus, Kim *et al.* (1993) demonstrated that anti-VEGF monoclonal antibodies greatly inhibited progression of human tumours in nude mice. The validity of this strategy has been confirmed by other studies using anti-sense cDNA (Saleh *et al.*, 1996; Oku *et al.*, 1998). Inhibition of VEGF action also caused dramatic reduction in metastasis formation by aggressive tumour cell lines (Warren *et al.*, 1995; Melnyk *et al.*, 1999). Non-invasive imaging of the vasculature revealed complete suppression of tumour-associated angiogenesis in animals treated with monoclonal anti-VEGF antibodies, verifying the previous observations and providing a direct proof that inhibition of angiogenesis is the mechanism by which tumours regress after anti-VEGF treatment (Borgström *et al.*, 1996).

Inhibition of VEGF receptor-mediated signal transduction has supplied further evidence for the role of VEGF signalling in tumour angiogenesis. Thus, retrovirus-mediated expression of a dominant-negative VEGFR-2 protein suppressed the growth of glioblastoma and other tumour cell lines *in vivo* (Millauer *et al.*, 1996). Other studies, where soluble extracellular domains of VEGFR-1 or VEGFR-2 were locally expressed in nude mice-borne tumours, demonstrated significant reduction not only in tumour growth but also in metastasis and mortality rate (Kong *et al.*, 1998; Goldman *et al.*, 1998).

Given their powerful anti-angiogenic potential, antibodies against VEGF or its receptors are very promising tools for anti-angiogenic therapy. Presently, one such "humanised" anti-VEGF antibody (Presta *et al.*, 1997) is undergoing phase II clinical trials. In addition, small chemical compounds that inhibit VEGFR-2 signal transduction (*e.g.* SU5416 and SU6668, Sugen, USA) are undergoing phase II clinical trials in cancer patients (Strawn *et al.*, 1996; Fong *et al.*, 1999; Laird *et al.*, 2000).

One important problem associated with this anti-angiogenic approach is the possibility that tumours, whose growth is suppressed as the result of neutralising the action of an angiogenic factor, might be able to activate, after prolonged therapy, alternative angiogenic pathways, thereby acquiring resistance to such a treatment (Ferrara and Alitalo, 1999). Therefore, use of a range of angiogenesis inhibitors along

with direct anti-endothelial agents may be necessary to achieve success in long-term inhibition of tumour growth (Maucery *et al.,* 1998).

1.4.3 Targeting endothelial cell-ECM interactions

Interaction of endothelial cells with the ECM is an important aspect of angiogenesis. Consequently, molecules mediating these interactions are potential targets for the anti-angiogenic strategy too. Integrin $\alpha_v\beta_3$, for instance, has a unique expression pattern in endothelial cells and smooth muscle cells of proliferating vessels in healing wounds and in growing tumours. Studies where the function of $\alpha_v\beta_3$ integrin was disrupted by use of $\alpha_v\beta_3$-integrin blocking antibodies indicate that tumour growth regression ensues due to induced endothelial cell apoptosis (Brooks *et al.,* 1994a; Brooks *et al.,* 1995). Another approach in inhibiting $\alpha_v\beta_3$-integrin-mediated endothelial cell survival and proliferation has been the use of synthetic peptides containing an exposed integrin recognition motif RGD, which can also be used to specifically target tumour-derived vasculature (Pasqualini *et al.,* 1997; Arap *et al.,* 1998).

Matrix metalloproteinases (MMPs) are a family of enzymes composed of 15 different membrane-bound zinc-endopeptidases, which are thought to be able to degrade almost all ECM components (Birkedal-Hansen *et al.,* 1993; Powell and Matrisian, 1996). Since invasive events (*e.g.* tumour cell or endothelial cell migration) require the ability to cause limited degradation of the ECM, the MMPs sequestered on the tumour cell/endothelial cell surface are believed to play a vital role in tumour angiogenesis, invasion and metastasis. The naturally occurring tissue inhibitors of metalloproteinases (TIMPs) have been shown to inhibit tumour angiogenesis, growth and metastasis (Wojtowicz-Praga *et al.,* 1997; Johnson *et al.,* 1994). Pharmaceutical companies have synthesised several low-molecular weight MMP inhibitors (*e.g.* Marimastat (British Biotechnologies, USA), COL-3 (Collagenex, USA)) with the purpose of preventing new blood vessels from being able to invade surrounding tissue (Holmgren and Bicknell, 1997).

1.4.4 Direct inhibition of endothelial cell proliferation

In order to grow progressively, tumours need to induce angiogenesis by the up-regulation of pro-angiogenic and down-regulation of anti-angiogenic factors. The discovery that tumours also generate angiogenesis inhibitors (*e.g.* angiostatin, O'Reilly *et al.*, 1994; endostatin, O'Reilly *et al.*, 1997) was therefore somewhat surprising. This finding, however, was consistent with the observation that the removal of a primary tumour can lead to the rapid growth of previously undetected metastases (Sugarbaker *et al.*, 1997; Woodruff, 1990). Therefore, it was proposed that tumours generally produce both stimulators and inhibitors of angiogenesis. However, the excess of the former in the proximity of the primary tumours stimulates angiogenesis, while the excess of the latter, by virtue of its longer half-life in the circulation, suppresses angiogenesis at distant sites and consequently the growth of the secondary tumours (O'Reilly et al., 1994).

Angiostatin and endostatin are the best examples of such inhibitory molecules. These fragments of larger non-anti-angiogenic proteins, putatively generated by the action of tumour-derived proteases (Gately *et al.*, 1996; Gately *et al.*, 1996), are currently the most potent anti-angiogenic factors, exhibiting strong anti-tumour activity *in vivo* (O'Reilly *et al.*, 1996; O'Reilly *et al.*, 1997). Mechanisms of action of the two potent tumour-derived inhibitors are being elucidated. It appears that angiostatin induces endothelial cell apoptosis by binding to and deactivating endothelial ATP synthase, thus blocking cellular energy generation (Moser *et al.*, 1999). Endostatin, in turn, was found to induce tyrosine kinase activity leading to apoptosis in FGF-treated endothelial cells (Dixelius *et al.*, 2000). Importantly, in animal experiments, endostatin did not induce acquired drug resistance after repeated cycles of therapy (Boehm *et al.*, 1997; Blezinger *et al.*, 1999). Owing to its very promising anti-angiogenic and anti-tumour performance, endostatin (EntreMed, USA) is now undergoing phase I clinical trials in cancer patients.

There are several examples of drugs currently undergoing clinical trials as possible inhibitors of angiogenesis that have been originally developed without the intention of inhibiting tumour angiogenesis. These include thalidomide and interferon α (Kerbel *et al.*, 2000). Therefore, it may be appealing to screen clinically used compounds for their potential anti-angiogenesis properties. On the other hand, the ability of anti-angiogenic drugs to affect tumour cell directly would be a desirable 'side' effect of anti-angiogenic therapy. Hyperforin may be one candidate for becoming such a

multipurpose drug. This plant derivative, which has long been used as a folk remedy for burns and skin injuries (Hänsel *et al.,* 1993), has lately gained a reputation as an effective anti-depressant (Linde *et al.,* 1996). It was also shown to inhibit the growth of gram-positive bacteria (Gurevich *et al.,* 1971; Schempp *et al.,* 1999). Furthermore, hyperforin has been reported to exert a potent antiproliferative action on mammalian cells *in vitro* (Schempp *et al.,* 2000). This anti-proliferative effect highlighted the possibility that hyperforin may be an anti-cancer agent, acting either directly on tumour cells, or indirectly on endothelial cells. This possibility was investigated as part of this thesis.

1.5 Aims of the study

Anti-angiogenic therapy is currently recognised as a powerful supplement to the other established anti-cancer modalities, which has the promise of low toxicity, reduced risk of drug resistance and increased anti-cancer efficacy (Folkman, 1999). Key molecular mediators of angiogenesis have been identified, among which the members of VEGF family and their cognate receptors are thought to play the most prominent role (Veikkola *et al.,* 2000; Saaristo *et al.,* 2000). Consequently, targeting VEGFR-mediated signalling of VEGF has become an integral element of the anti-angiogenic strategy in the anti-cancer research (Schlaeppi and Wood, 1999).

Lymphangiogenesis is another process that is now being recognised as playing an important role in tumour progression and dissemination. Its major known positive mediators VEGF-C and VEGF-D signal through the VEGFR-3 receptor which is expressed mostly on the lymphatic endothelium and on the vascular endothelium of angiogenic tumours. Thus, VEGFR-3 and its ligands may serve as targets for the prevention of tumour-induced lymphangiogenesis (Saaristo *et al.,* 2000).

The anti-proliferative properties of hyperforin suggest that it may have potential in the treatment of cancer, perhaps by blocking tumour growth directly, or by interfering with tumour-host interactions. For example, hyperforin may have the ability to block the proliferation of endothelial cells and thereby inhibit angiogenesis and lymphangiogenesis.

The task of my thesis work was to identify novel anti-tumour agents, which would block tumour cell proliferation, angiogenesis and/or lymphangiogenesis, thereby

preventing tumour growth and metastasis. Therefore, the specific goals of the original study were the following:

- Cloning, expression and purification of recombinant rat VEGF, VEGF-C and VEGF-D proteins;

- Characterisation of the recombinant proteins in terms of their ability to bind to and activate their cognate VEGFR receptors, stimulate proliferation of VEGFR-expressing cells and promote angiogenesis and lymphangiogenesis *in vivo*;

- Establishment of lymphatic endothelial cultures *in vitro* to check the possibility of inhibiting vascular and lymphatic endothelium proliferation with the identified substances;

- Selection and characterisation of indolinones capable of inhibiting VEGFR-2/VEGFR-3 signalling;

- Characterisation of anti-tumour properties of the plant-derived drug hyperforin;

- Use of the successful inhibitory substances for the inhibition of tumour growth and metastasis *in vivo*.

ജ്ഞയ

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All general chemicals were, unless otherwise stated, purchased from *Carl Roth GmbH + Co, Karlsruhe, Merck KgaA, Darmstadt,* or *Sigma Chemie GmbH, Deisenhofen* and were of the highest quality. All radiochemicals were supplied by *Amersham Pharmacia Biotech, Freiburg.*

2.1.2 Oligonucleotides

All the oligonucleotides were made by *MWG Biotech GmbH* and were all of an HPLC-purified grade.

2.1.3 Antibodies and cytokines

Antibodies and cytokines were purchased from the following companies:

DAKO Diagnostika GmbH: all secondary antibodies (HRP, biotin or FITC/PE-conjugated);

Invitrogen: anti-His (C-term) and anti-His (C-term)-HRP mouse monoclonal antibodies;

Novagen, Inc.: His-Tag mouse monoclonal antibody;

Pharmingen (BD): anti-rat CD31 (PECAM) and anti-phosphotyrosine mouse monoclonal antibodies;

PromoCell GmbH: recombinant hVEGF, aFGF, bFGF;

R&D Systems: recombinant hVEGF, aFGF, bFGF;

Research Diagnostics, Inc.: anti-Flt4 (mouse/rat) and anti-Flt4 (human) rabbit polyclonal antibodies;

Santa Cruz Biotechnology, Inc.: anti-Flt1 (H-225) (mouse, rat and human), anti-Flk1 (C-1158) (mouse, rat and human), anti-Flt4 (M-20) (mouse/rat) rabbit polyclonal antibodies;

Transduction Laboratories (Becton Dickenson): anti-phosphotyrosine antibody (monoclonal, PY20 and RC20:HRPO).

2.1.4 Bacteria

E. coli strain used:

DH 5α: supE44Δ*lac*U169(φ80*lac*ZΔM15)*hsdR17recA1 endA1 gyrA96 thi*-1 *relA*1

2.1.5 Cell lines and media

Cell lines and corresponding media used in the current study are listed in **Table 2.1**. Tissue culture media for mammalian cells were, unless otherwise stated, purchased from *Life Technologies GmbH, Karlsruhe.* Insect cell culture and expression media were obtained from *Invitrogen, the Netherlands.* All media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (*Life Technologies*). Trypsin was purchased from *Difco Laboratories, Detroit, USA*, and was diluted to 0.25% in 15 mM sodium citrate, 134 mM potassium chloride prior to use.

Cell line	Origin	Medium
Drosophila expression system		
S2	Schneider 2 Drosophila melanogaster cells (*Invitrogen*, R690-07)	DES medium (*Invitrogen*)
Endothelial cells		
HDMEC	Human dermal microvascular endothelial cells (*PromoCell*, c-12210)	ECGM MV (*PromoCell*, Heidelberg)
HUVEC	Human umbilical vascular endothelial cells (*PromoCell*, c-12200)	ECGM (*PromoCell*, Heidelberg)
PAE	Porcine aortic endothelial cells (Kroll and Waltenberger, 1997)	F-12, 10% FCS
PAE/VEGFR-1	PAE transfected with a human VEGFR-1 construct (Kroll and Waltenberger, 1997)	F-12, 10% FCS
PAE/VEGFR-2	PAE transfected with a human VEGFR-2 construct (Kroll and Waltenberger, 1997)	F-12, 10% FCS
PAE/VEGFR-3	PAE transfected with a murine VEGFR-3 construct	F-12, 10% FCS, 400 µg/ml neomycin
Tumour cell lines		
BSp73-ASML	Rat pancreatic carcinoma (Matzku *et al.*, 1983)	RPMI, 10% FCS
BSp73-1AS	Rat pancreatic carcinoma (Matzku *et al.*, 1983)	RPMI, 10% FCS
NM081	Rat mammary carcinoma (Kim, 1986)	DMEM, 10% FCS
MT450	Rat mammary carcinoma (Kim, 1986)	DMEM, 10% FCS
G	Rat prostatic carcinoma (Isaacs *et al.*, 1986)	RPMI, 10% FCS, 250 nM dexamethason
AT-6.1	Rat prostatic carcinoma (Isaacs *et al.*, 1986)	RPMI, 10% FCS, 250 nM Dexamethason
MTLN-3	Rat mammary carcinoma (Neri and Nicolson, 1981)	DMEM, 10% FCS
MTLy	Rat mammary carcinoma (Neri and Nicolson, 1981)	DMEM, 10% FCS
Other cell lines		
293	Human embryonic kidney cells (Xie *et al.*, 1996)	DMEM, 10% FCS
293/mVEGF	293 transfected with a murine VEGF construct (gift of Jaya Krishnan)	DMEM, 10% FCS, 600 µg/ml neomycin
293/mVEGF-C	293 transfected with a murine VEGF-C construct (gift of Jaya Krishnan)	DMEM, 10% FCS, 600 µg/ml neomycin

Table 2.1 *Cell lines and corresponding cell culture media.*

2.2 General Methods

The majority of protocols and recipes for commonly used buffers used in this project were taken from the laboratory manual of Sambrook *et al.* (1989) and Current Protocols in Molecular Biology (Ausubel *et al.,* 1989) unless otherwise stated.

2.2.1 Phenol/Chloroform extraction of nucleic acids

To remove unwanted protein contaminants from nucleic acids, an equal volume of Tris-buffered phenol and chloroform at a ratio of 1:1 (*v/v*) was added and the mixture vortexed. The two phases were separated by centrifugation at 13,000 rpm for 10 min. The upper aqueous nucleic acid-containing phase was transferred to a fresh reaction tube and subjected to a further round of extraction with phenol/chloroform (1:1, *v/v*).

2.2.2 Ethanol (or 2-propanol) precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was brought to 200 mM with 3 M Na-acetate (pH 4.8-5.0) and 2.5 volumes of $-20°C$ ethanol or 1 volume of 2-propanol were added. After 30 min to overnight incubation at $-20°C$ or 15 min at $-80°C$ (only ethanol precipitation), the precipitate was centrifuged at 13,000 rpm for 15-20 min. The pellet was washed with 70% ethanol, centrifuged for another 3 min to remove the salt and was then dried. DNA was re-suspended in either H_2O or TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

2.2.3 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. An $OD_{260} = 1$ is equivalent to 50 µg/ml double stranded DNA or 40 µg/ml RNA or 20 µg/ml single-stranded oligonucleotide. The OD_{280} is used as an indication of the purity of the nucleic acid; it should be approximately 50% of the OD_{260}.

2.2.4 Total RNA isolation from cells or tissue

Total RNA was prepared from the cells when they were 60-80% confluent in 15 cm culture dishes (*Greiner, Frickenhausen*). The medium was completely removed and cells were lysed by adding 1 ml of peqGOLD RNA Pure solution *(Peqlab Biotechnologie*

GmbH, Erlangen). To lyse tissue-derived cells, 100-mg snap-frozen tissue was placed directly into 1 ml peqGOLD solution and homogenised using an ultra-Turrax T25 (*IKA-Labortechnik*) homogeniser for 3-5 min. After an incubation period for 5 min at room temperature, 0.2 ml chloroform was added to the lysed cells or to the homogenised tissue mix and vortexed vigorously. Following a 3-10-min incubation at room temperature, the mixture was centrifuged for 5 min at 4°C at 13,000 rpm and the top aqueous RNA-containing phase was removed and transferred to a fresh tube. Again 0.2 ml chloroform was added and the extraction repeated as above. After one more round of the chloroform extraction (altogether 3 rounds were done), the RNA was precipitated by addition 1 ml isopropanol, subsequent vortexing, incubation for 5-10 min at room temperature and 15 min centrifugation at 4°C at 13,000 rpm. The RNA pellet was washed twice with 75% ethanol and re-suspended in 50 μl H_2O for determination of the RNA concentration. The RNA preparation was stored at - 80°C.

2.2.5 Reverse transcription polymerase chain reaction (RT-PCR)

2.2.5.1 First strand cDNA synthesis

To create the first strand of cDNA, the SuperScript II/RNase H Reverse Transcriptase kit (*Life Technologies*) was used. Total RNA (1-5 μg) was mixed with 1μl Oligo-(dT)$_{12-18}$ primer (500 μg/ml) (*Promega*) and bi-distilled water to give a 12 μl reaction volume. After incubation of the mixture at 70°C for 10 min and quick chilling on ice, 4 μl 5x First Strand buffer, 2 μl 0.1 M DTT and 1 μl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP, *Peqlab*) were added to the reaction. The reaction tube was then incubated for 2 min at 42°C, followed by the addition of 1μl (200 U) SuperScript II. Finally, the reaction was incubated at 42°C for 50 min. To inactivate the reaction, the tube was heated at 70°C for 15 min.

2.2.5.2 Amplification of first strand cDNA by PCR

In order to amplify a specific cDNA fragment the standard PCR reaction (see below) was employed using 1 μl of the first strand reaction and a specific set of amplification primers. The reaction was performed in a 50 μl volume.

2.2.6 Polymerase chain reaction (PCR) DNA amplification

PCR was performed using the Advantage-HF PCR kit (*Clontech Laboratories GmbH, Heidelberg*). Components of the reaction were mixed together as shown in **Table 2.2** and cycled in a PCR machine using the following parameters: 94°C, 15 sec, 68°C, 4 min for a total of 30-35 cycles.

Components	Volume, μl
HF polymerase buffer	5
HF polymerase dNTPs	16
10x HF dNTP mix	5
Forward and reverse primers (10 pmol/μl each)	2 (each)
Template DNA (10 ng/μl)	3
50x HF polymerase	1
Autoclaved distilled water	up to 50

Table 2.2 *Composition of a PCR reaction.*

2.2.7 Recombinant PCR

A modification of the common PCR protocol was utilised in order to introduce mutations into cDNA sequences essentially as described by Higuchi (1990). Typically this recombinant PCR method consisted of 3 independent PCRs (**Fig. 2.1**). Two first reactions where done in parallel. One reaction was performed with the following pair of oligonucleotides: the forward one was normal and the reverse one was designed in a way that the desired mutation was introduced into the central portion of the primer being flanked by approximately 20 bps of normal coding sequence on each side. This reaction resulted in a PCR product that consisted of a 3'-truncated DNA sequence containing the desired mutation. In the other reaction, the reverse primer was normal and the forward one was designed in a way that the desired mutation was introduced into the central portion of the primer being flanked by 20 bps of normal DNA sequence on each side. This reaction resulted in a PCR product that consisted of a 5'-truncated DNA sequence containing the desired mutation. The products of the two reactions purified and then mixed in a third PCR reaction in which they were allowed to anneal and extend in one cycle in the absence of oligonucleotides. Normal gene-specific primers were then added

and the reaction was resumed as usually. Thus, the PCR fragments retrieved from the third reaction largely consisted of full length mutated cDNA sequences.
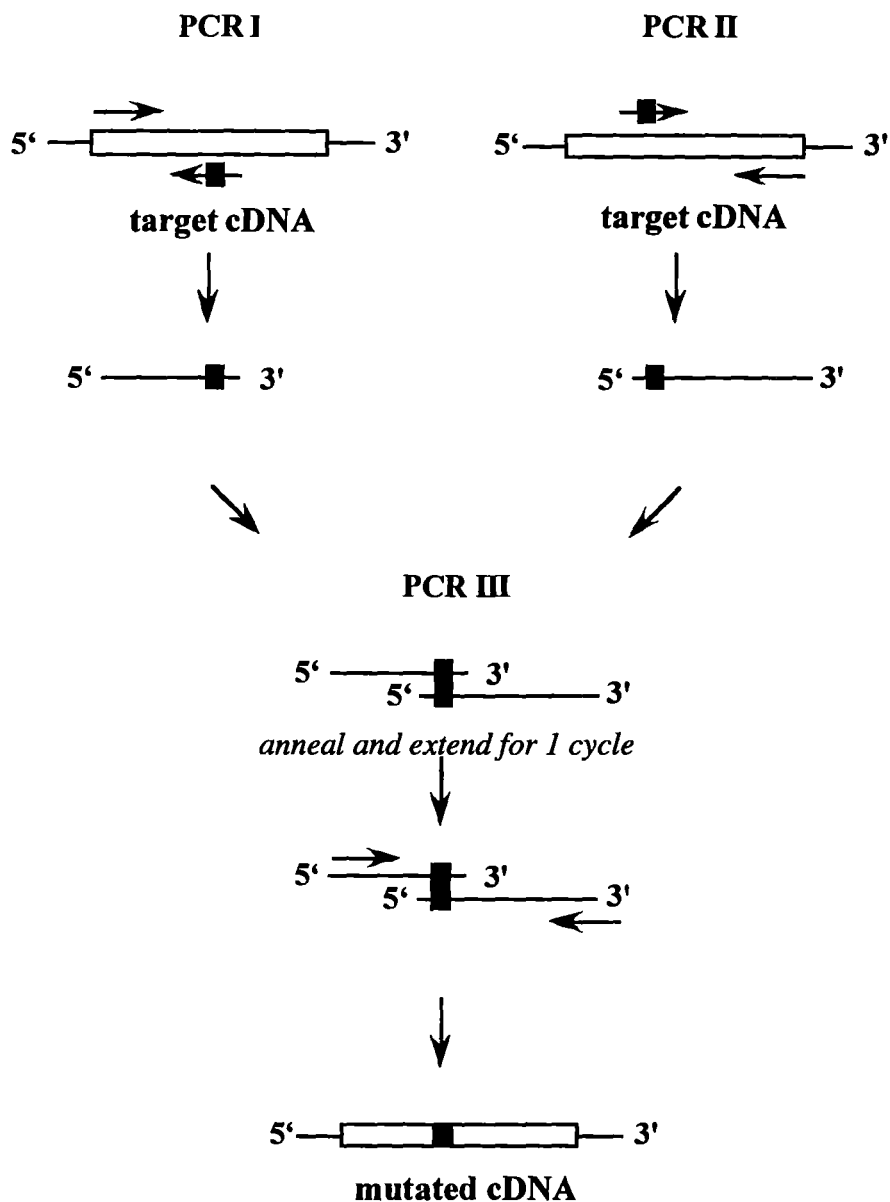
Figure 2.1 *Diagram illustrating the sequential steps of the recombinant PCR.*

2.2.8 Restriction endonuclease digestion of DNA

Usually 2-3 units of a restriction enzyme (*New England Biolabs GmbH, Frankfurt am Main,* or *Life Technologies*) were used for each µg DNA. DNA was digested at a concentration of 1 µg/10 µl in a buffer recommended by the supplier. Plasmid DNA solution was mixed with 0.1 volume restriction endonuclease. The reaction was carried out for 2 hours to overnight at 37°C (unless otherwise recommended by the supplier) and was stopped by an EasyPure kit (*Biozym Diagnostik, Hameln*) extraction. The quality of the digest was controlled by gel electrophoresis.

2.2.9 DNA ligation

All ligation reactions were performed using ligase and buffers supplied by the manufacturer (*Life Technologies*) in a total of 20 µl and incubated overnight at 14°C, followed by heat inactivation of the ligase at 70°C for 5 minutes before storing at − 20°C.

2.2.10 Sub-cloning

The cloned fragment of DNA was released from the vector using appropriate restriction endonucleases, purified by agarose gel electrophoresis and subsequently cloned into the new vector using compatible sites or through blunt end ligation.

2.2.11 Size separation of nucleic acid by agarose gel electrophoresis

The required amount of agarose (*SeaKem, Biozym Diagnostik, Hameln,* final concentration between 0.7 and 2%) was dissolved in 100 ml electrophoresis buffer (TBE: 90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Ethidium bromide was added at a concentration of 0.3 µg/ml. The molten gel was poured into a horizontal chamber. Combs with the appropriate number and size of the teeth were used to make the loading slots. Once the gel was set, electrophoresis buffer was added and the gel was run at 35-45 mA (50-100 V) at room temperature for the required time. Samples were loaded onto the gel in loading buffer (10 mM EDTA, 10% glycerol, 0.1% SDS, 0.02% bromophenol blue). DNA was visualised by transillumination with 302 nm ultraviolet radiation.

2.2.12 Isolation/purification of DNA from agarose gels

To isolate an appropriate DNA fragment from an agarose gel, Easy Pure DNA Purification Kit (*Biozym Diagnostik GmbH*) was used essentially as recommended by the manufacturer. Briefly, the DNA band of choice was cut out from the gel under long wave UV light with the aid of a scalpel. The gel piece containing DNA was melted at 65°C in the appropriate buffer and the DNA-binding resin was added. After two subsequent washing steps the resin with bound DNA was air-dried and the DNA eluted by addition of bi-distilled H_2O. The presence of the DNA fragment in solution was confirmed by agarose gel electrophoresis.

2.2.13 *E. coli* strain maintenance

A single colony of the DH5α was streaked onto a 2xTY-agar (Sambrook *et al.*, 1989). Then cells were grown overnight in incubator at 37°C and stored at 4°C for a month.

2.2.14 Growth of *E. coli* culture in liquid medium

An appropriate volume of 2xTY medium (Sambrook *et al.*, 1989) containing ampicillin (100 µg/ml) was inoculated with a single stock colony of *E. coli*. Cells were grown overnight in a 37°C incubator, with shaking at 250 rpm.

2.2.15 Preparation of competent bacteria (*E. coli*)

2.2.15.1 Chemically competent E. coli (calcium chloride method)

A single colony of *E. coli* DH5α was taken to inoculate 5 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and allowed to grow overnight at 37°C with shaking (250 rpm). Then 4 ml was removed and added directly to 400 ml of LB medium. The bacteria were grown to an OD_{590} of 0.375 and then incubated on ice for 10 min. The bacteria were sedimented by centrifugation (without brake) at 3,600 x g for 7 min at 4°C. The pellet was carefully re-suspended in 20 ml of ice cold 0.1 M $CaCl_2$ and allowed to stand on ice for a further 10-15 min. The cells were centrifuged again under the same conditions and re-suspended in a further 20 ml of ice cold $CaCl_2$. This process was repeated once more and the final pellet re-suspended in 2 ml of ice-cold $CaCl_2$ with 10% glycerol. After a short period on ice, the bacteria were dispensed in 100 µl aliquots in

pre-chilled reaction tubes and snap-frozen in liquid nitrogen before storing at – 80°C.

2.2.15.2 Electrocompetent E. coli

As above, a single colony of *E. coli* DH5α was taken to inoculate 1 ml of YENB medium (7.5 g/l Bacto yeast extract, 8.0 g/l Bacto Nutrient broth) and the culture grown overnight at 37°C with shaking. The use of this medium is very important as it eliminates all the steps needed to remove the salts. Salts are known to reduce the efficiency of electro-transformation and cause arcing in the electroporation cuvette. 500 ml of fresh YENB medium was inoculated with the 1 ml overnight culture. Bacteria were grown at 37°C with shaking and harvested when the OD_{600} was between 0.5 and 0.9. To harvest cells, the bacteria were chilled on ice and centrifuged at 4,000 x g for 10 min at 4°C. The medium was discarded and the pellet was washed in 100 ml of cold water twice and centrifuged as previously described. The supernatant was discarded and the bacteria re-suspended in 10 ml of cold 10% glycerol, centrifuged and the supernatant discarded. Cells were re-suspended in a final volume of 2 ml cold 10% glycerol. The cell number in the suspension was 1.5-3 x 10^{10} cells/ml. To freeze competent cells, they were aliquoted into reaction tubes (40 μl/tube and placed on dry ice until frozen. Frozen electrocompetent cells were stored at – 80°C and thawed on ice before use.

2.2.16 Transformation of *E. coli*

2.2.16.1 Chemically

This type of transformation was used for propagation of different plasmids as follows. A DNA sample (0.01-1 μg) was added to 45 μl $MgCl_2$/5 mM Tris-HCl, pH7.4, followed by 100 μl of the ice-thawed chemically competent cells. After brief vortexing and incubation on ice for 1 hour the cells were subsequently heat-shocked for 5 minutes at 37°C and incubated on ice for another 1 min. The transformed *E. coli* cells were finally mixed with 400 μl 2xTY medium, incubated at 37°C for 1 hour, plated onto 2xTY-agar plates supplemented with ampicillin (100 μg/ml) and grown overnight at 37°C.

2.2.16.2 Electroporation

This type of transformation was used for ligation reactions as follows. A 2 µl sample of ligation mixture was added to 40 µl of the ice-thawed electrocompetent cells. The resulting mixture was placed in a pre-cooled electroporation cuvette. The cells were electroporated at 1.8 kV using a Rad Gene Pulser (*BioRad GmbH, Munich*). The cells were then immediately re-suspended in 450 µl SOC medium (SOB medium supplemented with 0.36% glucose) pre-warmed to 37°C. After incubation at 37°C for 30 minutes with shaking, the transformed *E. coli* cells were finally plated onto 2xTY-agar plates containing ampicillin (100 µg/ml) and grown overnight at 37°C.

2.2.17 Plasmid DNA preparation

2.2.17.1 Small scale method - 1

A 1.5 ml overnight culture of *E. coli* cells harbouring an appropriate plasmid was transferred to a microfuge tube and centrifuged at 13,000 rpm for 5 min. The bacterial cell pellet was re-suspended in 90 µl GTE buffer (50 mM glucose, 25 mM Tris, pH8.0, 10 mM EDTA), followed by the addition of 180 µl SDS/NaOH lysis buffer (1% SDS, 0.2 M NaOH) and thorough mixing. Subsequently 135 µl potassium-acetate buffer (3 M potassium acetate, 2 M acetic acid) was added, followed by thorough mixing and centrifugation at 13,000 rpm for 5 min. The supernatant was decanted into a fresh tube containing 1 ml ethanol. After mixing the sample was centrifuged at 13,000 rpm for 10 min. The pellet was re-suspended in 100 µl distilled water, followed by the addition of 100 µl 5 M LiCl. After mixing and incubation on ice for 15-30 min, the sample was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and mixed with 500 µl ethanol. After centrifugation at 13,000 rpm for 10 min the pellet containing plasmid DNA was re-suspended in 20 µl TE (10 mM Tris-HCl, pH7.4, 1 mM EDTA, pH8.0) containing 10 µg/µl RNase.

2.2.17.2 Small scale method - 2

A 1.5-ml overnight culture of *E. coli* cells harbouring an appropriate plasmid was transferred to a microfuge tube and centrifuged at 13,000 rpm for 1 min. The bacterial cell pellet was re-suspended in 200 µl TELT buffer (50 mM Tris-HCl, pH8.0, 62.5 mM

EDTA, 2.5 M LiCl, 0.4% Triton X-100), followed by the addition of 20 μl lysozyme solution (10 mg/ml in TE buffer) and thorough mixing with a pipette. After incubation for 3 min at 96°C and subsequent cooling on ice for 5 min, the lysate was centrifuged for 8 min at 13,000 rpm. To precipitate DNA, the cleared lysate was transferred to a fresh tube, 100 μl isopropanol was added and the resultant mix was thoroughly vortexed. After centrifugation at 13,000 rpm for 5 min the pellet containing plasmid DNA was washed once with 70% ethanol and air-dried. The plasmid DNA was finally re-suspended in 20 to 50 μl TE buffer containing 10 μg/μl RNase.

2.2.17.3 Large scale plasmid preparation

A 200-ml overnight culture of *E. coli* cells harbouring an appropriate plasmid was centrifuged at 4,000 rpm for 10 min. The cell pellet was then used for the plasmid DNA preparation. Plasmid DNA was prepared on a large scale using either the Nucleobond AX kit PC 500 (*Machery-Nagel GmbH & Co. KG*) or the Quiagen Plasmid Maxi Kit (*Quiagen*) following the manufacturer's instructions. The resultant DNA preparation was further purified using phenol/chloroform extraction followed by ethanol precipitation.

2.2.18 Sequencing of double-stranded template DNA

2.2.18.1 Automated (fluorescence) DNA sequencing method

The VISTRA Thermo Sequenase Pre-mixed Cycle Sequencing *Kit (Amersham Pharmacia Biotech)* was used to routinely sequence cloned DNA on both strands. Briefly, 1 μg DNA was mixed with 4 pmol of an appropriate Texas-red-labelled primer (e.g. T7 Forward universal primer). From this mixture 6 μl was aliquoted into each termination vial ('G', 'A', 'T', 'C') containing 2 μl of the corresponding ddNTP mix (i.e. ddGTP, ddATP, ddTTP and ddCTP) containing all the necessary reaction components such as polymerase and dNTPs plus the actual ddNTPs. The reaction was cycled in a PCR machine using the following parameters: 94°C, 1 min, 1 cycle; 94°C, 30 sec, 50°C, 30 sec and 72°C, 30 sec for a total of 25 cycles. Upon completion, 3 μl loading buffer was added to each reaction mix and the volume of the reaction was reduced to 3 μl by drying in a vacuum centrifuge. The samples were loaded on a RapidGel-XL-6% gel (*Amersham*) and run with TBE buffer in a DNA Sequencer 725 (*Molecular Dynamics &*

Amersham) for 12 hours. The sequencing data were analysed using Molecular Dynamics software.

2.2.18.2 Manual (radioactive) DNA sequencing method

The Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit *(Amersham Pharmacia Biotech)* was used for the sequencing of DNA templates. The kit combines two revolutionary innovations for sequencing DNA. First, the label is incorporated into the DNA reaction products by the use of four (^{33}P)-ddNTP terminators and secondly, the use of an engineered Thermo Sequenase DNA polymerase that allows for efficient incorporation in cycling sequencing protocols. Sequencing of desired templates was carried out according to the manufacturer's guidelines. Approximately 1 µg of plasmid DNA was taken together with 3 pmol of an appropriate sequencing primer with 8 U of the Thermo Sequenase polymerase in a total volume of 20 µl. From this mixture 4.5 µl was aliquoted into each 'termination' PCR vial ('G', 'A', 'T', 'C') containing a mixture of 2 µl dGTP and 0.5 µl (^{33}P)-ddNTP and cycled in a PCR machine using the following parameters: 95°C, 30 sec, 55°C, 30 sec and 72°C, 1 min for a total of 40 cycles. The reactions were stopped by the addition of the kit stop solution. After a denaturation step (*i.e.* incubation at 70°C for 5 min), 3 µl samples were loaded in each lane and resolved over a 6% polyacrylamide 6 M urea TBE gel. Once the run had reached the desired length, the gel was removed, dried on Whatmann 3MM paper at 80°C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

2.2.19 Cell culture

All mammalian cells were maintained at 37°C in an incubator (*Forma Scientific, Labortect GmbH, Göttingen*) in 5% CO_2 and 95% air humidity. All cells were grown in petri dishes or flasks (*Greiner*) of varying sizes depending on the application. The cells were allowed to grow until a confluence of 80-90% had been reached, whereupon the cells were subsequently split by trypsinisation and re-seeded at a lower density. Trypsin treatment of cells was performed by removal of the culture medium from the cells, followed by one wash with Ca^{2+}/Mg^{2+} free PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM

Na_2HPO_4, 1.5 mM KH_2PO_4). After removal of PBS, 0.25% trypsin was applied to the cells and the cells incubated at 37°C until they became detached as observed under a low-powered microscope. Fresh medium was then directly applied and the cells re-plated at the desired density in new petri dishes. To prepare cells for storage, logarithmically growing cells from a large flask were trypsinised, harvested by the addition of medium, then the cells were centrifuged at 1,500 rpm. The medium was removed and the cells re-suspended in 90% FCS and 10% DMSO (*Fluka Chemie AG. Switzerland*) and placed in five 1 ml aliquots in cryovials. After incubation on ice for 1 hour, the cells were transferred to – 80°C for several hours before finally being stored in liquid nitrogen. To re-propagate cells, the vials were removed from the liquid nitrogen and placed at 37°C until most of the cells had thawed. The cells were then removed and mixed with 10 ml fresh medium (to remove the DMSO) followed by centrifugation and seeding on petri dishes in fresh medium. Schneider 2 (S2) Drosophila cells were obtained from *Invitrogen* and cultured at 22-24°C in a non-humidified incubator following the supplier's recommendations. All cells were routinely screened for mycoplasma contamination using the VenorGeM Mycoplasma Detection Kit (*Minerca Biolabs GmbH*, Berlin) according to the manufacturer' protocol and found to be negative.

2.2.20 Stable and transient transfection of cells

2.2.20.1 Calcium phosphate method

To transfect Schneider 2 (S2) cells both stably and transiently, the Calcium Phosphate Transfection kit (*Invitrogen*) was used. To prepare S2 cells for transfection, they were seeded in 3 ml complete DES expression medium at 10^6 cells/ml. When the cells reached a density of approximately $5x10^6$ cells/ml they were transfection competent. To prepare the transfection mix, Solution A (36 µl 2M $CaCl_2$, 19 µl 1µg/µl expression vector DNA, 1 µl selection vector (in the case of the stable transfection) and H_2O up to 300 µl) was added drop-wise to Solution B (300 µl 2x HBS) and incubated at room temperature for 30 min. After the combined solution was thoroughly mixed it was drop-wise added to the cells. The calcium phosphate medium was removed from the cells after 12-14 hours and replaced with fresh medium. After further incubation of the cells for 48 hours the cells were either analysed for the expression of the gene of interest (in the case of transient

transfections) or placed under appropriate antibiotic selection (400 µg/ml hygromycin) to generate stably transfected clones. After a period of 2-3 weeks, hygromycin-resistant S2 cell clones were pooled together and a mass culture expressing the desired recombinant protein was thus established.

2.2.20.2 Lipofection

To stably transfect PAE cells with the murine VEGFR-3 expression construct, GenePORTER Transfection Reagent (*Peqlab*) was used. The transfection protocol was supplied by the manufacturer and optimised for the given cell line. Briefly, a mixture of two solutions A and B (Solution A: 4 µg plasmid DNA in 500 µl serum-free F-12 medium; Solution B: 20 µl GenePORTER in 500 µl serum-free F-12 medium) was prepared and incubated for 30 min at room temperature.

The mix was added to 60% confluent PAE cells grown in 6-well dishes after the removal of medium from the cells. The cells were incubated with the transfection medium for 3-5 hours. After that 1 ml F-12/20% FCS medium was added and the cells were incubated for a further 12-14 hours. Following the addition of fresh F-12/10% FCS medium, the cells were incubated for another 24 hours. To generate clones, cells were plated into several petri dishes and cultured in selection medium (400 µg/ml neomycin) until resistant cells appeared. Individual clones were picked, expanded and analysed for the expression of the gene of interest by Western blot.

2.2.21 Purification of recombinant His-tagged proteins

Recombinant His-tagged proteins were purified from insect cell medium supernatants using Ni-NTA agarose (Quiagen) essentially as described by the manufacturer. Stable S2 transfectants expressing a particular His-tagged protein were cultured as recommended by the supplier. To facilitate the purification procedure, the cells were grown in serum-free insect medium (*Invitrogen*) containing no selective antibiotic for 6 days before the purification. The cell supernatant containing the His-tagged protein (as indicated by Western blot analysis) was collected after pelleting the S2 cells by centrifugation at 1,500 rpm for 20 min. The medium supernatant was then dialysed at 4°C overnight against 50 mM sodium phosphate buffer, pH7.4, 300 mM NaCl, 100 µM PMSF followed by the

addition of 20 mM imidazole. After dialysis, the pH of the medium was adjusted to 8.0 with 5M NaOH. Ni-NTA resin (*Quiagen*) was washed three times with Wash buffer (50 mM sodium phosphate buffer, pH8.0, 300 mM NaCl, 20 mM imidazole, 100 µM PMSF) and added to the dialysed medium at a ratio of 10 ml Ni-NTA 50% resin slurry per 280 ml dialysed medium. The mixture was loaded into 500-ml tubes and rotated for 4 hours to overnight on a tumbler to allow the resin to bind to the His-tagged proteins. Afterwards the mixture was poured into a 20-ml column and the "flow-through" fraction was collected. After washing the column three times with ice-cold wash buffer (25 ml each wash), the proteins were eluted with ice-cold elution buffer (50 mM sodium phosphate buffer, pH8.0, 300 mM NaCl, 300 mM imidazole, 100 µM PMSF) due to the high imidazole concentration. Ten 2-ml fractions were collected. Fractions which were positively evaluated in Western blot analysis were pooled and dialysed overnight at 4°C against PBS. Following protein concentration determination, the proteins were mixed with 50 µg BSA per every µg of the recombinant protein, aliquoted and lyophilised for long-term storage at − 80°C. For immediate use, an aliquot of the recombinant proteins was reconstituted with PBS at 100 µg/ml and further aliquoted to be stored at − 20°C. Repeated freeze-thaw cycling was avoided.

2.2.22 Determination of protein concentration

Protein concentration was determined using the DC Protein Assay Kit (*BioRad GmbH*, essentially a modification of the Biuret method of protein concentration determination) as recommended by the manufacturer.

2.2.23 Cell lysate preparation

Cells were grown to confluence and then removed from the petri dish by scraping using a rubber policeman, or by incubation with PBS (Mg^{2+} and Ca^{2+} free) containing 5 mM EDTA. The cells were counted with a cell counter chamber slide and re-suspended at a final concentration of 1×10^7 cells/ml in either reducing sample buffer (200 mM Tris-HCl pH 6.8, 250 mM sucrose, 2.5 mM EDTA, 0.1% (*w/v*) bromophenol blue, 2% SDS, 10% 2-mercaptoethanol or 100mM DTT) or non-reducing sample buffer (containing no 2-mercaptoethanol or DTT). Samples were boiled for 5 min and then sonicated for 15 to

20 sec to break down chromosomal DNA. 5×10^5 equivalent cell volumes were loaded into each gel slot.

2.2.24 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were electrophoretically separated on the basis of size using the method of Laemmli (1970). The resolving gel containing between 6 to 15% acrylamide (depending on the experiment) and 5% stacking gel were cast according to Sambrook *et al.* (1989). Samples were run into the stacking gel at 50 V, and then run at 100-200 V during the day or 30 V overnight.

2.2.25 Staining the SDS-PAGE gels

2.2.25.1 Coomassie staining

Gels were incubated with Coomassie stain solution (0.25% Coomassie brilliant blue, R-250, 50% methanol and 10% acetic acid) for 4-24 hours. To de-stain, the gels were incubated in 10% acetic acid; 10% methanol for 24 hours or longer with several changes of the de-stain solution.

2.2.25.2 Silver staining

Gels were silver stained using a Silver Staining Kit (*Amersham*) following the manufacturer's guidelines. The gels were then air-dried between two sheets of cellophane held in between two gel drying frames (*Promega GmbH, Mannheim*).

2.2.26 Western blotting

After proteins were separated by SDS-PAGE, they were electrically transferred onto Immobilon membrane (*Millipore*, type PVDF, pre-soaked in methanol) at 250-300 mA for at least 6 hours in transfer buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol and 0.05% (w/v) SDS). Following the completion of the transfer, in order to reduce unspecific binding of the antibodies to the PVDF membrane, the blot was incubated in TBST blocking solution (10 mM Tris-HCl, pH 8.1, 100 mM NaCl, 0.1% Tween 20) supplemented with either 4% (w/v) non-fat dried milk (for all but anti-phosphotyrosine antibodies) or 1% BSA (for anti-phosphotyrosine antibodies) at room temperature for 30

min with shaking. For detection of proteins of interest the membrane was further incubated in TBST blocking solution containing the appropriate primary antibody (at the manufacturer-recommended dilution) for 1 hour at room temperature or at 4°C for about 12 hours. After 3 washes in TBST, unless the primary antibodies were conjugated to HRP, the membrane was incubated in TBST solution containing a 1:1000 dilution of HRP-conjugated secondary antibody. Once the membrane has been washed 3-4 times 2 min each in TBST buffer, detection of specific proteins was achieved by enhanced chemiluminescence using ECL Western blotting detection reagents (*Amersham*) and ECL Hyperfilm (*Amersham*) following the manufacturer's instructions.

2.2.27 Stripping western blot membrane

To allow more than a single use of western blot membranes, the membranes were stripped in the following way. They were incubated with a Strip solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.75% 2-mercaptoethanol) at 55°C for 20 min with shaking. The membranes were then washed twice in the TBST for 2 min each time, blocked as usual and used for the normal western blot probing protocol.

2.3 Analytical Methods

2.3.1 *In vitro* tyrosine kinase assay

An ELISA-based protocol was used to screen potential RTK inhibitors *in vitro* essentially as described in Laird *et al.* (2000). In this assay, test substances were added to *in vitro* substrate phosphorylation reactions catalysed by recombinant GST-kinase fusion proteins (*KTB Tumorforschungs GmbH, Freiburg*). All tests were performed in duplicate. ELISA 96-well plates (*Immunon II, Dynex*) were coated with 20 μg (100 μl)/well substrate (poly-Glu,Tyr$_{4:1}$) diluted in 100 mM sodium bicarbonate, pH 9.6 buffer. Plates were coated at room temperature overnight in a humidified sandwich box. The substrate solution was then removed, the plates washed twice in TBS (10 mM Tris-HCl, pH8.1, 100 mM NaCl) buffer and blocked by incubation with 150 μl/well 5% BSA/TBS for a minimum of 30 min. Again the plates were washed twice with TBS. The reaction mix was prepared by combining (in each well) 50 μl test substance (2 or 20 μg/ml in 10%

DMSO), 25 µl 4x kinase dilution buffer (200 mM HEPES, 100 mM NaCl, 80 µM Na$_3$VO$_4$ and 0.04% BSA) containing an appropriate amount of recombinant GST-kinase and 25 µl 160 µM ATP (diluted in 40 mM MnCl$_2$). Hence, the final concentration of the test substances in the assay was either 1 or 10 µg/ml in 5% DMSO. The addition of ATP immediately triggered the reaction that was performed at 30°C for 90 min. To stop the reaction, 50 µl/well 30 mM EDTA was added. After the plates were washed twice with 0.05% Tween 20/TBS buffer, 100 µl/well anti-phosphotyrosine antibody diluted (1:500) in 0.05% Tween 20/TBS buffer (supplemented with 0.5% BSA, 0.025% non-fat dry milk, and 100 µM Na$_3$VO$_4$) was added and the plate incubated for 1 hour at 37°C. The plates were washed three times with 0.05% Tween 20/TBS. Secondary HRP-conjugated antibody (1:1000) in 0.05% Tween 20/TBS buffer (supplemented with 0.5% BSA, 0.025% non-fat dry milk, and 100 µM Na$_3$VO$_4$) was added. After another incubation for 1 hour at 37°C, the plates were washed three times with 0.05% Tween 20/TBS and 100 µl/well ABTS substrate (*Roche*) was added. After the green colour had developed, the plate was analysed by use of an ELISA plate reader (405 nm filter).

2.3.2 Cellular phosphorylation assay

In order to determine the degree of RTK phosphorylation, PAE, PAE/VEGFR-1, PAE/VEGFR-2 or PAE/VEGFR-3 were seeded into 15-cm tissue culture plates and grown to 50% confluence. Cells were then serum-starved with serum-free medium (supplemented with 0.2% BSA) for 16-24 hours (PAE, PAE/VEGFR-1 and PAE/VEGFR-3) or 72 hours (PAE/VEGFR-2). After 30 min pre-incubation with 5-ml serum-free medium containing 1 mM Na$_3$VO$_4$, or 1 hour – with 5 ml serum-free medium containing inhibitors (0, 0.5, 5 and 50 µM), 1 mM Na$_3$VO$_4$, and 5% DMSO, the cells were stimulated at 37°C for 5 min (VEGFR-1, VEGFR-2) or 8 min (VEGFR-2) with the corresponding RTK ligands (*e.g.* S2 transfectant conditioned medium or purified recombinant growth factors at 200-400 ng/ml). In the inhibitor experiments, the ligand-containing medium was also supplemented with the test inhibitor and 5% DMSO. Afterwards, the cells were quickly washed twice with ice-cold PBS (supplemented with 100 µM Na$_3$VO$_4$). Ice-cold modified RIPA buffer (30 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 0.5% (*v/v*) Triton X100, 0.5% (*w/v*) sodium desoxycholate, 10 mM

NaF) freshly supplemented with 1 mM PMSF, 0.1 U/ml aprotinin, 10 ng/ml leupeptin and 5 mM Na$_3$VO$_4$ was added to lyse the cells. The cells were scraped off the plates with a rubber-policeman and collected into centrifuge tubes on ice. The lysates were solubilised by pushing them through a syringe fitted with a 25G needle and centrifuged at 4°C at 13,000 rpm for 15 min to remove the insoluble matter. The cleared lysates were incubated with 4-μg appropriate anti-receptor antibody at 4°C overnight. To precipitate the receptor-antibody complexes, 30-40 μl/tube 50% slurry of protein A-sepharose (*Amersham*) was added and the incubation at 4°C was resumed for a further 2 hrs. The beads were then centrifuged for 1 min at 4°C and washed three times with ice-cold wash buffer (30 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 0.05% (v/v) Triton X100, 0.5% (w/v) sodium desoxycholate, 10 mM NaF) freshly supplemented with 1 mM PMSF, 0.1 U/ml aprotinin, 10 ng/ml leupeptin and 5 mM Na$_3$VO$_4$. The remaining wash buffer was removed by suction with a 1-ml syringe fitted with a 27G needle. Finally, the beads were re-suspended in 50 μl SDS-PAGE loading buffer, boiled and loaded on a 6% SDS-PAGE gel. After the proteins released from the beads had been resolved on the SDS-PAGE, they were blotted as described in the western blotting protocol. The blots were first analysed by probing with anti-phosphotyrosine antibodies, then the membranes were stripped and re-probed with specific anti-receptor antibodies to control loading of the samples.

2.3.3 Proliferation assay: (^{3}H)-thymidine incorporation

100 μl cells were seeded into each well of a 96-well cell culture plate at 10^5 cells/ml and incubated at 37°C for approximately 24 hours. The cells were then starved for 24 hours with 50 μl/well corresponding serum-free medium. To stimulate the cells, serial dilutions of 2x-concentrated recombinant growth factors in serum-free medium were added to cells at 50 μl/well. In the experiments with inhibitors, cells were pre-incubated for 2 hrs with serial dilutions (0-10 μM) of the inhibitors and then stimulated with 50 μl of either normal culture medium or serum-free medium containing a 2x-concentrated specific growth factor and serial dilutions of the 2x-concentrated inhibitor. To study anti-proliferative properties of hyperforin, cells were stimulated with 50 μl of normal culture medium containing serial dilutions (0-200 μg/ml) of 2x-concentrated hyperforin or of

10% DMSO.

Each sample was performed in triplicate or quadruplicate. After a 24-hour incubation, (^{3}H)-thymidine was added to the cells at 1 µCi/well and the incubation was resumed at 37°C for another 4-6 hours. To analyse the amount of incorporated radioactivity, the cells were trypsinised for 30 min and harvested onto a glass fibre filter (*Wallac Oy, Turku, Finland*) with the aid of a Harvester 96 cell harvester (*Tomtec, Hamden, USA*). The filter-immobilised radioactivity was counted using scintillation liquid and a MicroBeta TriLux Liquid Scintillation and Luminescence counter (*Wallac*).

2.3.4 Apoptosis detection assay

To detect apoptosis and quantify the pro-apoptotic effect of different substances, the Cell Death Detection ELISA^{PLUS} kit (*Roche Diagnostics GmbH, Mannheim*) was used. It is a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) produced after programmed cell death (apoptosis). Cells (10^5 cells/ml) were seeded into a 96-well cell culture plate at 50 µl/well and incubated at 37°C for approximately 24 hours. Then different 2x-concentrations of test substances diluted in normal growth medium were applied to the cells at 50 µl/well. Each sample was performed in duplicate. After 24-hour incubation apoptosis was detected and quantified following the kit manufacturer's recommendations.

2.3.5 Cell cycle analysis

Mg^{2+} and Ca^{2+}-free PBS was used throughout the experiment. Approximately 10^6 cells were harvested by trypsinisation and washed once with PBS. The cells were then re-suspended in 100 µl PBS supplemented with 5% FCS. The cells were fixed by the addition of 100 µl 8% formaldehyde solution in PBS (containing 2% methanol) and incubation for 10 min at room temperature. Afterwards cells were permeabilised by the addition of 33 µl 0.5% Tween 20 and further incubated for 10 min at room temperature. The cells were the washed once with 1 ml PBS supplemented with 5% FCS by centrifugation at 5,000 rpm for 3 min and re-suspended in 100 µl PBS supplemented with 5% FCS and 0.05% Tween 20. After the cellular DNA was stained by the addition of 1 µl

4 µg/ml Höchst 33258 solution, the cell cycle was analysed by measuring the DNA contents using a FACStar flow cytometer (Becton Dickinson).

2.3.6 Matrtigel-based *in vivo* angiogenesis assay

Matrigel, a soluble basement membrane extract of the murine EHS (Engelbreth-Holm-Swarm) sarcoma, is widely used in different angiogenesis assays (Passaniti *et al.*, 1992). To test the angiogenic properties of the recombinant rat VEGFs, different amounts of the recombinant rat $\Delta N\Delta C$/VEGF-C, $\Delta N\Delta C$/VEGF-C(Cys$_{152}$Ser) and VEGF proteins (*e.g.* 10, 100 and 1000 ng) were mixed with liquid matrigel (*Becton Dickenson*) at 4°C and injected subcutaneously into the back of experimental rats. Matrigel rapidly solidified at body temperature, trapping the factors to allow their slow release to surrounding tissues. After 14 days the matrigel was extracted and examined for the presence of newly grown vessels.

2.3.7 Induction of lymphangiomas *in vivo*

Lymphangiomas were induced in different strains of rats essentially as described by Mancardi *et al.* (1999). Briefly, incomplete Freund's adjuvant in PBS (1:1, *v:v*) was injected intraperitoneally with a subsequent boost after a 14-day period. After an additional period of 7 days, the rats were sacrificed and their peritonea examined for the presence of lymphangiomas.

2.3.8 Tissue embedding and preparation of sections

The methods described in this section are modifications of established protocols (*e.g.* Prophet *et al.*, 1994).

2.3.8.1 Paraffin sections

Freshly isolated organs or tissue were cut into 4-5 mm pieces, placed into cassettes and fixed in 4% paraformaldehyde (in PBS) in a refrigerator for a minimum of 72 hours. The cassettes with tissue samples were then washed in PBS and embedded into paraffin using a Shandon Hypercenter XP embedding machine (*Life Sciences International (Europe) Ltd, UK*). Following mounting, 6 µm sections were prepared from the tissue-

containing paraffin blocks using the RM 2155 microtome (*Leica Instruments GmbH, Nussloch*). After drying the sections overnight they were stored at room temperature for future use.

2.3.8.2 Frozen sections

Freshly isolated organs or tissue were placed into a metal tray filled with Polyfreeze tissue freezing medium (*Polysciences, Inc, Warrington, USA*). The latter was allowed to solidify by placing the tray onto dry ice. Frozen blocks were removed from the metal trays and used to cut 10 μm sections with the aid of a Cryocut 1800 cryostat microtome (*Leica*). To fix the tissue, the sections were first air-dried for 1 hour, then incubated in ice-cold acetone for 10 min. The sections were air-dried again for 1 hour and then stored at 4°C (for 1-2 months) or − 20°C (for a longer term).

2.3.9 Tissue section staining

2.3.9.1 Staining with hemotoxylin and eosin (H&E staining)

To perform H&E staining, the paraffin sections were used. Tissue was deparaffinised by successive washes in xylene (2 times, 5 min each), 100% ethanol (1 min), 95% ethanol (2 times, 1 min each), 70% ethanol (1 min). After washing the slides with tap water for 2 min, the slides were incubated in 0.1% hemotoxylin solution (*Sigma*) for 1 min and washed again with tap water for 5 min. Then the slides were incubated in alcoholic eosin Y solution (eosin Y, 0.1% w/v; phloxine B, 0.1%, w/v; ethanol, 90%, v/v) for 1 min, washed in 70% ethanol (2 times, 1 min each), 95% ethanol (2 times, 1 min each), 100% ethanol (2 times, 1 min each) and xylene (2 times, 1 min each). Stained tissue sections were covered with glass slips using Immu-mount mounting medium (*Life Sciences International*) and allowed to dry.

2.3.9.2 Masson's trichrome staining

Staining with Masson's trichrome was primarily used to distinguish vessel structures from matrigel. The staining procedure was performed using the Accustains Trichrome Stains (Masson) kit (*Sigma*) following the manufacturer's guidelines. Briefly, the tissue section slides were deparaffinised as above and incubated overnight in Bouin's solution

60

(*Sigma*). After extensive washes in running tap water the slides were stained in Working Weigert's Iron Hematoxylin Solution (*Sigma*) for 5 min, washed in running tap water for 5 min and rinsed in deionised water. Staining with Biebrich Scarlet-Acid Fucshin for 5 min was performed and the slides were rinsed again in running tap water. After the subsequent 5-min staining with Working Phosphotungustic/Phosphomolybdic Acid Solution, the slides were placed for 5 min in Aniline Blue Solution. After the final 2-min incubation in 1% acetic acid the slides were rinsed in water, dehydrated through 70% ethanol (2 times, 1 min each), 95% ethanol (2 times, 1 min each), 100% ethanol (2 times, 1 min each) and cleared in xylene (2 times, 1 min each). The slides were covered with Micromount xylene-based mounting medium (*Surgipath*, Richmond, USA) and glass slips after which they were allowed to dry.

2.3.9.3 Immmunohistochemistry (IHC)

In order to detect expression of specific proteins in tissue sections, IHC was performed with minor modifications essentially as described elsewhere (Prophet *et al.*, 1994). In brief, paraffin-embedded tissue sections where deparaffinised as above, then extensively washed in PBS. Frozen sections where placed in PBS to dissolve the freezing medium, then also extensively washed in PBS. To unmask antigens, when necessary, sections were heated in a microwave with antigen unmasking solution (*Vector Laboratories, Inc., Burlingame, USA*) for 20 sec, then incubated with the hot antigen unmasking solution at room temperature for 10-15 min, washed twice with water and once with PBS. Tissue sections were then outlined with the DAKO pen (*DAKO*) (which allows small aliquots (100-300 μl/ section) of antibodies to be used) and covered for 15 min with blocking solution (10% normal goat serum in PBS). After a brief wash with PBS, primary antibody solution in PBS was applied and sections were incubated at room temperature for 1 hour. To remove unbound antibodies, the sections were washed three times with PBS (2 min each). The sections were then incubated for 15 min with 3% (v/v) H_2O_2 (in PBS) to quench the intrinsic peroxidase activity, then washed three times with more PBS (2 min each). Biotinylated secondary antibody in PBS was then applied to the sections for 30 min and washed off with PBS (3 times, 2 min each). Streptavidin-biotin-peroxidase complex was prepared 30 min in advance (Peroxidase standard kit, *Vector Laboratories*).

It was applied to the sections for 30 min and then washed off with PBS (3 times, 2 min each). Colour development was achieved by using the AEC peroxidase substrate kit (*Vector Laboratories*). The sections were incubated with the kit solutions (which were mixed according to the manufacturer's instructions) for up to 20 min until the red colour had developed. Then the sections were briefly rinsed with water and counter-stained with 0.1% hemotoxylin for 2-4 min. Excessive hemotoxylin dye was removed by extensive washing with water. The slides were finally covered with glass slips using Immu-mount mounting medium (*Life Sciences International*) and allowed to dry horizontally for 10 min before they were observed and photographed under the microscope.

2.3.10 Endothelial sprouting: *in vitro* angiogenesis assay

A modified method of Korff and Augustin (1999) was used to measure the potential of certain angiogenesis-related RTK inhibitors to suppress the growth and differentiation of blood endothelial cells *in vitro*. Endothelial cell spheroids were generated at 37°C overnight by suspending endothelial cells (750 cells/well) in appropriate culture medium containing 0.24% (*w/v*) methylcellulose (4000 centipoises) and seeding them in non-adherent round bottom 96-well plates. Once the endothelial cell spheroids had formed, 24 to 48 spheroids were collected with a pipette into a centrifuge tube, centrifuged at 1,500 rpm for 3 min and re-suspended in 0.5 ml culture medium containing 0.48% (*w/v*) methylcellulose, 100 ng/ml VEGF, 2% DMSO and 20 μM of the corresponding test substance. After that 0.5 ml collagen (made by mixing on ice 8 volumes acidic collagen extract from rat tails (equilibrated to 4 mg/ml), 1 volume of Hanks' balanced salt solution, neutralising with 1M NaOH and adding 1 volume FCS) was added. After gentle mixing, the spheroid-containing gel was rapidly transferred into pre-warmed 24-well plates and allowed to polymerise for 1 min. Growth medium (200 μl) was pipetted on top of the gel. After 3 days of incubation the outgrowth of capillary-like structures from the spheroids (endothelial cell sprouting) was documented.

2.3.11 *In vivo* tumour growth inhibition experiments

All studies with laboratory animals were performed in the accredited animal facility of the Institut für Toxicologie und Genetik, Forschungszentrum Karlsruhe GmbH and were approved by the local Ethical Review Board.

2.3.11.1 Inhibition of tumour growth by indolinones

In order to check the anti-tumour effect of indolinones, groups of 8 male and female BD10 rats (1AS cell line) or Wistar Furth (MT450 cell line) rats were injected subcutaneously with 5×10^5 tumour cells in PBS. Drug treatment was initiated either on the next day or 15 days after tumour cell injection. The animals received 4 mg/kg/day (1AS tumour experiments) or 8 mg/kg/day (MT450 tumour experiments) indolinone solution in 100% DMSO (AE87, eight per group; AE106, eight per group; MAZ51, eight per group) or the carrier alone (100% DMSO, eight per group). Daily injections of the drug/control were administered intraperitoneally for 2-4 weeks. Tumours were measured with a micrometer calliper every fifth day throughout the study (Sleeman, 1999).

2.3.11.2 Inhibition of tumour growth by hyperforin

In order to check the possible anti-tumour effect of hyperforin, female Wistar Furth rats were injected subcutaneously with 5×10^5 MT450 cells in PBS. Drug treatment was initiated 15 days after tumour cell injection, a time at which tumours were readily detectable and could easily be measured. In one experiment, the animals received 100 μl of 2 mM drug solution in 10% DMSO (hyperforin, eight per group; paclitaxel, eight per group) or the solvent (10% DMSO, eight per group). In the other experiment, the animals received 100 μl of 4 mM drug solution in nanoemulsion (hyperforin, eight per group) or the carrier alone (nanoemulsion E80 Mig 23201: 2.8 mg/ml E80 lipid, 7 mg/ml miglyol 812, 0.1 mg/ml tocopherol; eight per group). Hyperforin and paclitaxel were kindly supplied by Dr. Christoph Schempp. Daily injections of the drug/control were administered subcutaneously at the site of the tumour cell injection for two weeks. Tumours were measured with a micrometer calliper every fourth-fifth day throughout the study (Sleeman, 1999).

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Chapter 3

Results

PART I

Creation of a cellular system for analysing anti-tumour substances with anti-angiogenic and/or anti-lymphangiogenic properties

For cancer therapy, it would be desirable to identify substances capable of blocking either tumour growth directly or inhibiting tumour-host interactions such as angiogenesis and lymphangiogenesis. The members of VEGFR tyrosine kinase family are expressed on the surface of the vascular and lymphatic endothelial cells, where they play pivotal roles in both physiologic and pathologic angiogenesis and lymphangiogenesis. Antibodies or synthetic compounds that block VEGFR-2 activation, for instance, have been shown to exert anti-angiogenic effect, emphasising the role of this receptor in angiogenesis (Witte *et al.*, 1998; Vajkoczy *et al.*, 1999; Drevs *et al.*, 2000). Targeting the receptors of the VEGFR family has, therefore, the potential to interfere with the tumour-induced angiogenesis and lymphangiogenesis (Saaristo *et al.*, 2000).

Small chemical compounds such as indolinones have been shown to block different RTKs including VEGFR-2 (Mendel *et al.*, 2000; Sun *et al.*, 2000). I therefore decided to look through a panel of synthetic indolinones made in the Institut für Organische Chemie, Karlsruhe University, in order to identify VEGFR-2 and VEGFR-3 inhibitors. In order to screen for indolinones interfering with VEGFR signalling, a cellular system in which the functional VEGF receptors could be expressed in an appropriate cellular context and which could be specifically activated was necessary. Activation of VEGFRs with specific ligands in the presence of inhibitory substances would allow one to measure the effects of the VEGFR inhibition such as decreased receptor phosphorylation and ensuing deficiency in endothelial cell survival, proliferation, migration, and differentiation.

To create such a system, I aimed to produce specific ligands for VEGFRs, namely VEGF, VEGF-C and VEGF-D, in a *Drosophila* expression system (DES). To do this, cDNAs encoding the VEGF proteins are to be cloned into a specific DES vector. When stably transfected into *Drosophila* cells, the vectors are expected to express the desired proteins at high levels. This can subsequently permit purification of the recombinant VEGF proteins via an engineered C-terminal polyhistidine tag. The expressed growth factors should be used to activate their cognate receptors expressed

on endothelial cells. These factors could therefore be employed to screen for potential inhibitors, and also possibly be useful for establishing endothelial cells in culture for other studies.

VEGF-C and VEGF-D can bind to and activate two receptors VEGFR-2 and VEGFR-3 (Joukov *et al.*, 1997; Stacker *et al.*, 1999). Therefore, as a tool to discriminate between activation of these two receptors, I also aimed to make mutated VEGF-C and VEGF-D proteins in which one of the cysteines would be replaced by serine to resemble the published VEGFR-3-specific VEGF-C mutant (Joukov *et al.*, 1998). These mutated forms should be specific activators of VEGFR-3, and thus only induce LE proliferation. These properties would be useful for the establishment of LE in culture.

3.1 Cloning, expression and purification of recombinant rat VEGFs

The rat system was chosen for the current study due to the availability of well-characterised rat tumour models that can be relevant in studying eventual anti-tumour effects of the anti-angiogenic substances. Therefore, in order to screen the compounds on the basis of their ability to inhibit activation of VEGFRs mediated by their specific ligands, rat VEGF-C, VEGF-D and VEGF were cloned and subsequently expressed. The *Drosophila* expression system was chosen to produce the recombinant rat VEGFs as it has been reported to be both simple and reliable, yielding large amounts of the recombinant protein and supporting its post-translational modifications.

3.1.1 Cloning rat VEGFs

To create biologically active recombinant rat VEGF-C and VEGF-D proteins, parts of their respective cDNAs encoding the VEGF-homology domain (VHD) were cloned into the pAc5.1 expression vector (*Invitrogen*) to permit expression of the proteins in *Drosophila* S2 cells. The encoded proteins represent the fully processed forms of VEGF-C lacking the N- and C-termini that exhibit highest affinity for VEGFR-3 and also activate VEGFR-2 ($\Delta N\Delta C$ proteins). Additional constructs, in which point mutation converting the codon encoding cysteine 152 (or cysteine 141, in case of VEGF-D) to serine was introduced into the $\Delta N\Delta C$/VEGF-C and $\Delta N\Delta C$/VEGF-D constructs to create $\Delta N\Delta C$/VEGF-C(Cys$_{152}$Ser) and $\Delta N\Delta C$/VEGF-D(Cys$_{141}$Ser), respectively, were produced as it has been reported that such a mutation abrogates the

ability of the fully processed form of VEGF-C to activate VEGFR-2 (Joukov *et al.*, 1998). The leader sequence of the *Drosophila* BiP protein was engineered at the N-terminus of the ΔNΔC constructs to ensure secretion of the recombinant protein into the culture medium. To produce rat recombinant VEGF, a cDNA encoding rat VEGF$_{190}$ isoform was cloned into the pAc5.1 expression vector, too. All constructs contained six consecutive histidine residues at the C-terminus of the protein to permit easy detection and purification. The cloning was done in steps that are described below (**Table 3.1**).

Construct	Description
pAc5.1/His/VEGF	pAc5.1 encoding rat VEGF
pAc5.1/His/VEGF-C	pAc5.1 encoding full-length rat VEGF-C
pAc5.1/His/VEGF-D	pAc5.1 encoding full-length rat VEGF-D
pAc5.1/His/BiP/ΔNΔC/VEGF-C	pAc5.1 encoding mature rat VEGF-C
pAc5.1/His/BiP/ΔNΔC/VEGF-C(Cys$_{152}$Ser)	pAc5.1 encoding Cys mutant of mature rat VEGF-C
pAc5.1/His/BiP/ΔNΔC/VEGF-D	pAc5.1 encoding mature rat VEGF-D
pAc5.1/His/BiP/ΔNΔC/VEGF-D(Cys$_{141}$Ser)	pAc5.1 encoding Cys mutant of mature rat VEGF-D

Table 3.1 *DES constructs used to produce recombinant rat VEGFs.*

3.1.1.1 Cloning rat VEGF-C, VEGF-D and VEGF

Firstly, the full-length VEGF-C and VEGF-D constructs were made. To this end, their corresponding cDNAs were amplified by PCR from the commercially available rat spleen marathon cDNA (*Clontech*) using gene-specific oligonucleotide pairs as follows: *VEGF-C* (5'-G CGA ATT C<u>GG ACC GGC CTC CTC GCT CCC</u>-3' and 5'-G CAC CGG <u>TGT TCA GAT GTG GTC TTT TCC AAT ATG</u>-3'), *VEGF-D* (5'-GC GAA TTC <u>AAA CAA CTG CTT AGC CAT CAG TGG</u>-3' and 5'-GC ACC GGT <u>AGG GGA CAG TGA AAA GAC CAT TGA</u>-3'). The amplified cDNAs therefore contained cDNA sequences (underlined) flanked by restriction endonuclease consensus sequences (*i.e.* EcoRI and AgeI) allowing their subsequent cloning into EcoRI/AgeI sites of DES vectors. Thus after restricting the PCR products, *VEGF-C* and *VEGF-D* were successfully cloned into pAc5.1/V5-HisA vector (*Invitrogen*) as shown in **Figure 3.1**.

Rat *VEGF* was cloned in a similar same way (**Fig. 3.1**) using the following primer pair: 5'-GCG AAT TC<u>A ACC ATG AAC TTT</u> CTG CTC TCT TGG-3' and 5'-G

CAC CGG T<u>CC GCC TTG GCT TGT CAC ATC TGC</u>-3'. To ensure that *VEGF-C,*
VEGF-D and *VEGF* were cloned in frame with the polyhistidine tag as well as to rule
out any possible PCR mistakes, coding regions for the recombinant proteins in the
resultant constructs (pAc5.1/His/VEGF, pAc5.1/His/VEGF-C and pAc5.1/His/VEGF-
D) were thoroughly sequenced on both strands.
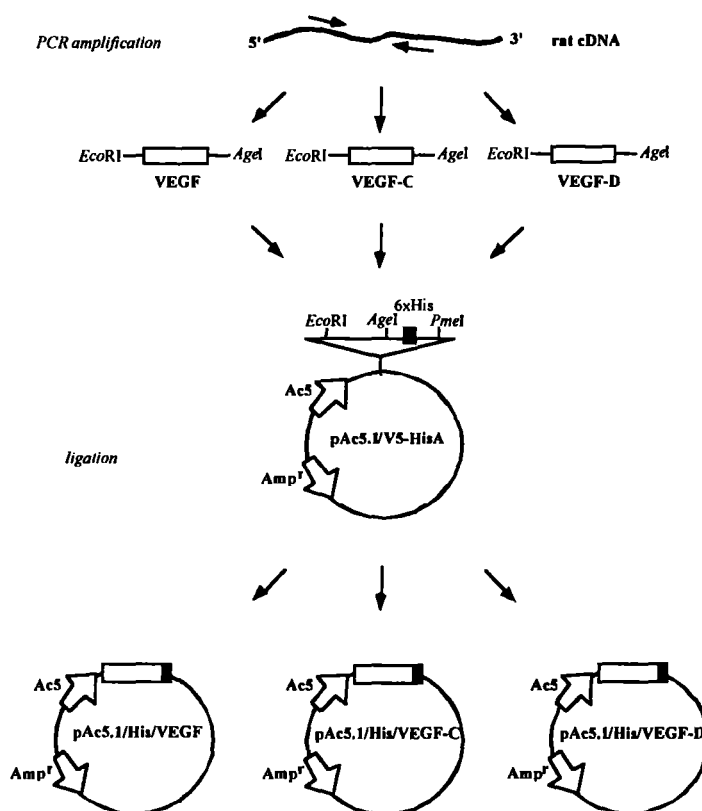
Figure 3.1 *Cloning rat VEGF-C, VEGF-D and VEGF cDNAs.*
Legend: Ac5, Ac5 promoter; Amp^r, ampicillin resistance gene; 6xHis, polyhistidine
region (see text for details).

3.1.1.2 Cloning rat ΔNΔC/VEGF-C and ΔNΔC/VEGF-C(Cys₁₅₂Ser)

To clone *ΔNΔC/VEGF-C* cDNA into pAc5.1/V5-HisA, the pAc5.1/His/VEGF-C
construct was used. cDNA corresponding to the VHD of VEGF-C was amplified by
PCR using the following oligonucleotide pairs: 5'-GCGAATTC GAT CTC AAT
<u>ATG AAG TTA TGC ATA TTA CTG GCC GTC GTG GCC TTT GTT GGC CTC</u>
<u>TCG CTC GGG</u> ACC GGT <u>GAC ACT GTA AAA CTT GCT GCT GC</u>-3' and 5'-
CCGGTTTAAAC TCA ATG GTG ATG GTG ATG ATG <u>AAT AAT TGA ATG</u>
<u>AAC TTG TCT GTA AAC</u>-3'. The resultant PCR product encompassing *Eco*RI
restriction site, *BiP Drosophila* signal sequence (dot-underlined), *Age*I restriction site,

ΔNΔC/VEGF-C (underlined), His-tag-coding sequence and *Pme*I restriction site was subsequently cloned into *Eco*RI/*Pme*I site of the pAc5.1/V5-HisA vector as described in **Figure 3.2**. The final construct was called pAc5.1/His/BiP/ΔNΔC/VEGF-C.

Recombinant PCR technique was then utilised to introduce the point mutation (*i.e.* change of Cys$_{152}$ to a Ser) into the ΔNΔC/VEGF-C polypeptide (**Fig. 3.2**). In the first step, PCR fragments were obtained in two independent reactions using the pAc5.1/His/BiP/ΔNΔC/VEGF-C construct and the following primer pairs: *reaction I* (**50-mer1** (5'-C ACA AAC ACC TTC TTT AAA CCT CCA <u>AGC</u> GTG TCC GTC TAC AGA TGT GGG G-3') and **29-mer** (5'-CCG GTT AAA CTC AAT GGT GAT GGT GAT G-3')); *reaction II* (**50-mer2** (5'-C CCC ACA TCT GTA GAC GGA CAC <u>GCT</u> TGG AGG TTT AAA GAA GGT GTT TGT G-3') and **22-mer** (5'-GGG ACC GGT GAC ACT GTA AAA C-3')), the target point mutation is underlined. *In the second step*, the resultant PCR fragments were combined in another PCR to obtain, with the use of the 22-mer and 29-mer oligonucleotides, the final product containing the mutated *ΔNΔC/VEGF-C* gene. PCR product of the latter reaction containing *ΔNΔC/VEGF-C(Cys$_{152}$Ser)* gene sequence was digested with *Age*I and *Pme*I and sub-cloned in place of *ΔNΔC/VEGF-C* into pAc5.1/His/BiP/ΔNΔC/VEGF-C. The final construct was thereafter called pAc5.1/His/BiP/ΔNΔC/VEGF-C(Cys$_{152}$Ser).

To ensure that *ΔNΔC/VEGF-C* as well as *ΔNΔC/VEGF-C(Cys$_{152}$Ser)* were cloned in frame with the polyhistidine tag as well as to identify any PCR mistakes, the corresponding *ΔNΔC/VEGF-C* and *ΔNΔC/VEGF-C(Cys$_{152}$Ser)* regions were sequenced.

3.1.1.3 Cloning rat *ΔNΔC/VEGF-D* and *ΔNΔC/VEGF-D(Cys$_{141}$Ser)*

To clone *ΔNΔC/VEGF-D* cDNA into the DES vector, the pAc5.1/His/VEGF-D construct was used. Using the *VEGF-D* gene sequence as a template, *ΔNΔC/VEGF-D* was amplified by PCR using the following oligonucleotide pairs: 5'-GCACCGGT <u>ACC AGA TTT GCG GCA ACT TTC TAT</u> G-3' and 5'-CCGGTTAAAC TCA ATG GTG ATG GTG ATG ATG <u>GAT AAT TGA ATA AGG ATG CCG GGG</u>-3'. The resultant PCR product containing *Age*I restriction site, *ΔNΔC/VEGF-D* (underlined), His-tag-coding sequence and *Pme*I restriction site was subsequently

cloned in place of *Age*I-*ΔNΔC/VEGF-C-Pme*I into pAc5.1/BiP/ΔNΔC/VEGF-C. The final construct was thereafter called pAc5.1/His/BiP/ΔNΔC/VEGF-D.
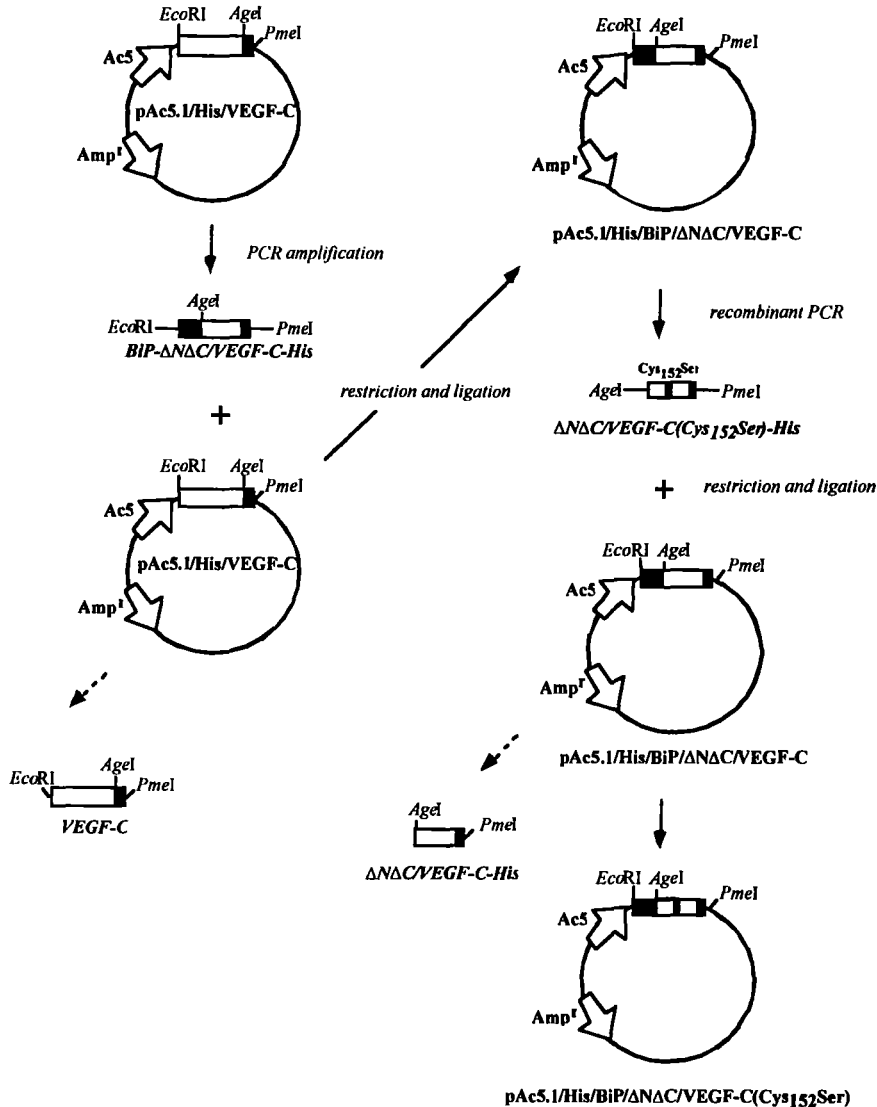
Figure 3.2 *Cloning rat* ΔNΔC/*VEGF-C and* ΔNΔC/*VEGF-C(Cys₁₅₂Ser) cDNAs.* *Legend:* Ac5, Ac5 promoter; Amp^r, ampicillin resistance gene; 6xHis, polyhistidine region (see text for details).

Recombinant PCR technique was also employed to introduce the point mutation into the ΔNΔC/VEGF-D polypeptide. *Firstly,* PCR fragments were obtained using pAc5.1/His/BiP/ΔNΔC/VEGF-D and the following primer pairs: *pair I* (**49-mer1**, 5'-CC AAC ACA TTT TTC AAG CCC CCT <u>AGC</u> GTA AAT GTC TTC CGG TGT GGA GG-3' and **29-mer**, 5'-CCG GTT AAA CTC AAT GGT GAT GGT GAT G-3'), *pair II* (**49-mer2**, 5'-CC TCC ACA CCG GAA GAC ATT TAC <u>GCT</u> AGG GGG

70

CTT GAA AAA TGT GTT GG-3' and **21-mer**, 5'-GGG ACC GGT ACC AGA TTT GCG-3'), the target point mutation is underlined. *Secondly*, they were combined in another PCR to produce, with the use of the 29-mer and 21-mer oligonucleotides, the final product containing the mutated triplet. The resulting PCR fragment containing $\Delta N\Delta C/VEGF\text{-}D/Cys_{141}Ser$-specific sequence was digested with AgeI and PmeI and sub-cloned in place of $\Delta N\Delta C/VEGF\text{-}D$ into pAc5.1/His/BiP/$\Delta N\Delta C$/VEGF-D. The final construct was called pAc5.1/His/BiP/$\Delta N\Delta C$/VEGF-D(Cys$_{141}$Ser). The $\Delta N\Delta C/VEGF\text{-}D$ and $\Delta N\Delta C/VEGF\text{-}D(Cys_{141}Ser)$-specific cDNA regions were sequenced.
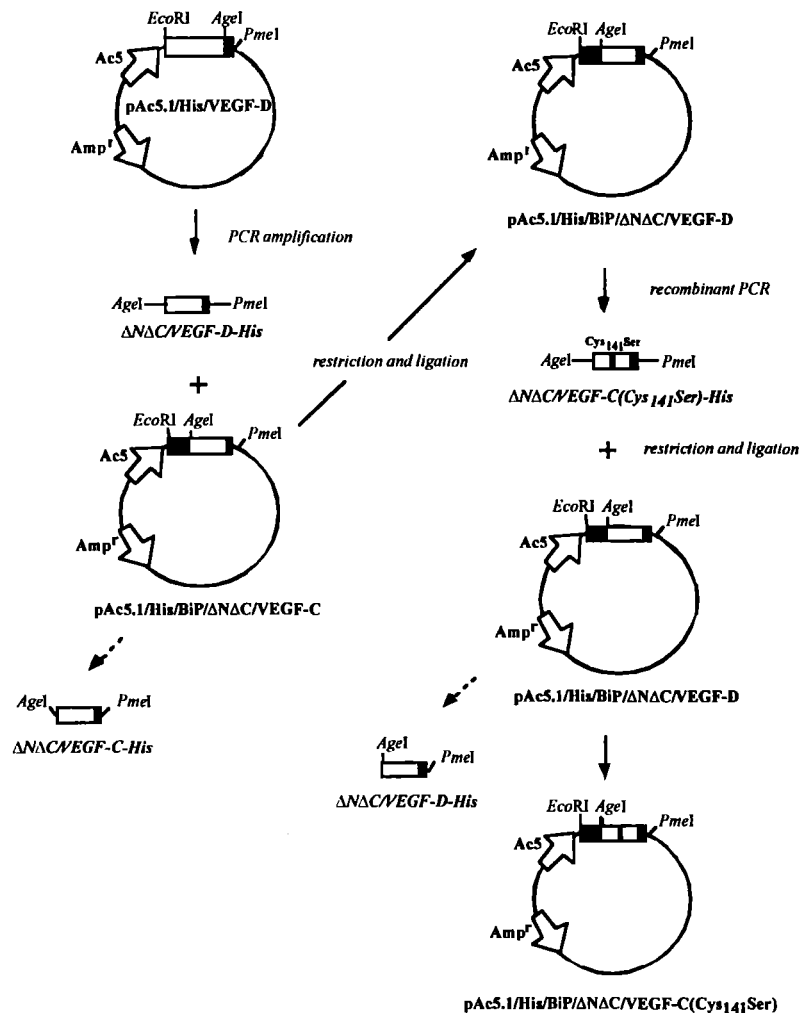
Figure 3.3 *Cloning rat $\Delta N\Delta C/VEGF\text{-}D$ and $\Delta N\Delta C/VEGF\text{-}D(Cys_{141}Ser)$ cDNAs.*
Legend: Ac5, Ac5 promoter; Amp^r, ampicillin resistance gene; 6xHis, polyhistidine region (see text for details).

3.1.2 Expression and purification of recombinant VEGFs

Drosophila S2 cells were transfected with the expression constructs shown in **Table 3.1** along with the selection plasmid pHYGRO (*Invitrogen*) in order to create stable transfectants secreting large amounts of the recombinant growth factors. Mass cultures of the stably transfected S2 cells were subsequently analysed for the presence of the secreted recombinant proteins in their medium supernatant by use of anti-His-tag antibodies (**Fig. 3.4**). The analysis showed that the recombinant rat VEGF, ΔNΔC/VEGF-C and ΔNΔC/VEGF-D proteins migrated as bands of approximately 19 kDa, 13 kDa, and 13kDa, respectively. These molecular weights approximately correspond to the ones predicted on the basis of the primary protein structure. The presence of the lower molecular weight band in some ΔNΔC/VEGF-C and NΔC/VEGF-C(Cys$_{152}$Ser) samples can be attributed to the alternative signal peptide cleavage site found 10 aminoacids downstream of the standard cleavage site in the ΔNΔC sequence of VEGF-C (Kirkin *et al.*, submitted). A signi ficant proportion of the proteins, when analysed on a non-reducing SDS-PAGE, was found to be present as covalent dimers (**Fig. 3.4**).

The recombinant proteins were purified on a large scale using Ni-NTA agarose matrix (*Quiagen*). The typical yield of a purified recombinant protein was between 5-20 mg per litre culture medium. The identity of the recombinant proteins was confirmed using both anti-His-tag and specific anti-VEGF-C and VEGF-D antibodies on western blots (**Fig. 3.5**).

3.2 Characterisation of recombinant rat VEGF proteins

The cloned rat VEGFs are fusion proteins that were expressed in a heterologous non-mammalian system and therefore may differ from the naturally occurring proteins. In order to determine whether the biological activity of the recombinant rat proteins was comparable to analogous preparations described in the literature, a number of experiments were performed.

3.2.1 Activation of VEGF receptors

Once the homodimeric VEGFs bound their specific receptor(s), they should in turn be able to cause receptor dimerisation. Dimerised VEGFRs can then transphosphorylate their tyrosine residues becoming competent to transduce the signal to other downstream cellular effectors (Petrova *et al.*, 1999). Consequently, the ability
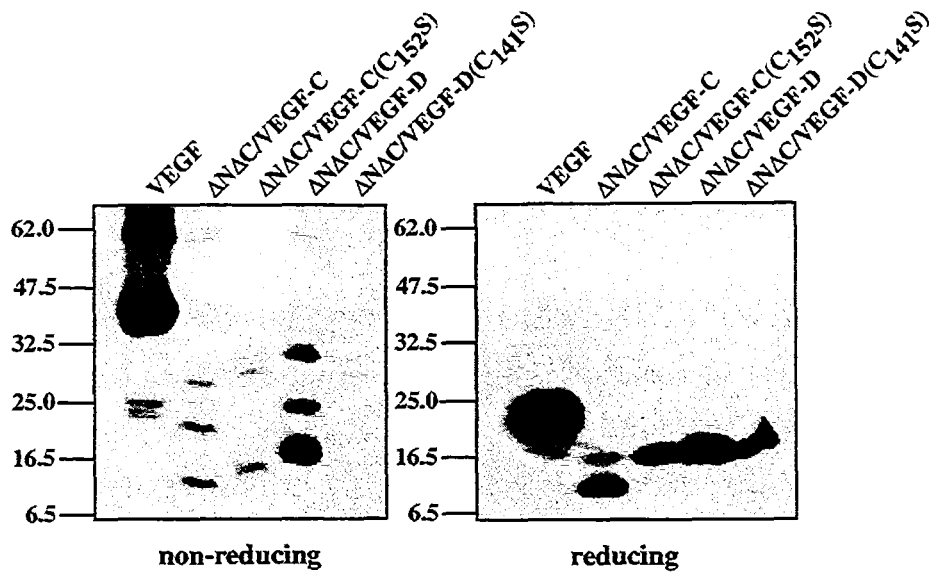
Figure 3.4 *Detection of the His-tagged recombinant VEGFs in the S2-transfectant medium supernatants.* Conditioned medium from the indicated S2 transfectants expressing the vectors shown in **Table 3.1** was incubated with Strataclean resin. Resin pellets were then boiled and resolved on a 12% SDS-PAGE (under either non-reducing or reducing conditions) followed by western blotting. The blot was probed with anti-His-tag antibody.
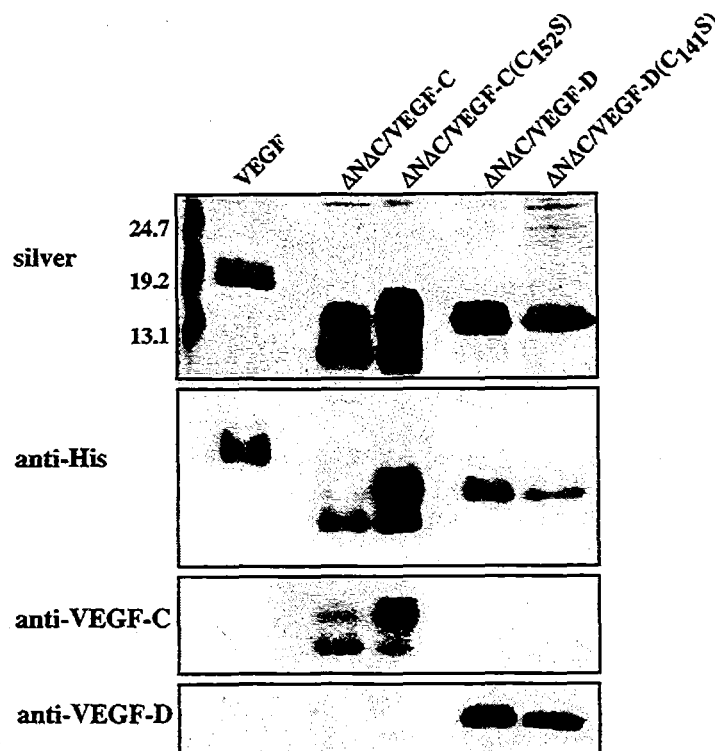
Figure 3.5 *Identity and purity of the purified recombinant VEGFs.* Purified proteins (5 μg) were resolved on a reducing 12% SDS-PAGE and subsequently analysed by silver staining and western blots probed with either anti-His or specific (anti-VEGF-C and anti-VEGF-D) antibodies.

to demonstrate the induction of phosphorylation of the corresponding receptor tyrosine kinase by the recombinant growth factor can serve as a proof for the eventual biological activity of the recombinant protein. To this end, a series of cellular phosphorylation experiments were performed.

3.2.1.1 VEGF, ΔNΔC/VEGF-C and ΔNΔC/VEGF-D conditioned medium

Firstly, the ability of conditioned medium from the stable S2 transfectants to induce phosphorylation of VEGF receptors was assessed using porcine aortic endothelial cells (PAE) cells expressing either VEGFR-2 (PAE/VEGFR-2, Kroll and Waltenberger, 1997) or VEGFR-3 (PAE/VEGFR-3). To produce PAE/VEGFR-3, PAE cells were transfected with a vector encoding a murine form of VEGFR-3 (a kind gift of Dr. Krishnan) and stably transfected cell lines were established as described in Materials and Methods.

In the cellular VEGFR phosphorylation assays, PAE/VEGFR-2 and PAE/VEGFR-3 cells were incubated with 3-day-conditioned medium taken from either S2 transfectants expressing recombinant rat VEGF, ΔNΔC/VEGF-C, and ΔNΔC/VEGF-D, respectively, or from non-transfected cells. Following the short period of induction, the cells were lysed, and the VEGFRs were immunoprecipitated to be analysed on reducing SDS-PAGE for the amount of receptor phosphorylation. As shown in **Figure 3.6**, the conditioned medium from either rat ΔNΔC/VEGF-C- or ΔNΔC/VEGF-D-expressing S2 cells (*lanes 5 and 7*, respectively) was able to induce phosphorylation of both VEGFR-2 and VEGFR-3, whereas VEGF-conditioned medium was able to induce VEGFR-2 but not VEGFR-3 phosphorylation (*lane 4*). Conditioned medium of non-transfected S2 cells was not able to induce phosphorylation of either receptor (*lane 3*). These results suggest that recombinant rat VEGFs, similar to their published human analogues, can specifically bind to and activate their cognate VEGF receptors.

3.2.1.2 ΔNΔC/VEGF-C(Cys$_{152}$Ser) and ΔNΔC/VEGF-D(Cys$_{141}$Ser) conditioned medium

In parallel, it was important to answer the question whether the specific cysteine mutations in the mature VEGF-C and VEGF-D proteins do abrogate their VEGFR-2-binding ability, similarly to the described human VEGF-C mutant (Joukov *et al.*, 1998). Therefore, PAE/VEGFR-2 and PAE/VEGFR-3 cells were incubated with
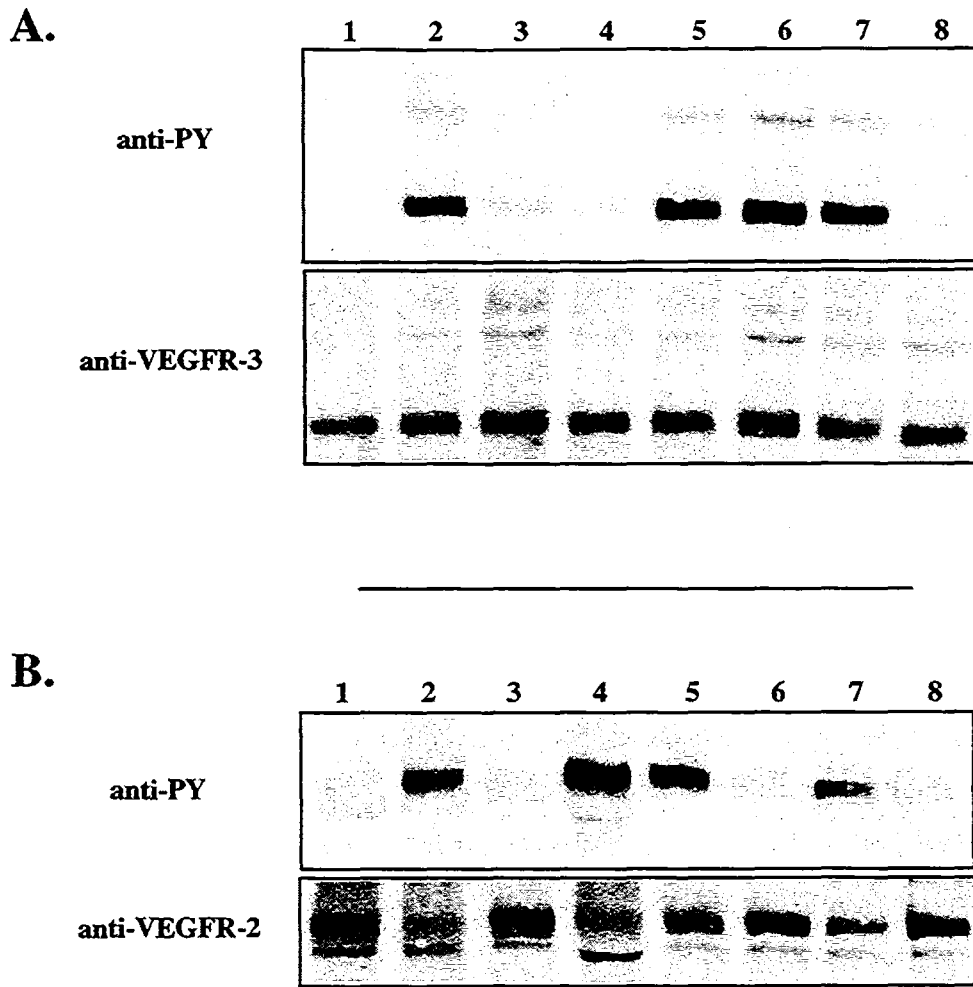
Figure 3.6 *Activation of VEGFR-2 and VEGFR-3 by conditioned medium of S2 cells expressing recombinant rat VEGFs.* PAE-VEGFR-3 (A) and PAE-VEGFR-2 (B) cells were stimulated with conditioned medium containing different recombinant VEGFs. VEGF receptors were immunoprecipitated from the cellular lysates, resolved on a reducing 6% SDS-PAGE, blotted and probed with anti-phosphotyrosine (anti-PY) antibody. Stripped membranes re-probed with anti-receptor antibodies were used as a loading control. *Lanes:* **1.** serum-free F-12 growth medium (*negative control*); **2.** murine VEGF-C (A) or VEGF (B) (*positive control*); **3** non-transfected S2 cells; **4.** rat VEGF; **5.** rat $\Delta N\Delta$/VEGF-C **6.** rat $\Delta N\Delta C$/VEGF-C(Cys$_{152}$Ser); **7.** rat $\Delta N\Delta C$/VEGF-D; **8.** rat $\Delta N\Delta C$/VEGF-D(Cys$_{141}$Ser).

conditioned medium from S2 cells expressing the VEGF-C and -D mutants and analysed for increased receptor phosphorylation as before. **Figure 3.6** illustrates the outcome of this experiment. Conditioned medium of $\Delta N\Delta C$/VEGF-C(Cys$_{152}$Ser) transfectants could only induce VEGFR-3 but not VEGFR-2 phosphorylation (*lane 6*), whereas $\Delta N\Delta C$/VEGF-D(Cys$_{141}$Ser)-containing conditioned medium was void of any VEGFR activity (*lane 8*). These data suggest that while the rat Cys$_{152}$Ser mutant has similar properties to the described human $\Delta N\Delta C$/VEGF-C(Cys$_{156}$Ser), a similar mutation in rat $\Delta N\Delta C$/VEGF-D protein destroys the ability to bind any of the studied VEGF-D receptors.

3.2.1.3 Purified recombinant rat VEGF proteins

To prove that the recombinant growth factors in the conditioned medium really were responsible for the induced receptor phosphorylation observed in previous experiments, I investigated whether VEGFR-2 and VEGFR-3 activation can be reproduced with recombinant His-tagged proteins purified from conditioned medium via a Ni-NTA agarose column. As shown in **Figure 3.7**, treatment of PAE/VEGFR-2 and PAE/VEGFR-3 cells with serum-free F-12 growth medium containing 50 ng/ml rat VEGF (*lane 3*) or 200 ng/ml $\Delta N\Delta C$/VEGF-C (*lane 4*) had similar effect as the corresponding conditioned medium. However, unlike the $\Delta N\Delta C$/VEGF-D conditioned medium, the purified $\Delta N\Delta C$/VEGF-D protein, when dissolved in serum-free F-12 growth medium, failed to induce phosphorylation of its receptors (*lane 6*) suggesting loss of activity upon purification. The purified $\Delta N\Delta C$/VEGF-C(Cys$_{152}$Ser) mutant preserved its specific VEGFR-3 activating property (*lane 5*). These data suggest that recombinant VEGF-C in conditioned medium from S2 transfectants is the specific activator of VEGFR-2 and VEGFR-3. In the case of $\Delta N\Delta C$/VEGF-D, although purified protein was not biologically active, clearly the conditioned medium contained active VEGF-D. Thus, for both VEGF-C and VEGF-D, conditioned medium was predominantly used in subsequent experiments for convenience.
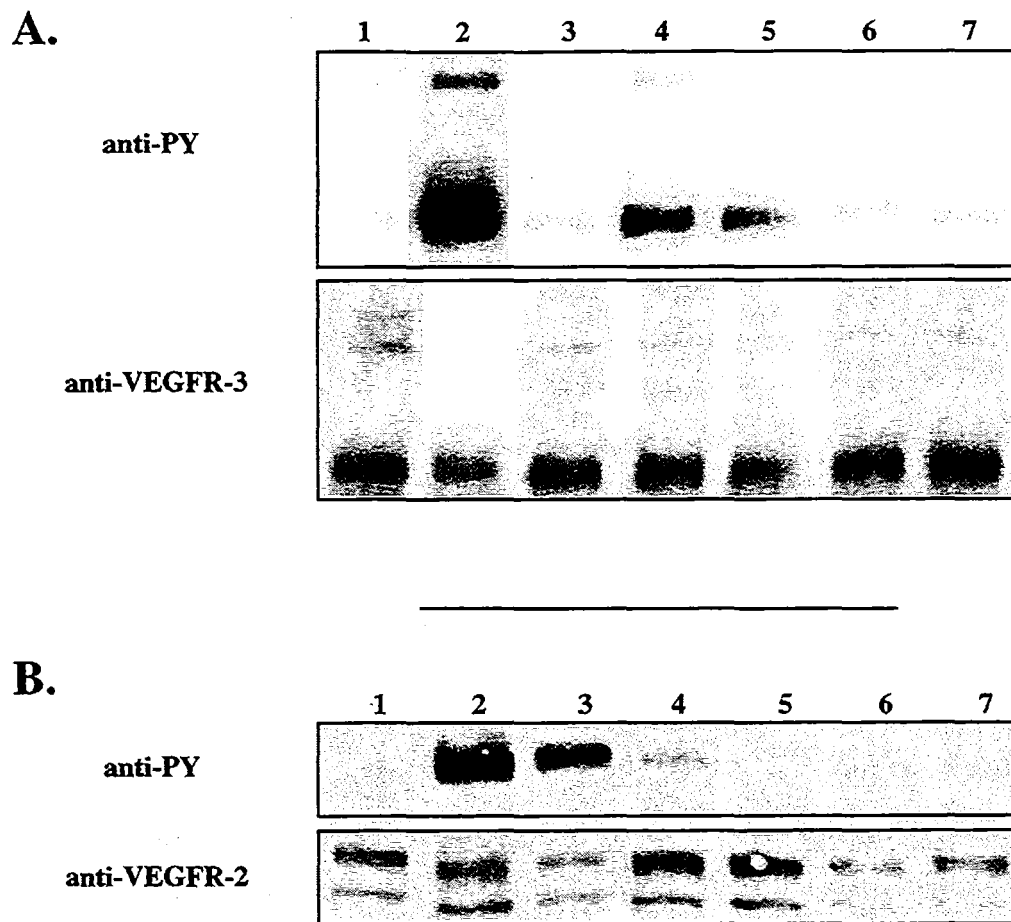
Figure 3.7 *Activation of VEGFR-2 and VEGFR-3 by purified recombinant rat VEGFs.* PAE/VEGFR-3 (A) and PAE/VEGFR-2 (B) cells were stimulated with either purified recombinant rat VEGFs re-suspended in F-12 growth medium or conditioned medium from 293 transfectants expressing murine VEGFs (*positive control*). VEGF receptors were immunoprecipitated from the cellular lysates, resolved on a reducing 6% SDS-PAGE, blotted and probed with anti-phosphotyrosine (anti-PY) antibody. Stripped membranes re-probed with anti-receptor antibodies were used as a loading control. *Lanes:* **1.** serum-free F12-growth medium (*negative control*); **2.** murine VEGF-C (A) or VEGF (B) (*positive control*); **3.** rat VEGF (50 ng/ml); **4.** rat ΔNΔC/VEGF-C (200 ng/ml); **5.** rat ΔNΔC/VEGF-C(Cys$_{152}$Ser) (200 ng/ml); **6.** rat ΔNΔC/VEGF-D (200 ng/ml); **7.** rat ΔNΔC/VEGF-D(Cys$_{141}$Ser) (200 ng/ml).

3.2.2 Stimulation of vascular endothelial cell proliferation

One of the important consequences of VEGF signals is the induction of cellular proliferation. Human dermal microvascular cells (HDMEC) are primary endothelial cells that were used to check the ability of recombinant rat VEGFs to stimulate their proliferation. A proliferation assay based on measuring incorporation of (^{3}H)-labelled thymidine into DNA in the course of DNA replication was used for this purpose. **Figure 3.8** shows that HDMECs incubated for 24 hours with culture medium containing increasing concentrations of purified rat $\Delta N\Delta C$/VEGF-C exhibited a consistent increase in proliferation, as was reflected by higher amounts of incorporated radioactivity. Consistent with published data (Joukov *et al.*, 1997), recombinant VEGF was 20-to 30-fold more active as compared to $\Delta N\Delta C$/VEGF-C. Also, the $\Delta N\Delta C$/VEGF-C($Cys_{152}Ser$) mutant was only weakly active, as was described for the human VEGF-C mutant (Joukov *et al.*, 1998). Due to inactivity of the purified VEGF-D proteins in the cellular phosphorylation assay, their ability to induce HDMEC proliferation was not studied. The results of this experiment suggest that the purified recombinant rat VEGFs are biologically active.

3.3 Establishment of rat lymphatic endothelium (LE) in culture

The ability to maintain LE in culture would provide a powerful tool for screening anti-lymphangiogenic substances *in vitro*. It would also permit the study of interactions between tumour cells and LE *in vitro* as well as to measure the effects of anti-lymphangiogenic substances on tumour-induced lymphangiogenesis. Unfortunately, whilst there are several blood vascular endothelial cell culture methods available, as yet there has not been a single report on the generation of LE cells from peripheral tissues *in vitro*. The biological activity of the recombinant rat VEGF-C protein demonstrates that it should be able to induce lymphangiogenesis and LE proliferation. I therefore attempted to utilise the lymphangiogenic properties of the recombinant rat $\Delta N\Delta C$/VEGF-C and the VEGFR-3-specific mutant $\Delta N\Delta C$/VEGF-C($Cys_{152}Ser$) to establish LE in culture. Another strategy was followed in parallel, where adjuvant-induced lymphangiomas were used as a source for LE.
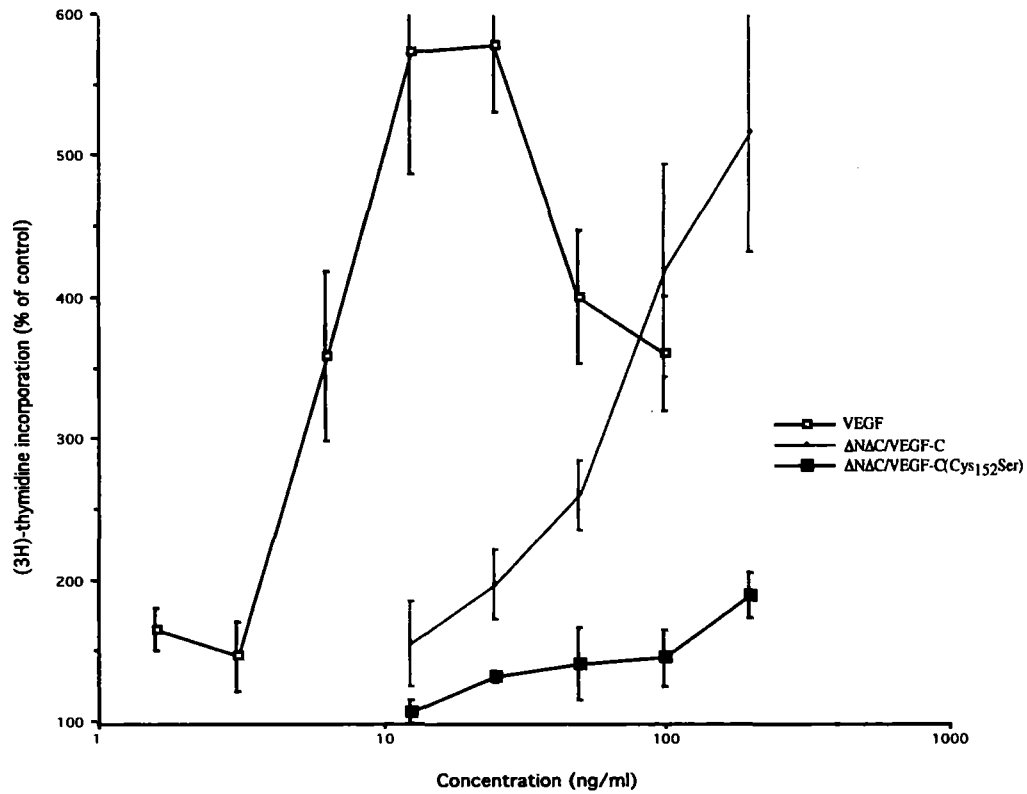
Figure 3.8 *Stimulation of HDMEC proliferation by recombinant rat VEGFs.* Starved HDMEC were stimulated for 24 hours with increasing concentrations of the indicated recombinant VEGFs. A pulse of tritium was given to the cells for the last 4-6 hours of the stimulation after which the cells were harvested and the amount of incorporated radioactivity was measured. Data are expressed as percent of non-stimulated cell proliferation (% of control).

3.3.1 Use of recombinant ΔNΔC/VEGF-C *in vivo*

VEGF-C and VEGF-D are the only lymphangiogenic proteins described to date (Jeltsch *et al.*, 1997; Oh *et al.*, 1997; Veikkola *et al.*, 2001). The recombinant rat ΔNΔC/VEGF-C and ΔNΔC/VEGF-C(Cys$_{152}$Ser) proteins, which were shown to be biologically active *in vitro*, were therefore used in an attempt to obtain lymphatic endothelial cells in culture. Matrigel, a soluble basement membrane extract of the EHS (Engelbreth-Holm-Swarm) sarcoma, containing different concentrations of the recombinant ΔNΔC/VEGF-C and ΔNΔC/VEGF-C(Cys$_{152}$Ser) was injected subcutaneously into the back of rats. Matrigel containing recombinant rat VEGF was also injected for controlling purposes. It was anticipated that the entrapped lymphatic endothelial growth factor would diffuse into the surrounding tissue, induce lymphangiogenesis and later permit the extraction of the LE into culture, as shown in **Figure 3.9.**

1   2   3
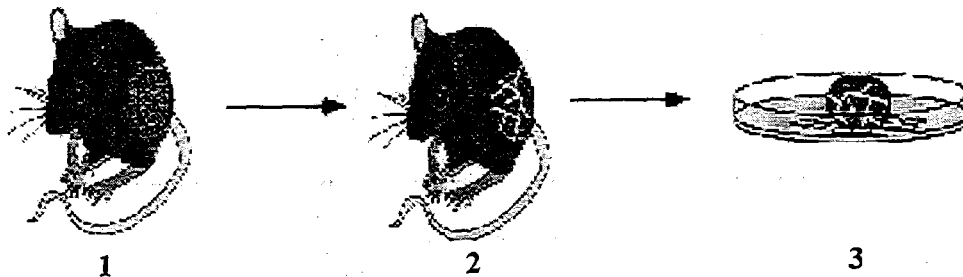
Figure 3.9 *Use of recombinant ΔNΔC/VEGF-C and ΔNΔC/VEGF-C(Cys$_{152}$Ser) to establish LE in culture.* Recombinant proteins are entrapped within an inert support, for instance matrigel. The support is then implanted subcutaneously into the back of an experimental animal (1). The entrapped growth factors diffuse into the surrounding tissue, inducing lymphangiogenesis (2). The support containing newly formed lymphatic vessels is extracted and used to obtain the LE in culture. The cells, which have grown into the support and in tissue culture, are analysed for the expression of LE-specific markers such as VEGFR-3.

Two weeks after the matrigel injection, the matrigel plugs were extracted, fixed, cut into sections and subsequently analysed for the presence of new vessel-like structures within the gels. Masson's trichrome staining that allows unambiguous discrimination of vessel structures from the surrounding matrix (Grant *et al.*, 1993) was employed to detect any newly grown vessels and VEGFR-3-specific anti-serum was used to detect any putative lymphatic capillaries within or in the proximity of the matrigel plugs. It was observed that none of the recombinant growth factors

specifically induced lymphangiogenesis or angiogenesis *in vivo* (data not shown). Several attempts were made with different batches of the purified recombinant proteins but none of them succeeded. Intriguingly, during these experiments it was noted that about 14 days after subcutaneous injection of matrigel containing no growth factors, vacuoles formed which were lined with cells (**Fig. 3.10**). These cells stained positively for VEGFR-3 (**Fig. 3.10, C and D**), suggesting their possible lymphatic endothelial origin. Cells were extracted from such matrigel plugs and put into culture. However, such cultures were heavily overgrown with fibroblast-like cells, which did not allow further characterisation of the cells.

3.3.2 Use of adjuvant-induced lymphangiomas

It has recently been published that the intraperitoneal injection of incomplete Freund's adjuvant in mice results in the appearance of lymphangiomas on the surface of the diaphragm and the liver (Mancardi *et al.,* 1999). The lymphangiomas were positive for VEGFR-3 and cells derived from the tumours expressed endothelial cell-specific markers. In order to find out whether such lymphangiomas can be induced in rats and whether it is possible to use them as a source of LE cells, the published experiment was repeated in rats. As described for mice, white tumours were found on the surface of the liver and the diaphragm in all the rats that received the adjuvant (**Fig. 3.11a**). Tumours were extracted and analysed by immunohistochemistry. They were found to contain VEGFR-3 (**Fig. 3.11b**) and CD31 (endothelial cell marker, data not shown) positive cells. The lymphangioma cells were extracted and put into culture. However, when cultured they could not be propagated for more than few passages (**Fig. 3.11c and d**) after which they apparently became senescent as indicated by loss of proliferation and gross changes in cellular morphology (data not shown). It seems therefore that in order to grow *in vitro* cells derived by either method, the LE cells would need to be immortalised. Due to time constraints, this aspect of the work had to be discontinued.
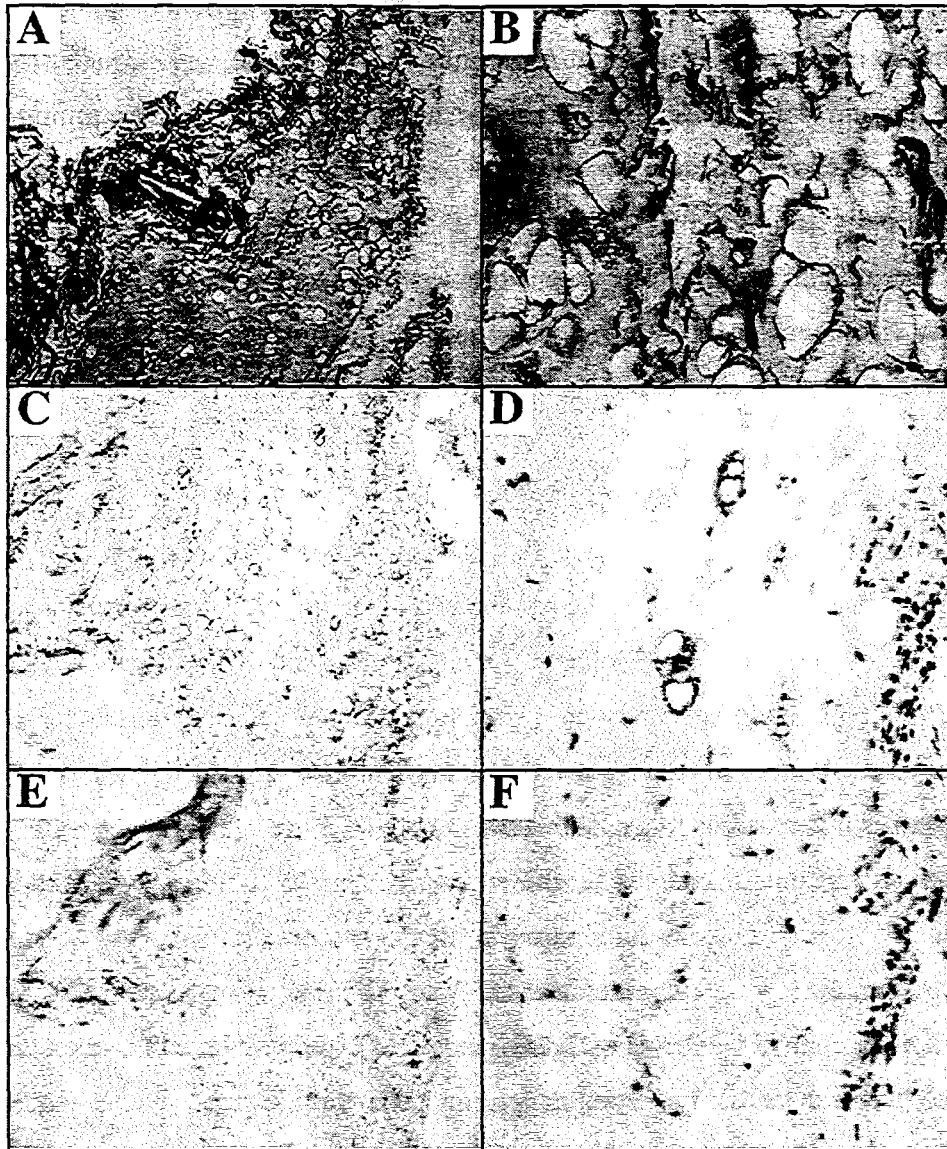
Figure 3.10 *Appearance of growth factor-free matrigel 14 days post-implantation.* Sections A (low magnification) and B (higher magnification), stained with Masson's trichrome, show the appearance of the vacuoles inside the matrigel. Sections C and D demonstrate VEGFR-3 positive staining of the vacuoles with VEGFR-3-specific rabbit polyclonal antiserum. Sections E and F (secondary antibody control) are given for comparison.
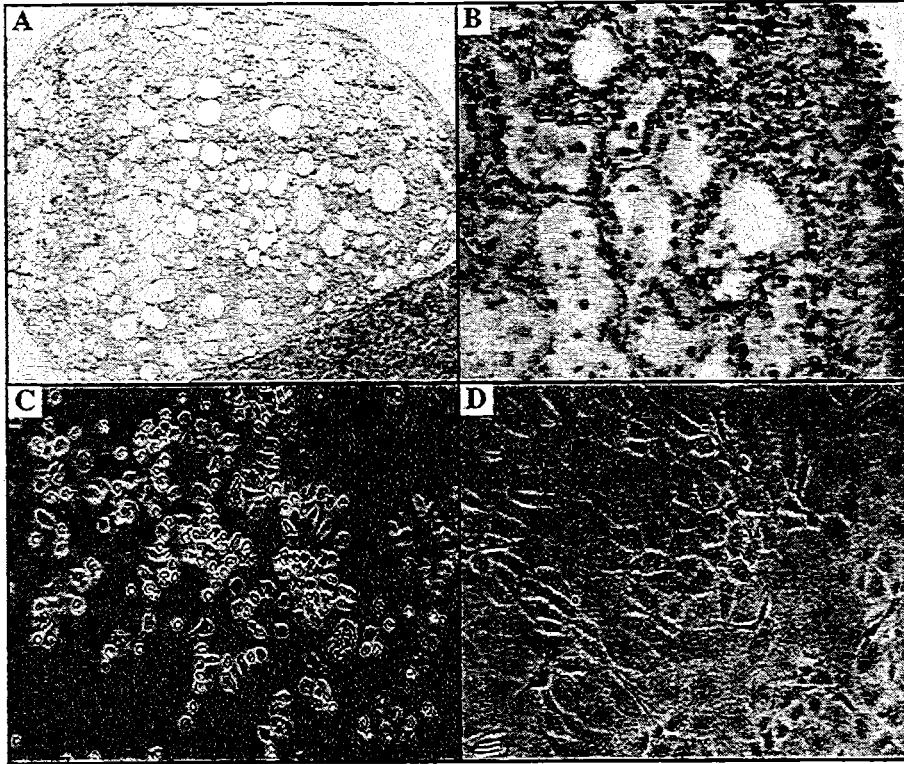
Figure 3.11 *Adjuvant-induced lymphangioma and lymphangioma-derived cells.* White tumour masses were observed on the surface of the diaphragm and the liver in 100% of animals. Panel A shows H&E staining of a paraffin section of the well-defined lymphangioma resting on the surface of the liver. Panel B shows a frozen section of the lymphangioma stained with anti-VEGFR-3 rabbit polyclonal serum. Panels C-D demonstrate the cells derived from the adjuvant-induced lymphangioma (C: a 12-hour-old primary cell colony, phase-contrast microscopy; D: a 7-day-old cells, phase-contrast microscopy).

PART II

Selection and characterisation of anti-tumour substances with anti-angiogenic and/or anti-lymphangiogenic properties

Inappropriate RTK signalling is implicated in a variety of pathological conditions such as tumour growth and tumour-induced angiogenesis and lymphangiogenesis (Saaristo *et al.*, 2000). Compounds that mimic ATP structure such as those based on an oxindole core called indolinones have been shown to inhibit activity of a number of RTKs such as those implicated in angiogenesis (Sun *et al.*, 1998; Sun *et al.*, 1999; Mendel *et al.*, 2000; Sun *et al.*, 2000). Structure *vs.* activity analysis for these compounds demonstrated that the oxindole occupies the kinase site that binds the adenine of ATP. In contrast, the moieties that extend from the oxindole core interact with residues in the hinge region between the kinase lobes, thereby determining the selectivity and affinity for the individual receptor (Mohammadi *et al.*, 1997). This principle can be employed to develop indolinones with the ability to inhibit a range of RTKs involved in different aspects of tumour progression.

As a step in this direction, a panel of potential kinase-inhibiting indolinones synthesised by the group of Professor Dr. Athanassios Giannis, Institut für Organische Chemie, Karlsruhe University, using chemical synthesis (**Fig. 3.12**) was screened for their potential to inhibit VEGFRs and other tumour-related RTKs. The potential VEGFR inhibitors were subjected to further selection using the cellular system I established (see Part I), in which activation of VEGFR family members and its inhibition can be studied. The most potent inhibitors of VEGFR-2 and VEGFR-3 were also tested for their direct anti-tumour effect. These indolinones were ultimately used to inhibit tumour growth *in vivo*.

Hyperforin is another candidate anti-cancer drug with possible direct anti-tumour and anti-angiogenic properties. Besides being an effective anti-depressant, it possesses potent anti-proliferative properties (Schempp *et al.*, 1999 and 2000) and therefore may target tumour and proliferating endothelial cells. The anti-tumour effect of hyperforin was therefore explored.
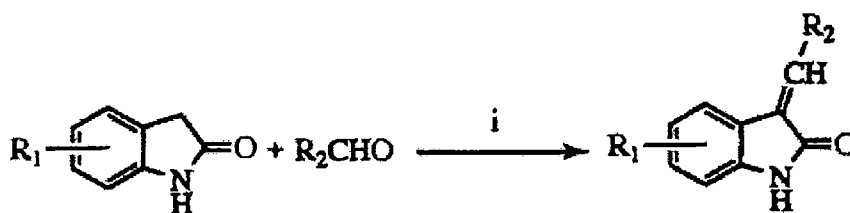
Figure 3.12 *Principle of 3-substituted indolin-2-one (indolinone) synthesis.*
Legend: i. piperidine/ethanol/reflux/5 hrs; R1. indoline-2-one; R2. target substitution group (*taken from Sun et al., 1998*).

3.4 Selection of indolinones inhibiting activation of tumour-related RTKs

In order to select the most useful compounds from the panel of synthesised indolinones, a screening procedure was developed. First, the substances were assessed for their ability to inhibit substrate phosphorylation catalysed by recombinant GST-fusion receptor tyrosine kinases *in vitro*; and second, they were checked for their ability to block activation of VEGFRs in my established cellular system where they are present in their natural conformation.

3.4.1 Primary screening of indolinones for their inhibitory activity

In order to screen the panel of indolinones for their potential as RTK inhibitors, an *in vitro* tyrosine kinase assay was established, which had previously been described to be a valuable tool for the crude selection of other RTK inhibitors (Laird *et al.*, 2000). In this assay, indolinones were added at 2 concentrations (*i.e.* 1 μg/ml and 10 μg/ml) to *in vitro* substrate phosphorylation reactions catalysed by recombinant GST fusion kinases containing only the catalytic domain of the respective kinases (purchased from *KTB Tumorforschungs GmbH,* Freiburg). The amount of the subsequent substrate phosphorylation was then measured by use of anti-phosphotyrosine antibodies and secondary antibodies linked to the horse radish peroxydase. The amount of the chromogenic substrate hydrolysed by the latter enzyme, as assessed with a spectrophotometer, directly correlates with the amount of substrate phosphorylation catalysed by the GST fusion kinase.

In the screen, 32 indolinones were tested for their ability to inhibit the substrate phosphorylation catalysed by six different tyrosine kinases. The chosen recombinant GST fusion proteins constitute analogues of some cellular RTKs that have been

implicated in angiogenesis (VEGFR-2, Tie-2), lymphangiogenesis (VEGFR-3) and tumorigenesis (EGFR, ErbB2, and IGFR1; Eccles, 2000; Wells, 1999; Happerfield *et al.*, 1997). As shown in **Figures 3.13 - 3.18** and summarised in **Table 3.2**, at 10 μg/ml many of the indolinones could inhibit one or more tyrosine kinases by more than 50%. However, because the primary goal of the screening was the identification of preferential inhibitors of VEGFR family members, the 15 compounds that at 10 μg/ml showed inhibition of either VEGFR-2 or VEGFR-3 (or both) by more than 50% (asterisk, **Table 3.2**) were selected for further studies.

No	Name	VEGFR-3 (%)	VEGFR-2 (%)	Tie2 (%)	EGFR (%)	ErbB2 (%)	IGFR1 (%)
1.	157(2B)	+4	+45	+42	-21	+4	+9
		-34	+18	-4	-74	-34	-44
2.	A4*	-15	-21	+20	-36	-7	-27
		-26	-60	-33	-74	-24	-67
3.	A5*	-10	-3	+3	-37	-13	-6
		-27	-71	-16	-81	-38	-89
4.	A10	+2	+5	-10	-10	+2	-4
		-9	+2	-4	-39	-23	-22
5.	A12	-11	+16	0	-20	-17	-4
		-27	-47	+6	-76	-30	-66
6.	AE86	+6	0	-13	-24	+7	-22
		+5	-21	-21	-37	-3	-34
7.	AE87*	-9	-46	+1	-70	-45	-63
		-74	-77	-65	-86	-84	-94
8.	AE89	-7	+25	-2	-2	+2	+4
		-2	+18	-2	-7	-7	+8
9.	AE90	-24	+2	-3	-2	-3	-13
		-46	-20	-11	-8	-18	-43
10.	AE91	-23	+9	-6	-11	+1	-8
		-25	-7	-9	-28	-7	-13
11.	AE92	-3	+21	-7	-13	-8	-5
		-18	-4	-5	-30	-27	-26
12.	AE94	-7	-1	-11	-28	+7	-15
		-15	0	-9	-37	-22	-30
13.	AE95	-5	+18	-2	-14	+11	+10
		-1	+26	+19	-11	-4	+3
14.	AE96	-5	+18	-6	-5	+1	+5
		-9	+17	-4	-13	-18	-7
15.	AE99	-8	+12	-1	-5	-13	+3
		-11	+11	+4	-26	-21	-11
16.	AE100	-1	+15	-9	-3	-9	+2
		-21	+29	-3	+3	-18	-27
17.	AE101	-8	+6	+1	-7	-8	-2
		-26	-6	+9	-19	-14	-29
18.	AE105	-6	+11	-17	-16	+2	-10
		-32	-36	-16	-52	-23	-39
19.	AE106*	+1	+21	-5	-5	+4	-10
		-57	-58	-28	-64	-41	-71
20.	AE108	-21	+18	-5	+2	-2	+2
		-23	-38	-17	-29	-17	-19

21.	H10*	+3	+22	-8	-5	-5	+7
		-33	-58	-11	-53	-36	-65
22.	H11*	-12	0	-15	-53	-26	-24
		-67	-68	-15	-73	-63	-64
23.	H12*	-20	1	+3	-41	-23	-16
		-61	-71	-10	-84	-56	-78
24.	H13*	-49	-72	-27	-78	-38	-83
		-80	-87	-72	-97	-91	-94
25.	H42*	-23	-17	-7	-48	-8	-30
		-32	-75	+2	-82	-30	-82
26.	MAZ40*	-14	-11	-3	-30	-10	-34
		-44	-57	-10	-71	-32	-71
27.	MAZ49*	-9	-8	-40	-2	-22	-45
		-56	-65	-69	-55	-54	-78
28.	MAZ51*	-4	-8	-7	-25	-11	-10
		-35	-74	-27	-58	-39	-68
29.	MAZ51-2*	-18	-13	0	-9	-10	-33
		-51	-74	-8	-73	-58	-81
30.	MAZ60*	-18	-77	+6	-68	-50	-80
		-48	-84	-35	-72	-65	-86
31.	MAZ60-2*	-13	-75	+11	-70	-53	-88
		-50	-84	-26	-79	-68	-93
32.	MAZ61*	-5	-19	+7	-14	-5	-27
		-34	-87	-23	-80	-46	-82

Table 3.2 *Inhibitory action of 32 indolinones on different recombinant GST-receptor tyrosine kinases.* Data are expressed as percent of control (1st value: 1 μg/ml; 2nd value: 10 μg/ml); shaded cells indicate the inhibition of 50% or higher; the compounds showing inhibition of at least one VEGFR by more than 50% are marked with an asterisk.

3.4.2 Screening of indolinones for inhibition of cellular VEGFR activity

In the next step, it was necessary to determine whether the indolinones selected on the basis of the *in vitro* kinase assay could also inhibit VEGFR phosphorylation in a cell-based assay. PAE cell lines transfected with VEGFR-1, VEGFR-2 or VEGFR-3 were used for this purpose.

3.4.2.1 Cellular VEGFR-3 phosphorylation assay

<u>a) Inhibition of VEGF-C-mediated VEGFR-3 activation</u>

Stimulation of PAE/VEGFR-3 cells with VEGF-C conditioned medium induced tyrosine phosphorylation of VEGFR-3 (see Part I). Using this system, the 9 indolinones which inhibited GST-VEGFR-3 phosphorylation, were further screened for their ability to inhibit VEGFR-3 phosphorylation in PAE cells. PAE/VEGFR-3 cells were stimulated with VEGF-C conditioned medium in the presence of increasing concentrations of the test indolinones. Cells stimulated without inhibitors were used a
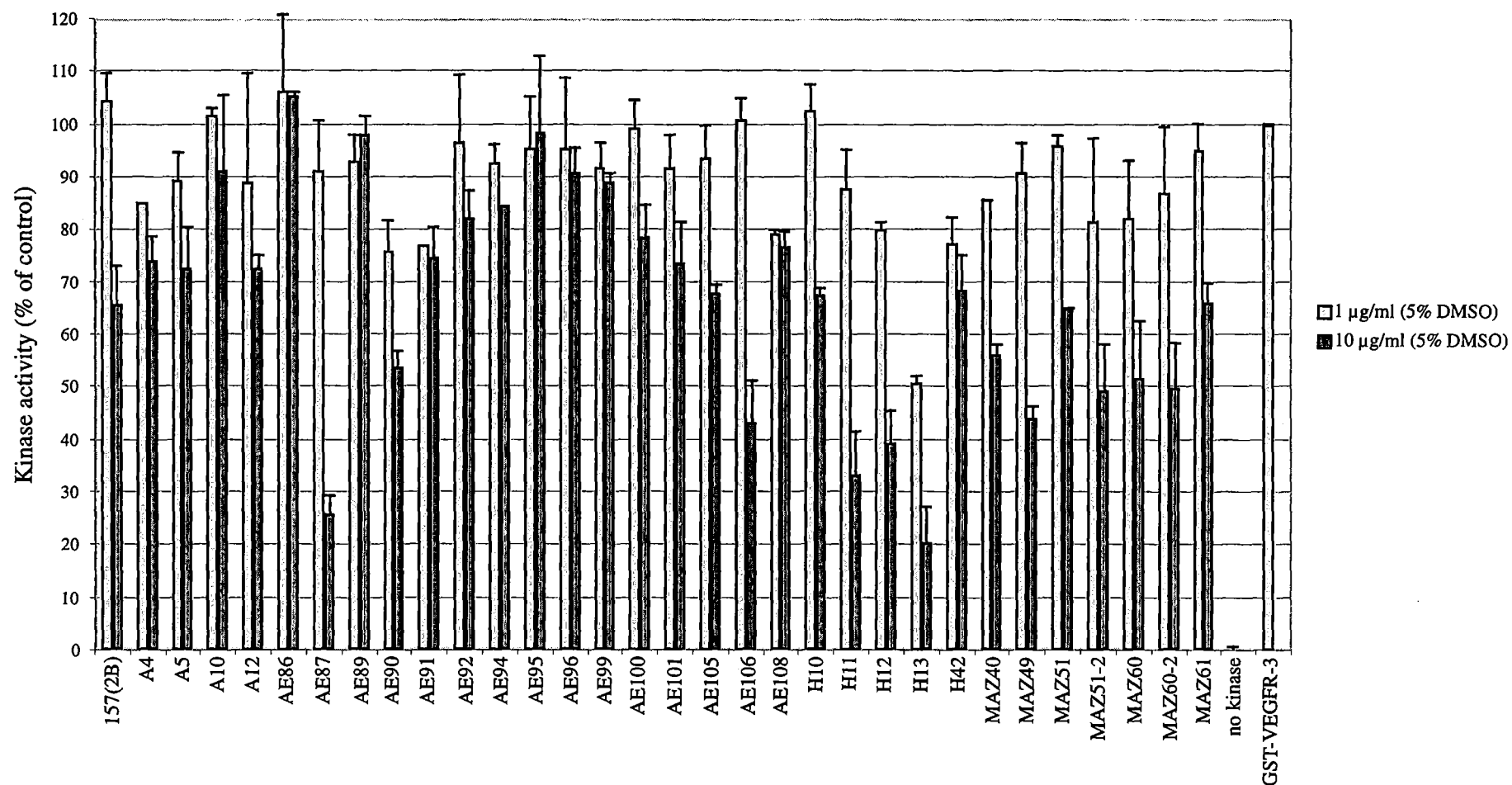
Figure 3.13 *Inhibition of GST-VEGFR-3 activity by indolinones. In vitro* substrate phosphorylation reactions catalysed by recombinant GST-VEGFR-3 kinase were performed in the presence of 1μg/ml or 10 μg/ml indicated indolinones and 5% DMSO. The amount of substrate phosphorylation was measured using a colorimetric assay. Data are expressed as % of substrate phosphorylation in the presence of 5% DMSO only (% of control).
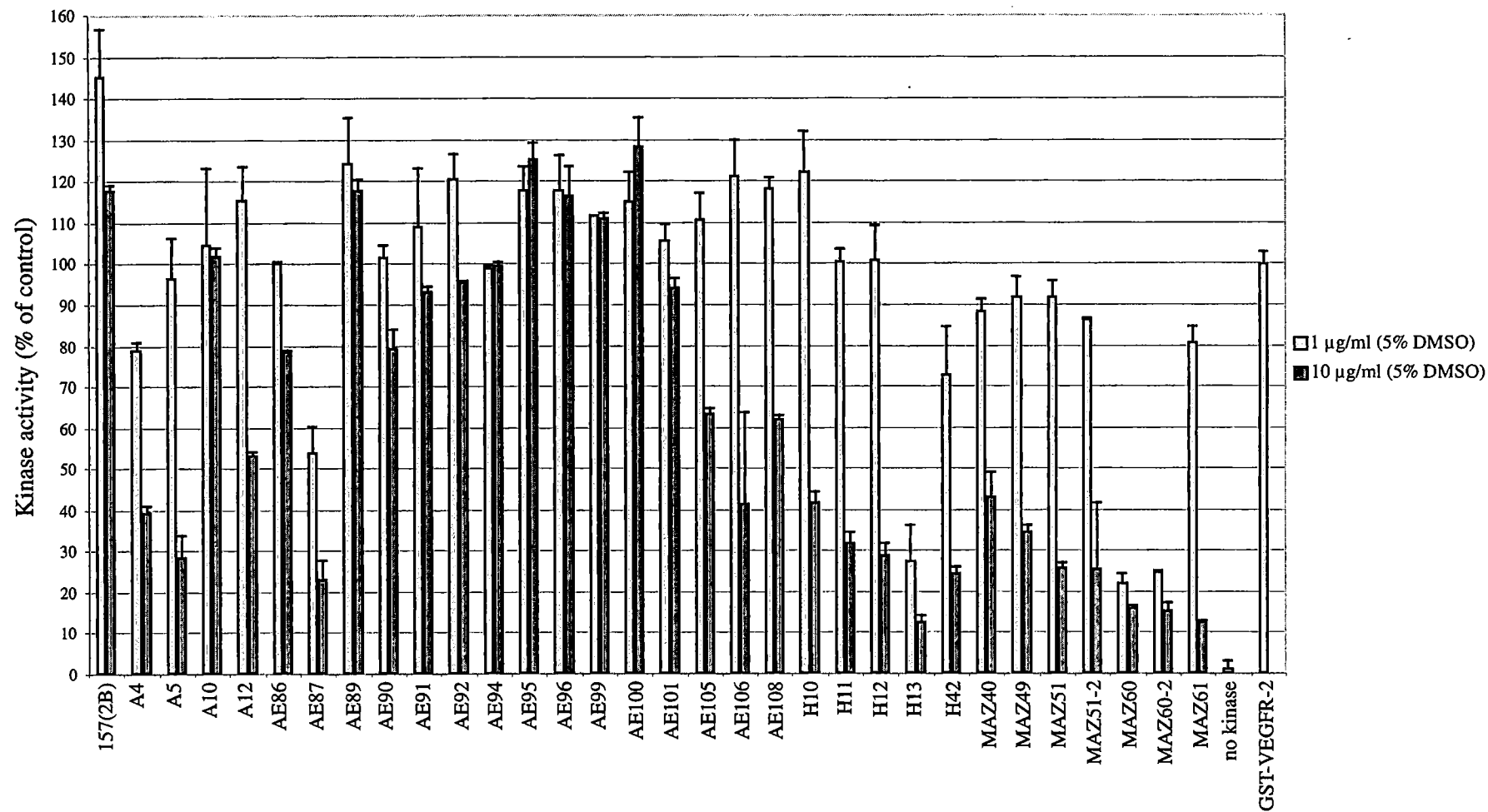
Figure 3.14 *Inhibition of GST-VEGFR-2 activity by indolinones.* *In vitro* substrate phosphorylation reactions catalysed by recombinant GST-VEGFR-2 kinase were performed in the presence of 1μg/ml or 10 μg/ml indicated indolinones and 5% DMSO. The amount of substrate phosphorylation was measured using a colorimetric assay. Data are expressed as % of substrate phosphorylation in the presence of 5% DMSO only (% of control).
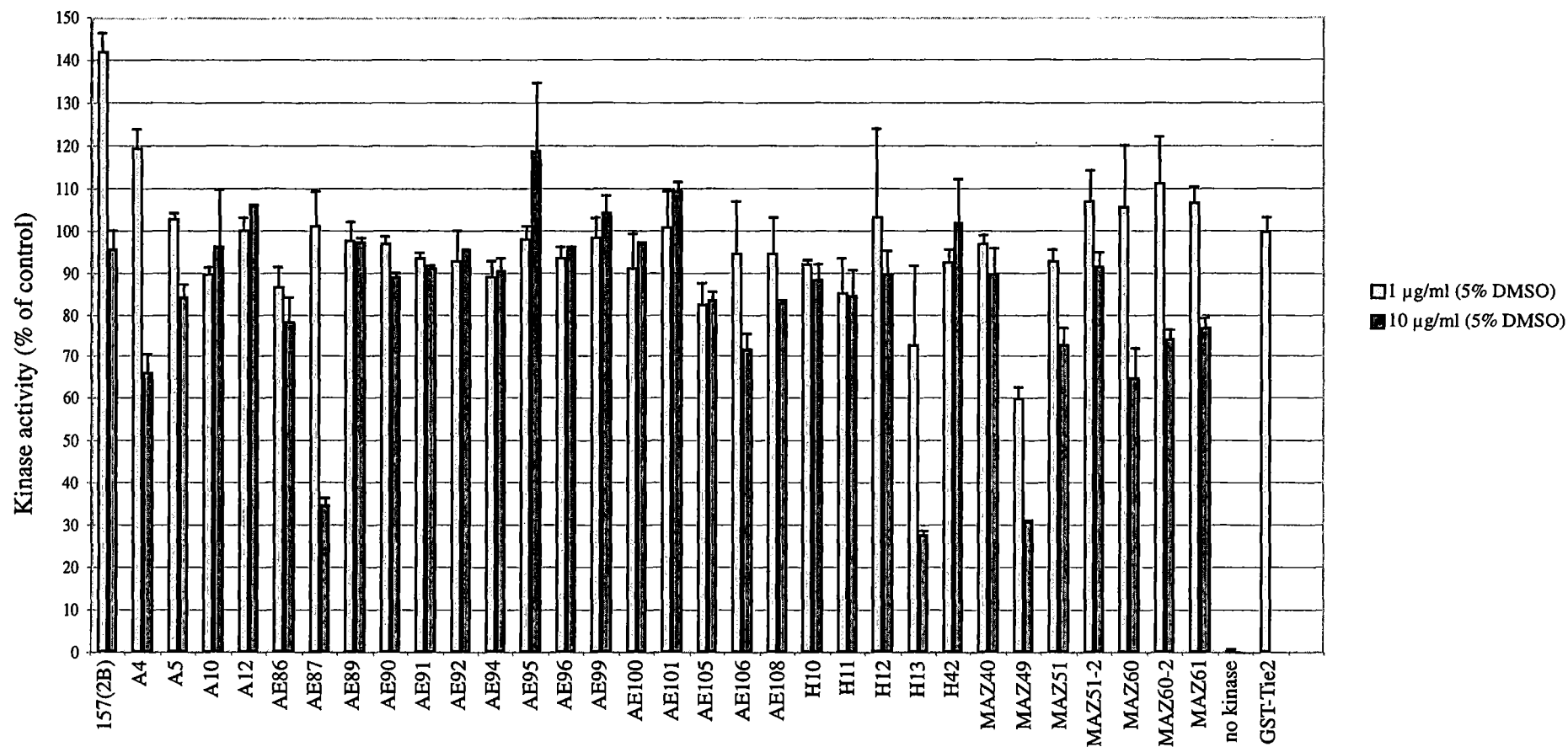
Figure 3.15 *Inhibition of GST-Tie2 activity by indolinones.* *In vitro* substrate phosphorylation reactions catalysed by recombinant GST-Tie2 kinase were performed in the presence of 1μg/ml or 10 μg/ml indicated indolinones and 5% DMSO. The amount of substrate phosphorylation was measured using a colorimetric assay. Data are expressed as % of substrate phosphorylation in the presence of 5% DMSO only (% of control).

Kinase activity (% of control)

120
110
100
90
80
70
60
50
40
30
20
10
0

157(2B) A4 A5 A10 A12 AE86 AE87 AE89 AE90 AE91 AE92 AE94 AE95 AE96 AE99 AE100 AE101 AE105 AE106 AE108 H10 H11 H12 H13 H42 MAZ40 MAZ49 MAZ51 MAZ51-2 MAZ60 MAZ60-2 MAZ61 no kinase GST-EGFR

□ 1 μg/ml (5% DMSO)
■ 10 μg/ml (5% DMSO)
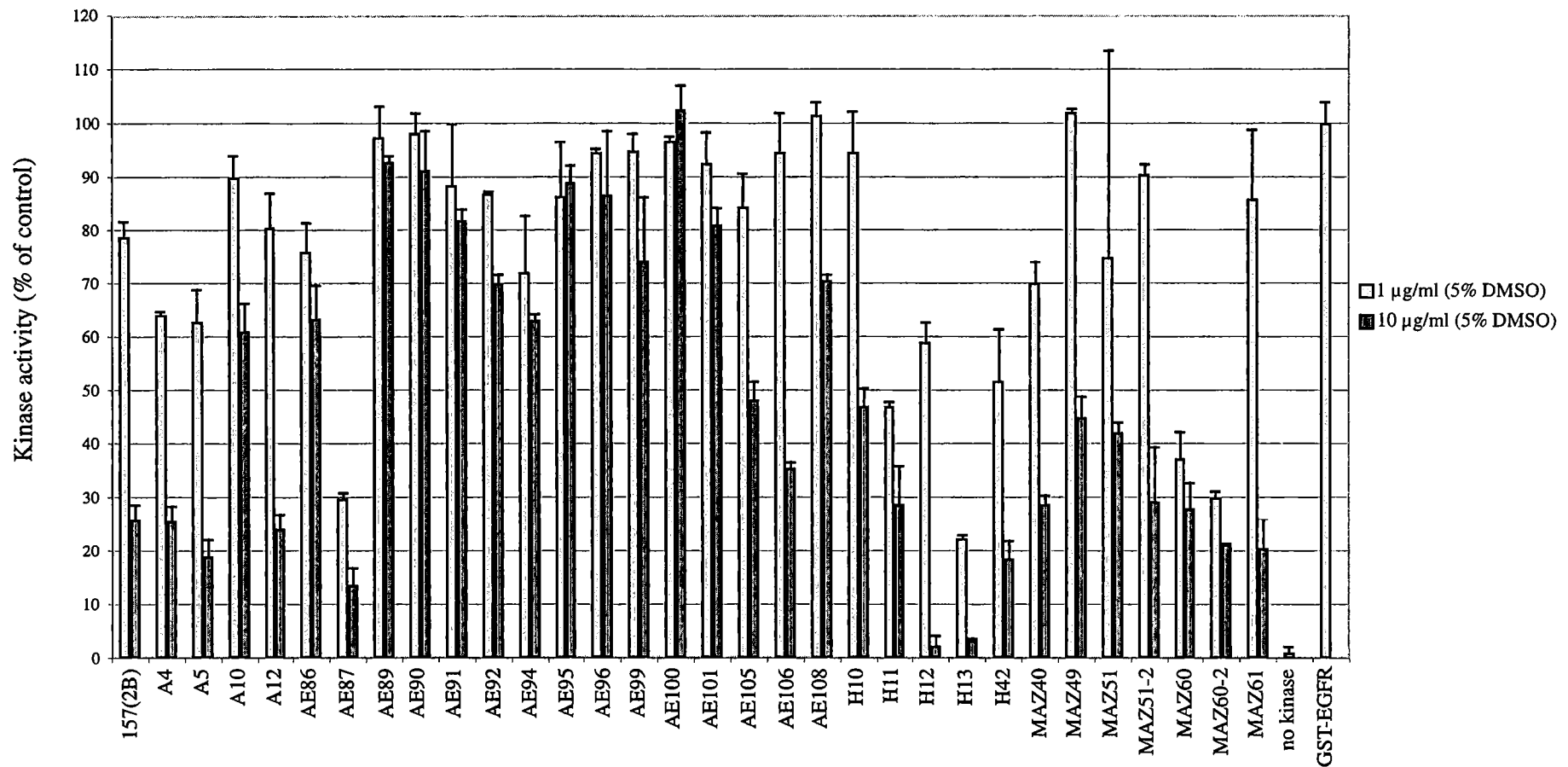
Figure 3.16 *Inhibition of GST-EGFR activity by indolinones. In vitro* substrate phosphorylation reactions catalysed by recombinant GST-EGFR kinase were performed in the presence of 1μg/ml or 10 μg/ml indicated indolinones and 5% DMSO. The amount of substrate phosphorylation was measured using a colorimetric assay. Data are expressed as % of substrate phosphorylation in the presence of 5% DMSO only (% of control).
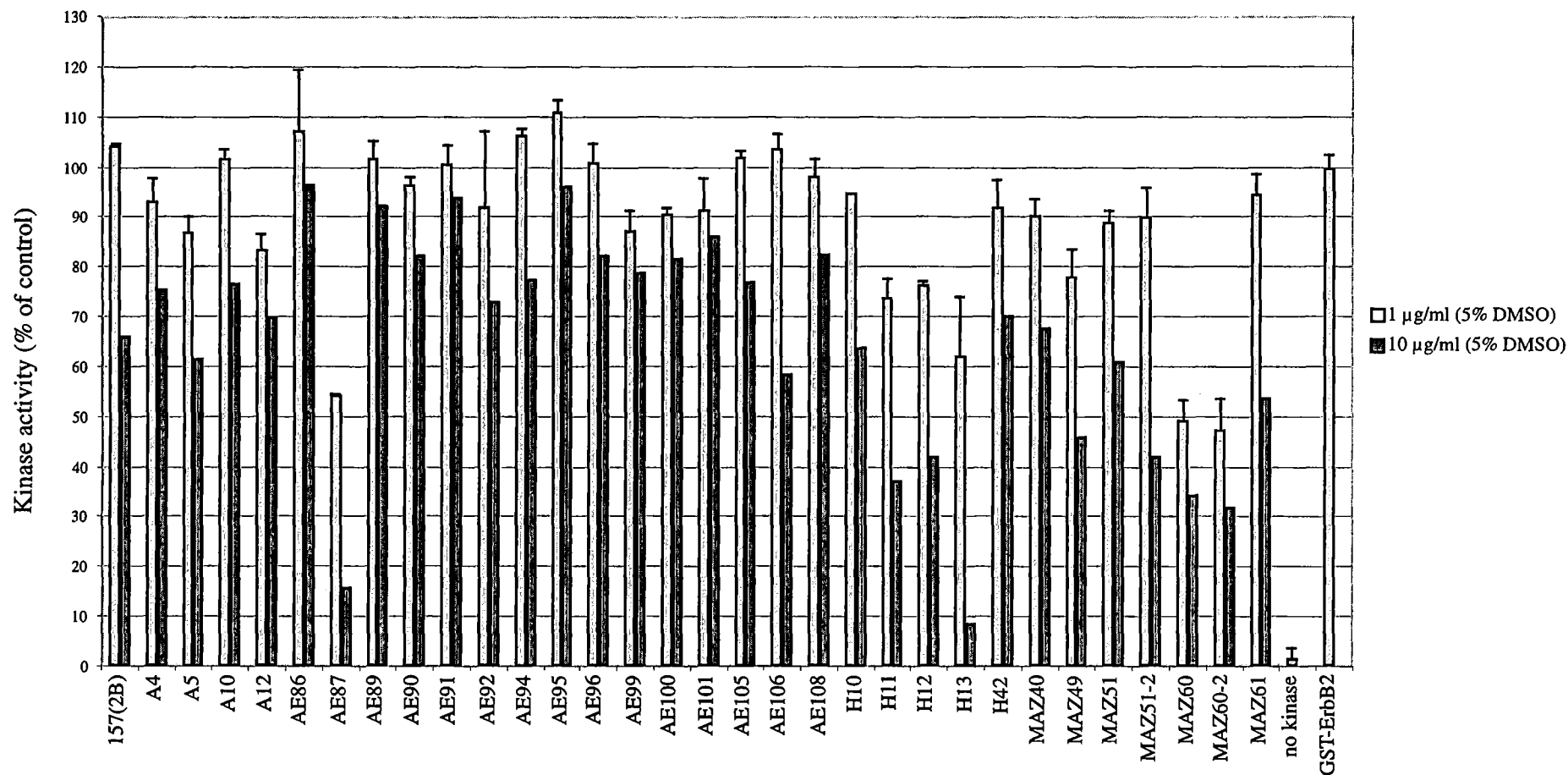
Figure 3.17 *Inhibition of GST-ErbB2 activity by indolinones. In vitro* substrate phosphorylation reactions catalysed by recombinant GST-ErbB2 kinase were performed in the presence of 1μg/ml or 10 μg/ml indicated indolinones and 5% DMSO. The amount of substrate phosphorylation was measured using a colorimetric assay. Data are expressed as % of substrate phosphorylation in the presence of 5% DMSO only (% of control).
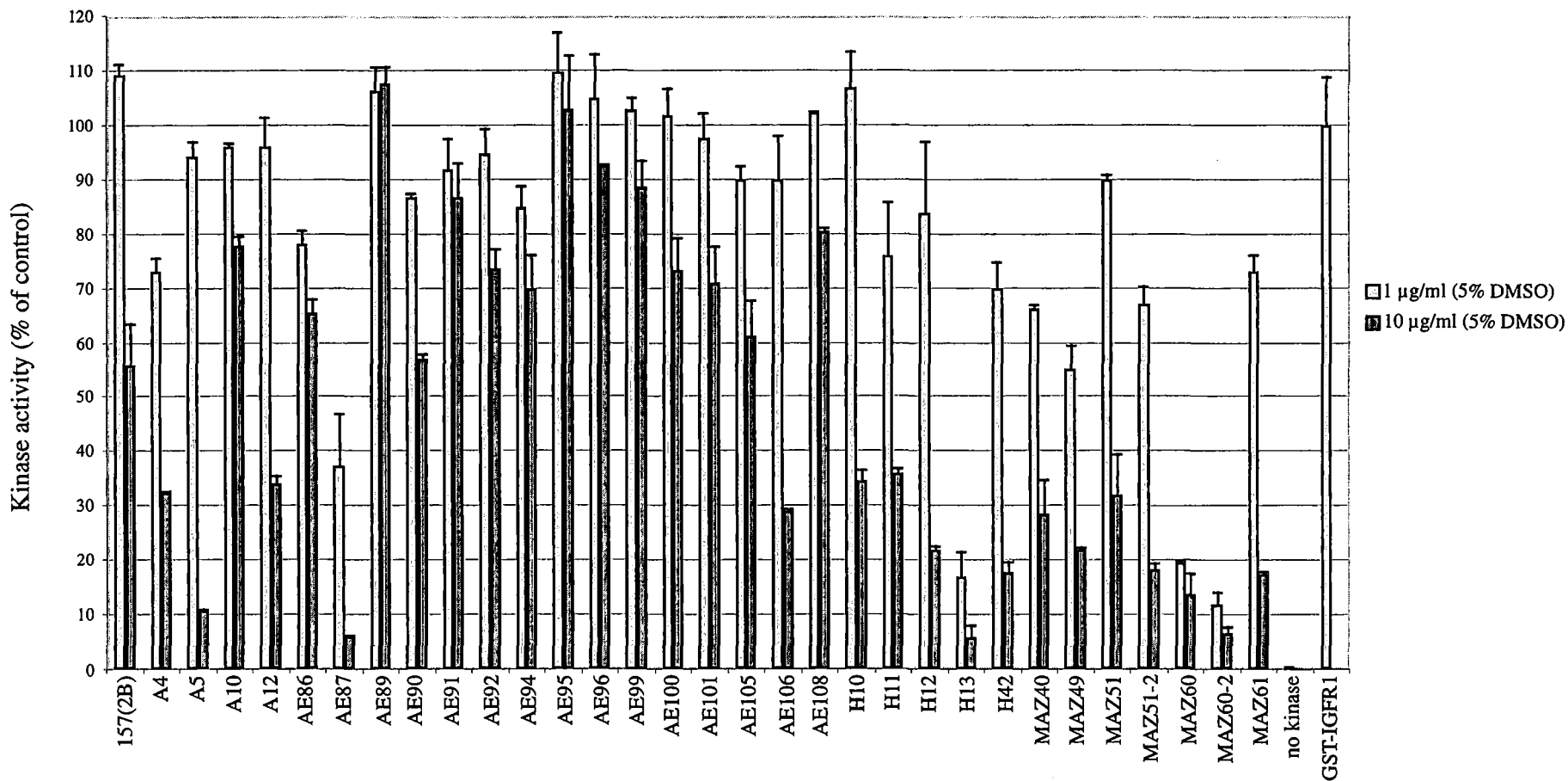
Figure 3.18 *Inhibition of GST-IGFR-1 activity by indolinones. In vitro* substrate phosphorylation reactions catalysed by recombinant GST-IGFR-1 kinase were performed in the presence of 1µg/ml or 10 µg/ml indicated indolinones and 5% DMSO. The amount of substrate phosphorylation was measured using a colorimetric assay. Data are expressed as % of substrate phosphorylation in the presence of 5% DMSO only (% of control).

positive control. As shown in **Figure 3.19**, only some of the tested indolinones could exert their inhibitory action in such a cellular assay. Among those that inhibited VEGFR-3 phosphorylation in a dose-dependent manner were AE87 (blocked VEGFR-3 phosphorylation at 50 µM), AE106 (at 5 µM), MAZ51 (at 5 µM) and MAZ51-2 (at 5 µM).

b) Inhibition of VEGF-D-mediated VEGFR-3 activation

AE87, AE106, MAZ51 and MAZ51-2 could block VEGFR-3 activation in PAE cells stimulated with VEGF-C conditioned medium. To verify that this inhibition is receptor-specific and independent of the ligand, the same experiment was repeated using VEGF-D conditioned medium. **Figure 3.20** demonstrates that the inhibitory effect of AE87, AE106, MAZ51 and MAZ51-2 on VEGFR-3 phosphorylation is equal irrespective of whether VEGFR-3 is activated by VEGF-D (**Fig. 3.20, B**) or by VEGF-C (**Fig. 3.20, A**).

3.4.2.2 Cellular VEGFR-2 phosphorylation assay

PAE/VEGFR-2 cells were used to check whether the 15 inhibitors of VEGFR-2 picked by the *in vitro* kinase assay could also block VEGFR-2 phosphorylation in PAE cells stimulated with VEGF conditioned medium. As before, PAE/VEGFR-2 cells were stimulated with VEGF conditioned medium in the presence of increasing concentrations of the test indolinones. Cells stimulated without inhibitors were used a positive control.

The experiment clearly demonstrated that only few of the indolinones (namely, MAZ51 and MAZ51-2) could significantly inhibit VEGFR-2 phosphorylation in the cellular context (**Fig. 3.21**). Interestingly, in comparison to the PAE/VEGFR-3 system, the inhibition of VEGFR-2 phosphorylation by MAZ51 and MAZ51-2 could only be achieved at a relatively high concentration (50 µM). Even at 50 µM AE87 and AE106 could inhibit VEGFR-2 phosphorylation only partially. These findings suggest that AE106, MAZ51 and MAZ51-2 act as inhibitors that may specifically block VEGFR-3 and not VEGFR-2 at the concentrations tested. Additionally, MAZ51 and MAZ51-2 seem to be the most potent inhibitors of the screened array of indolinones.
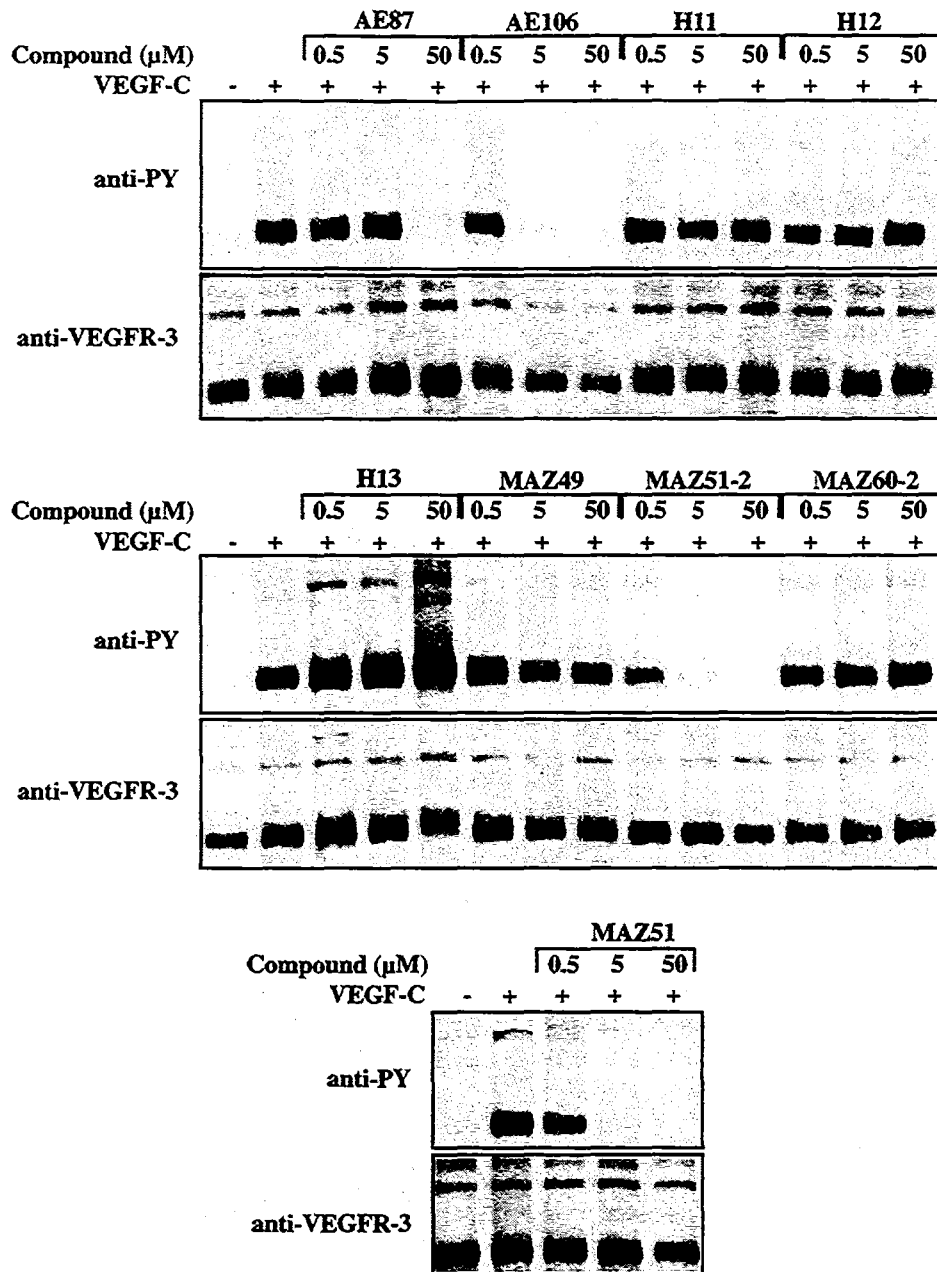
Figure 3.19 *Inhibition of cellular VEGFR-3 phosphorylation by indolinones.* PAE/VEGFR-3 cells were stimulated with VEGF-C conditioned medium in the presence of increasing concentrations of the indicated indolinones. VEGFR-3 receptors were immunoprecipitated from the cell lysates, resolved on a reducing 6% SDS-PAGE, blotted and probed with anti-phosphotyrosine (anti-PY) antibody. Stripped membranes reprobed with anti-receptor antibody were used as a loading control.

A.

B.
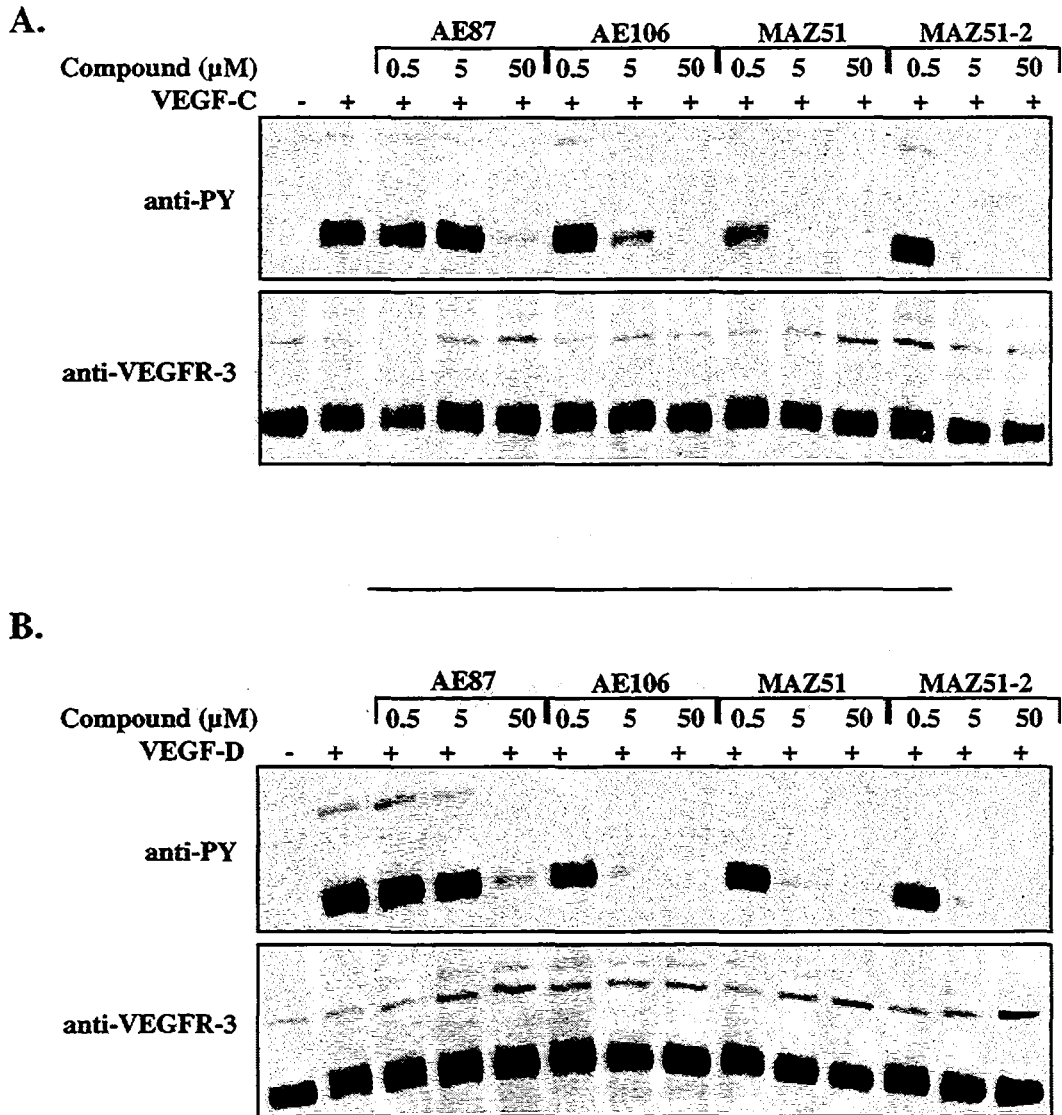
Figure 3.20 *Inhibition of VEGF-C- and VEGF-D-mediated VEGFR-3 phosphorylation by AE87, AE106, MAZ51 and MAZ51-2.* PAE-VEGFR-3 cells were stimulated with VEGF-C (A) or VEGF-D (B) conditioned medium in the presence of the indicated concentrations of the indolinones. VEGFR-3 receptors were immunoprecipitated from the cell lysates, resolved on a reducing 6% SDS-PAGE, blotted and probed with anti-phosphotyrosine (anti-PY) antibody. Stripped membranes re-probed with anti-receptor antibodies were used as a loading control.
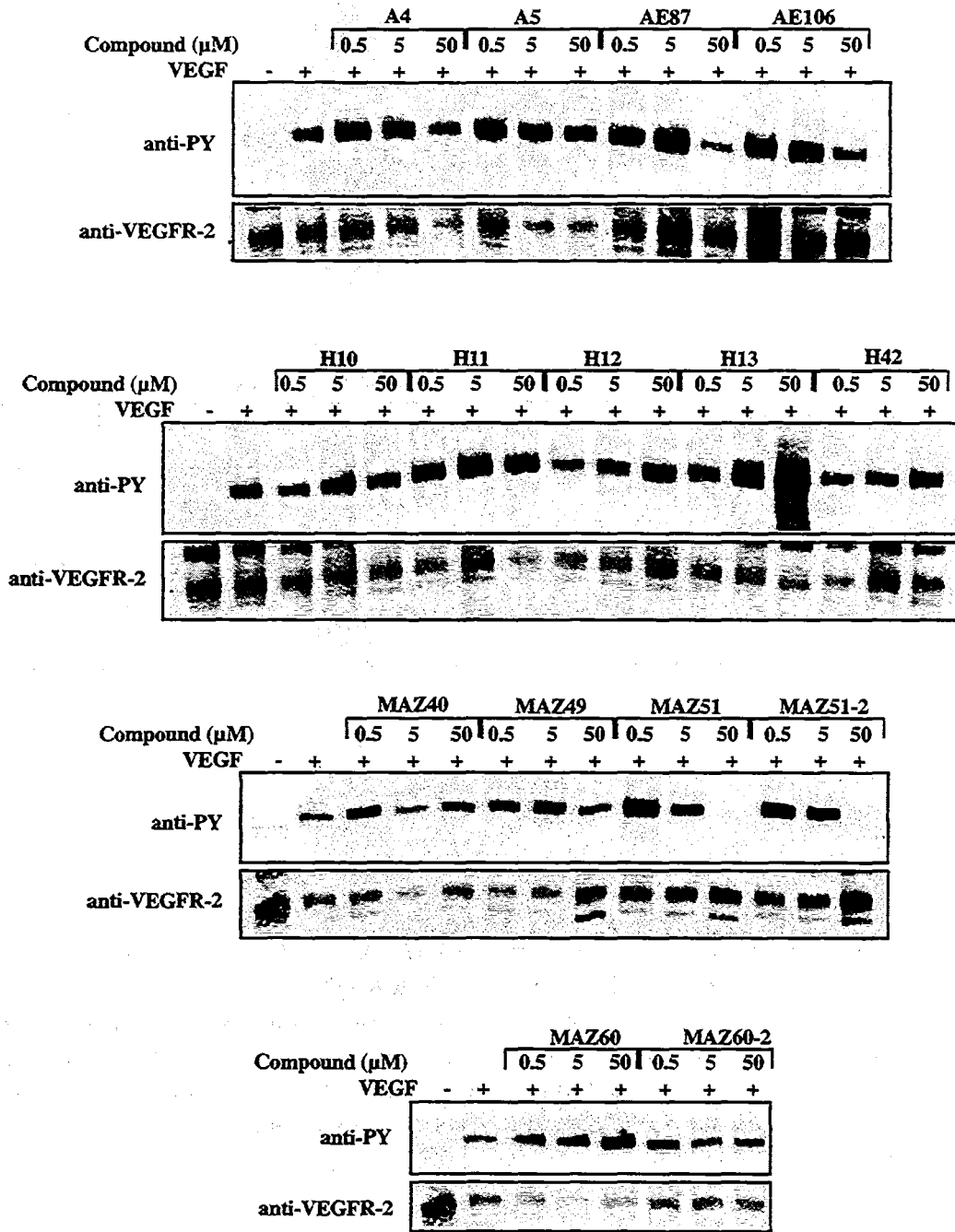
Figure 3.21 *Inhibition of cellular VEGFR-2 phosphorylation by indolinones.* PAE/VEGFR-2 cells were stimulated with VEGF conditioned medium in the presence of increasing concentrations of the indicated indolinones. VEGFR-2 receptors were immunoprecipitated from the cell lysates, resolved on a reducing 6% SDS-PAGE, blotted and probed with anti-phosphotyrosine antibody (anti-PY). Stripped membranes re-probed with anti-receptor antibodies were used as loading control.

	AE87			AE106			MAZ51			MAZ51-2		
Compound (µM)	0.5	5	50	0.5	5	50	0.5	5	50	0.5	5	50
VEGF -	+	+	+	+	+	+	+	+	+	+	+	+

anti-PY

anti-VEGFR-1
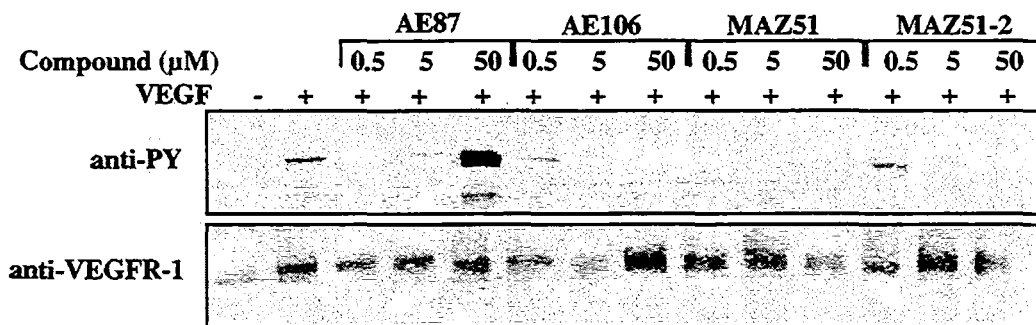
Figure 3.22 *Effect of AE87, AE106, MAZ51 and MAZ51-2 on phosphorylation of cellular VEGFR-1.* PAE-VEGFR-1 cells were stimulated with VEGF conditioned medium in the presence of increasing concentrations of the indolinones. VEGFR-1 receptors were immunoprecipitated from the cell lysates, resolved on a reducing 6% SDS-PAGE, blotted and probed with anti-phosphotyrosine (anti-PY) antibody. Stripped membranes re-probed with anti-receptor antibodies were used as a loading control.

3.4.2.3 Cellular VEGFR-1 phosphorylation assay

It is apparent that 4 of the indolinones analysed in the cellular assays differentially block VEGFR-2 and VEGFR-3 activation. VEGFR-1 is another member of VEGFR family known to play an important role in angiogenesis (Fong *et al.*, 1995). The possible inhibitory effect of the 4 most potent indolinones on the activation of VEGFR-1 was therefore tested using PAE cells expressing VEGFR-1 (PAE/VEGFR-1, Kroll and Waltenberger, 1997). Similar to the previous experiments, PAE/VEGFR-1 cells were stimulated with the VEGFR-1 ligand VEGF in the presence of three different concentrations of AE87, AE106, MAZ51 and MAZ51-2. As shown in **Figure 3.22**, AE106, MAZ51 and MAZ51-2 inhibited VEGFR-1 phosphorylation even at a 5-μM concentration. In contrast, AE87 seems to stimulate phosphorylation of VEGFR-1 in a dose-dependent manner. The data suggest that at similar concentrations AE106, MAZ51 and MAZ51-2 are capable of inhibiting VEGFR-1 and VEGFR-3 phosphorylation, but inhibit VEGFR-2 phosphorylation less potently. In contrast, AE87 is a weak inhibitor of VEGFR-3, which is able to stimulate VEGFR-1 phosphorylation.

3.5 Characterisation of the effect of AE87, AE106, MAZ51 and MAZ51-2 on angiogenesis, lymphangiogenesis and tumour growth

From my previous data, it is clear that AE87, AE106, MAZ51 and MAZ51-2 (**Fig. 3.23**) might have the potential to block tumour growth directly or by inhibiting angiogenesis/lymphangiogenesis. I therefore investigated the effects of these substances on endothelial cell and tumour cell growth in culture. I then checked *in vivo* whether the growth of solid rat tumours could also be blocked by these indolinones.

3.5.1 Anti-angiogenic properties of AE87, AE106, MAZ51 and MAZ51-2

3.5.1.1 Inhibition of endothelial cell proliferation

Triggering of endothelial cell proliferation is a major event in angiogenesis and lymphangiogenesis in which the members of VEGFR have been shown to play a central role (Saaristo *et al.*, 2000). Consequently, the ability to inhibit endothelial cell proliferation indicates an anti-angiogenesis potential of a given substance. AE87, AE106, MAZ51 and MAZ51-2 were therefore tested for their inhibitory effect on proliferation of endothelial cells using an endothelial cell proliferation assay. In this

experiment, HUVEC and HDMEC cells were starved and then stimulated by the addition of serum-containing culture medium. Serum contains a variety of growth factors necessary for endothelial cell proliferation. Before and during the stimulation, increasing concentrations of indolinones were present in the medium. After 24-hour incubation time, tritiated thymidine was added to the cells for several hours. The amount of the incorporated into DNA tritium was measured and proliferation of indolinone-treated versus non-treated cells was determined. As depicted in **Figure 3.24**, in both types of cells, AE106, MAZ51 and MAZ51-2 exerted a potent anti-proliferative action on endothelial cells with an estimated IC_{50} between 3-4 μM (HDMEC, **Figure 3.24, B**) and 7-9 μM (HUVEC, **Figure 3.24, A**). Consistent with the weaker inhibitory action of AE87 on VEGFR-2 and VEGFR-3, AE87 was only weakly inhibitory in the endothelial cell proliferation assay (IC_{50}, >15-20 μM).
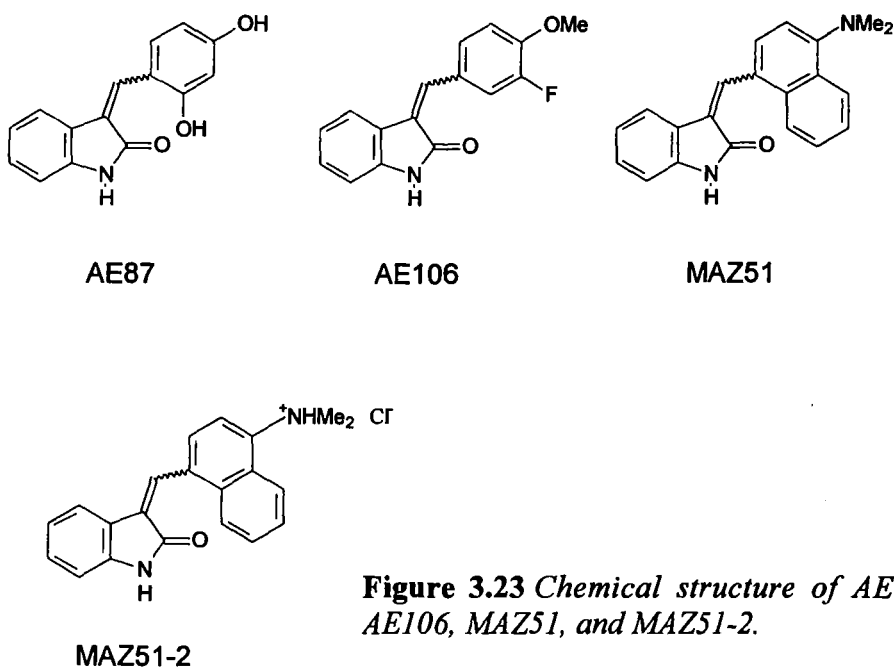
AE87   AE106   MAZ51

Figure 3.23 *Chemical structure of AE87, AE106, MAZ51, and MAZ51-2.*

MAZ51-2

3.5.1.2 Differential inhibition of VEGF- and FGF-mediated vascular endothelial cell proliferation

It is possible that angiogenesis-related RTKs other than VEGFR family members may be inhibited by AE87, AE106, MAZ51 and MAZ51-2, which would also result in suppression of endothelial cell proliferation. Indeed, these inhibitors had some activity against other RTKs in *in vitro* kinase assays (see **Table 3.2**) and also FGFR (Ralph Mazitschek, personal communication). The experiment in 3.5.1.1 in which

serum was used to stimulate endothelial cells could not differentiate between which RTK(s) was being inhibited by the various indolinones used. The effect of the indolinones on endothelial cell proliferation stimulated by defined factors was therefore tested.

Starved HDMEC cells were stimulated with 100 ng/ml VEGF, 32.5 ng/ml aFGF or 12.5 ng/ml bFGF diluted in serum and growth factor-free culture medium. Before and during the stimulation increasing concentrations of the indolinones were present in the medium. After 24-hour incubation time, (^{3}H)-thymidine was added to the cells for 4-6 hours. The amount of tritium incorporated into DNA was measured and proliferation of indolinone-treated versus non-treated cells was determined. As shown in **Figure 3.25**, only AE106 could potently inhibit the FGF receptor signalling in HDMEC (IC_{50}, 1.5-2 μM). This effect was comparable to the inhibition of VEGF-specific proliferation by AE106 (IC_{50}, 1 μM). In contrast, MAZ51 and MAZ51-2 did not cause considerable suppression of FGF-mediated proliferation, whereas its VEGFR-specific effect as reflected by inhibition of VEGF-stimulated proliferation was quite pronounced (IC_{50}, 2.5-3 μM). Although AE87 did inhibit FGF-stimulated proliferation (IC_{50}, >9 μM), its inhibitory effect on VEGFR signalling was about two times stronger (IC_{50}, 5 μM). In conclusion, under the tested conditions, MAZ51 and MAZ51-2 did not affect FGFR, AE87 acted as a weak FGFR inhibitor, whereas AE106 potently blocks FGFR function. In contrast, VEGF-stimulated proliferation, which is mediated via VEGFR-1 and VEGFR-2, could be inhibited by all the indolinones, with again AE87 being the weakest inhibitor of VEGFR-2 function.

3.5.1.3 Induction of apoptosis in endothelial cells

Inhibition of RTK signalling and thus EC proliferation observed in 3.5.1.1 and 3.5.1.2 by the indolinones may lead to cell cycle arrest or induction of programmed cell death (apoptosis) – both would be detectable by inhibition of proliferation. To get an insight into whether the observed anti-proliferative effect of the four indolinones is associated with programmed cell death in the endothelial cells, an *in vitro* apoptosis assay was performed. As before, starved HDMEC cells were stimulated with normal culture medium in the presence of different concentrations of the indolinones. After 24 hour culturing the cells were lysed and the induction of programmed cell death was quantified by specific detection of apoptosis-associated cytoplasmic mono- and
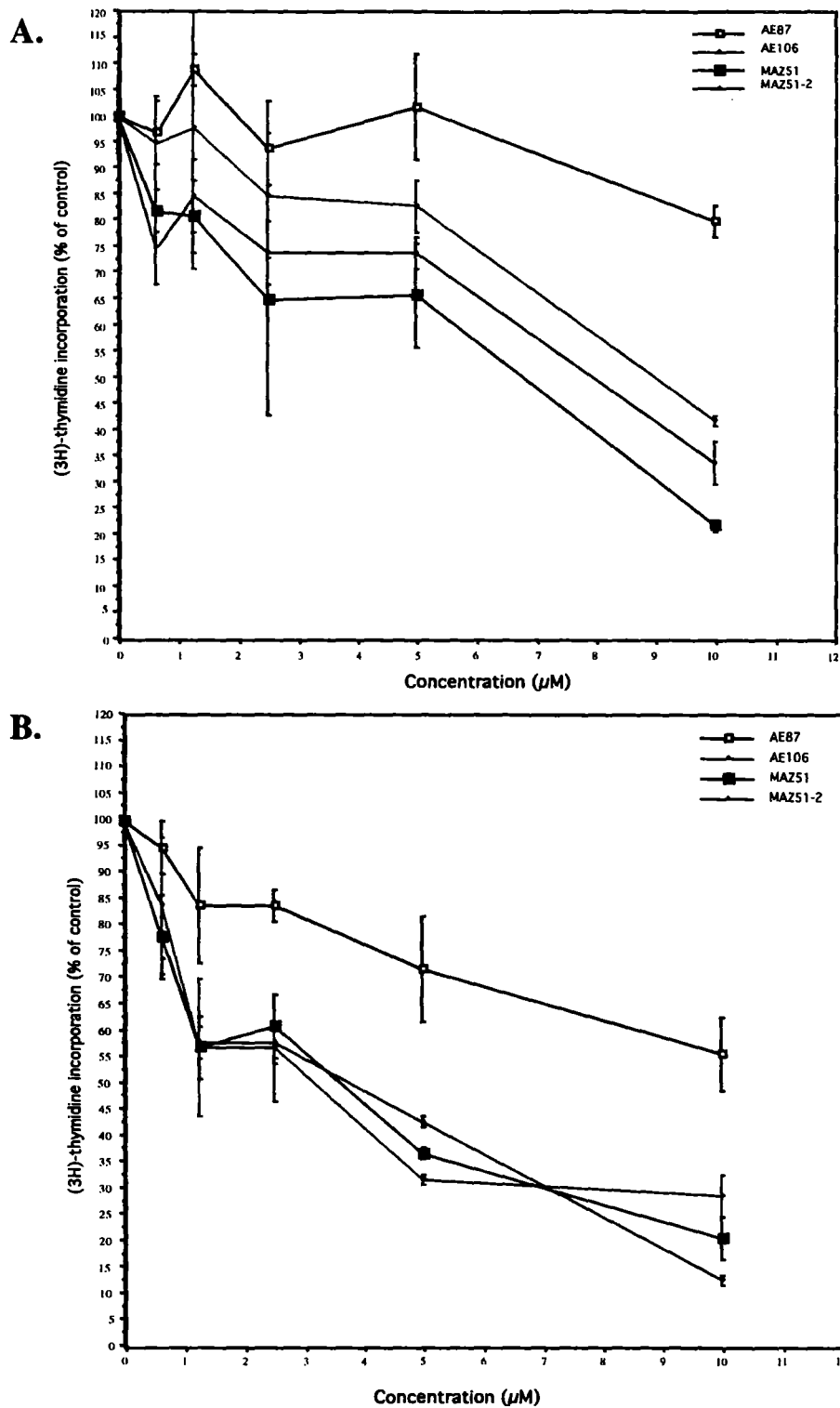
Figure 3.24 *Inhibition of vascular endothelial cell proliferation by AE87, AE106, MAZ51 and MAZ51-2.* Starved HUVEC (A) and HDMEC (B) cells were stimulated by normal culture medium and cultured for 24 hours in the presence of the indicated concentrations of the test compounds. A pulse of tritium was given to the cells for the last 4-6 hours of the stimulation after which the cells were harvested onto a filter and the amount of incorporated radioactivity was measured. Data are expressed as percent of proliferation of non-treated cell (% of control).

A.

AE87

Legend:
- VEGF (100 ng/ml)
- aFGF (32.5 ng/ml)
- bFGF (12.5 ng/ml)

Y-axis: (3H)-thymidine incorporation (% of control)
X-axis: Concentration (μM)

B.

AE106

Legend:
- VEGF (100 ng/ml)
- aFGF (32.5 ng/ml)
- bFGF (12.5 ng/ml)

Y-axis: (3H)-thymidine incorporation (% of control)
X-axis: Concentration (μM)

103
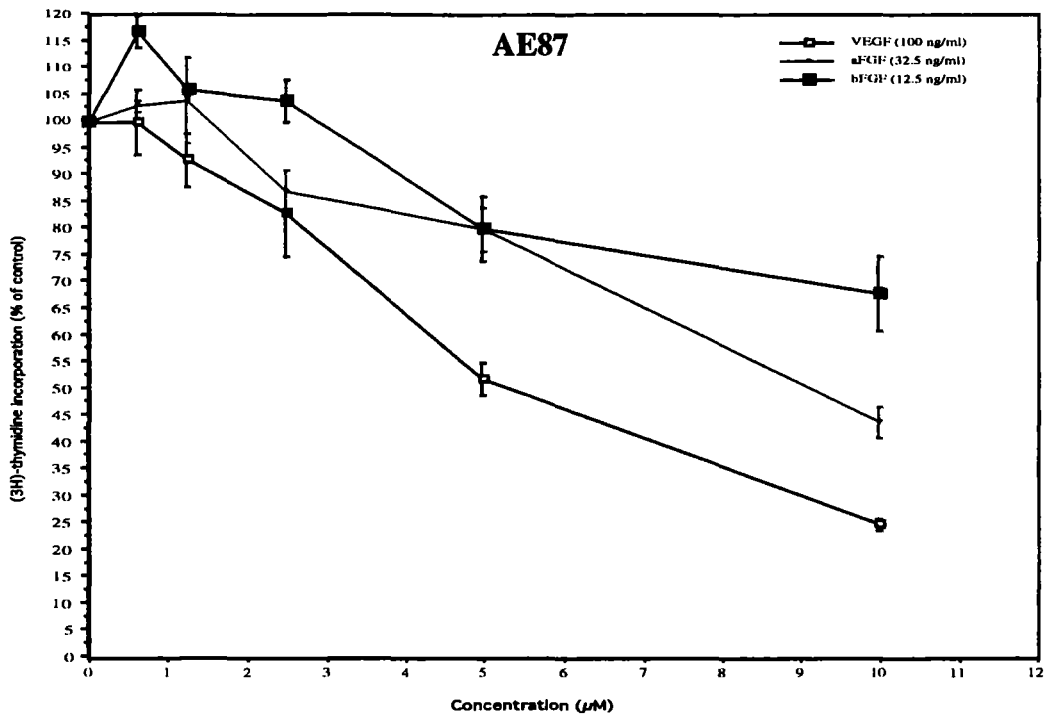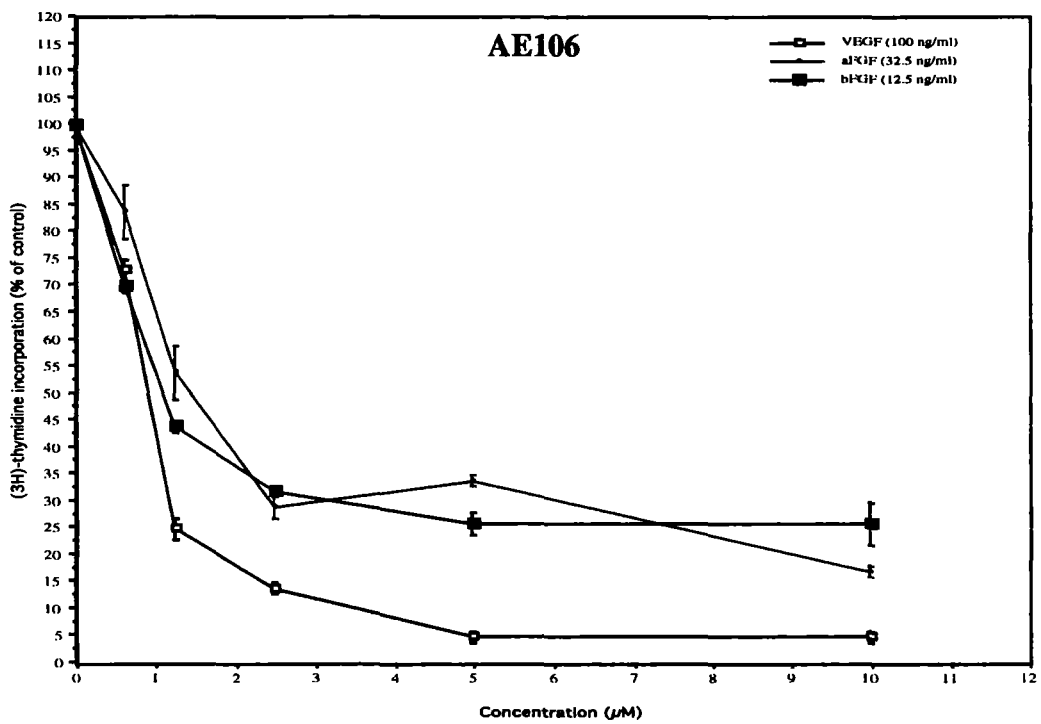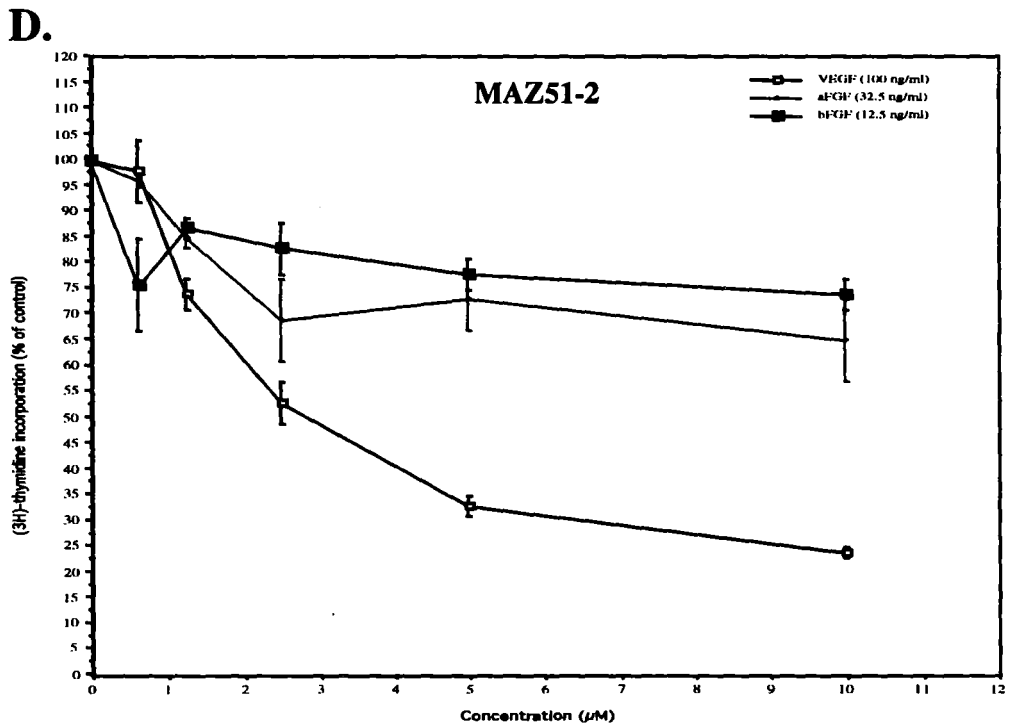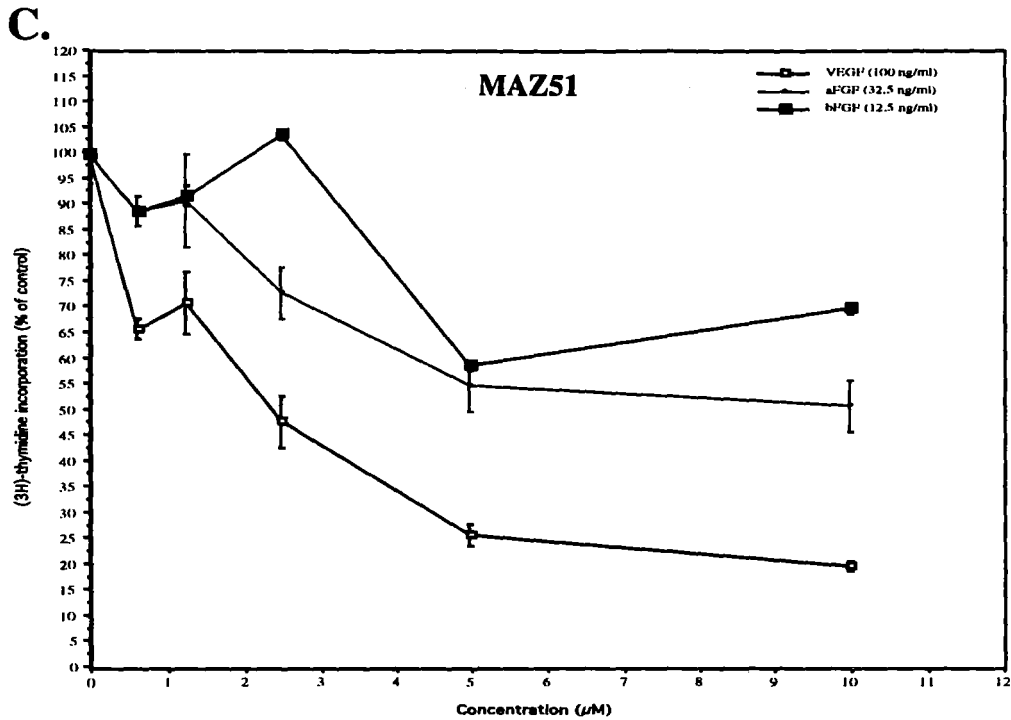
Figure 3.25 *Differential inhibition of VEGF- and FGF-stimulated endothelial cell proliferation by indolinones.* Starved HDMEC cells were stimulated for 24 hours with 100 ng/ml VEGF, 32.5 ng/ml aFGF or 12.5 ng/ml bFGF in the presence of the indicated concentrations of AE87 (A), AE106 (B), MAZ51 (C), MAZ51-2 (D). A pulse of tritium was given to the cells for the last 4-6 hours of the stimulation after which the cells were harvested and the amount of incorporated radioactivity was measured. Data are expressed as percent of non-treated cell proliferation (% of control).

oligonucleosomes with an antibody coupled to peroxidase. The amount of the chromogenic substrate produced in the subsequent colorimetric reaction catalysed by the antibody-coupled enzyme was quantified using a spectrophotometer at 405 nm. Thus the OD_{405} reading directly correlates with the amount of mono- and oligonucleosomes in the cell lysates. The experiment revealed (see **Fig. 3.26**) that MAZ-51 and MAZ51-2 had a strong dose-dependent apoptogenic effect in HDMEC, whereas AE87 and AE106 induced very little cell death.

3.5.1.4 Inhibition of endothelial cell sprouting

Endothelial cell sprouting is independent of cell proliferation and caused by elongation of individual endothelial cells upon receipt of an appropriate angiogenic stimulus (*e.g.* VEGF). It was therefore important to check whether the identified anti-angiogenic indolinones could also block endothelial sprouting. To this end, the effect of AE87, AE106, MAZ51 and MAZ51-2 on the formation of sprouts during the culturing of HUVEC spheroids in collagen gels was assessed. Endothelial spheroids were obtained by culturing HUVECs in a round-bottomed plate in the presence of an anti-adhesive substance (*i.e.* methylcellulose). Upon 3-day stimulation with VEGF, the collagen-embedded spheroids gave rise to long endothelial processes such as those shown in **Figure 3.27** (a, arrows). However, when 10 µM AE106 (**Fig. 3.27, c**), MAZ51 (**Fig. 3.27, d**), or MAZ51-2 (**Fig. 3.27, e**) was present in the collagen gels such sprouting was dramatically suppressed. Consistent with its overall weak inhibitory properties AE87 exhibited the weakest effect in the spheroid cultures, as some sprouts still persisted after 3 days of culture (**Fig. 3.27, b**).

3.5.2 Anti-tumour properties of AE87, AE106, MAZ51

Angiogenesis is a very important aspect of tumour progression and metastasis. My previous experiments demonstrate that this process is likely to be blocked by the indolinones AE87, AE106, MAZ51, MAZ51-2. However, these inhibitors also are active against other RTKs in *in vitro* assays (**Table 3.1**). They may therefore also interfere with tumour growth directly. I therefore next investigated (a) whether tumour cell proliferation is directly affected by the indolinones, and (b) whether tumour growth *in vivo* can be reduced or stopped by the inhibitors. According to all the experiments performed previously, MAZ51 and MAZ51-2 possess virtually

Relative absorbance (405 nm)

0.40

0.35

0.30

0.25

0.20

0.15

0.10

0.05

0.00

-0.05

AE87
AE106
MAZ51
MAZ51-2

0 1 2 3 4 5 6 7 8 9 10 11 12
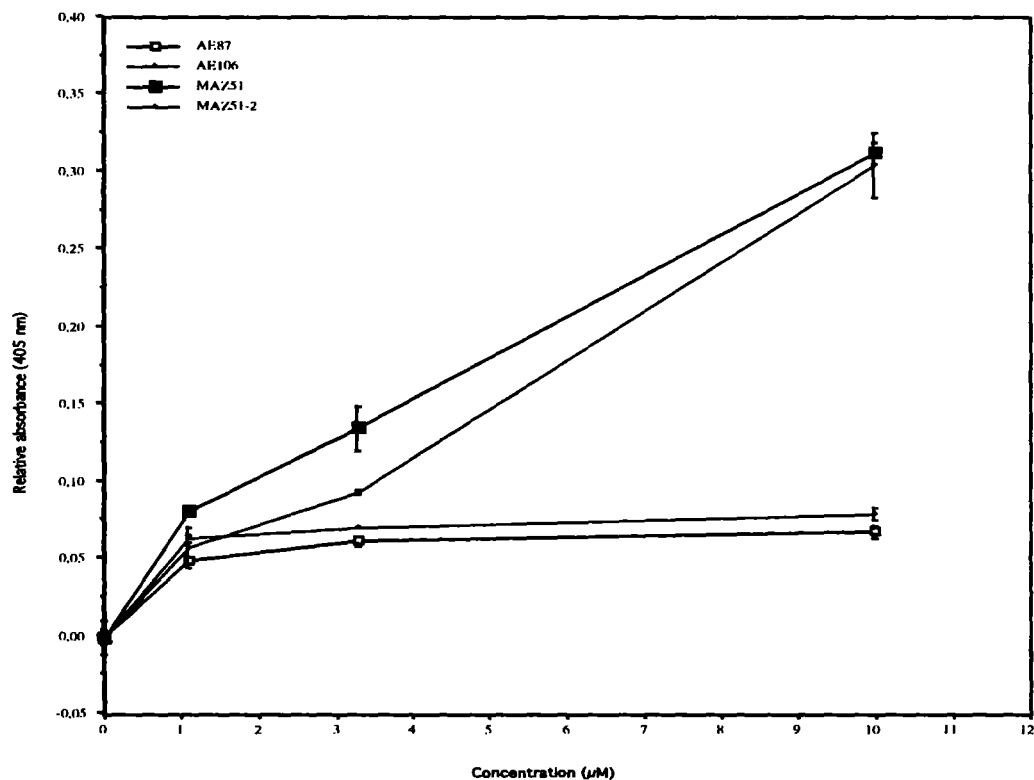
Concentration (μM)

Figure 3.26 *Induction of apoptosis in HDMEC by the indolinones.* Starved HDMEC cells were stimulated with normal culture medium for 24 hours in the presence of different concentrations of AE87, AE106, MAZ51 and MAZ51-2. Apoptosis was quantified using anti-DNA-peroxidase antibody. The amount of chromophoric substrate produced in a chromogenic reaction catalysed by peroxidase was measured photometrically at 405 nm and correlated with the amount of apoptosis-associated cytoplasmic mono- and oligonucleosomes present in the cell lysate. Data are expressed as relative absorbance at 405 nm, using non-treated cell sample as a blank.

identical inhibitory properties. Moreover, these two indolinones have very similar chemical structure. Only MAZ51 was therefore used in the following tumour experiments.

Tumour cell line	Metastatic potential
Bsp73 rat pancreatic carcinoma system	
1AS	-
ASML	+
Dunning rat prostatic carcinoma system	
G	-
AT6.1	+
13762NF rat mammary carcinoma system	
MTLN3	+
MTLY	+
Other mammary carcinomas	
NM081	+
MT450	+

Table 3.3 *Rat tumour cell lines used for the study of the direct anti-tumour effect of indolinones. Legend:* -, non-metastatic cell lines; +, cell lines with different metastatic potential. Data are taken from Nestl *et al.* (2001).

3.5.2.1 Inhibition of tumour cell proliferation in vitro

In order to determine the direct effect of the indolinones on tumour cells, a panel of rat tumour cell lines which differ in their metastatic potential was used (see **Table 3.3**). In the experiment, the indicated rat tumour cells were cultured for 24 hours in standard growth media supplemented with 2.5 μM or 10 μM AE87, AE106, and MAZ51, respectively. Cells which were grown in medium containing 1% DMSO (solvent control) were used as a control. To determine proliferation rate, tritiated thymidine was present in the cell medium for the last 4-6 hours of incubation. Then the amount of incorporated tritium was quantified and interpreted as a measure of cellular proliferation. As shown in **Figure 3.28**, AE87 did not have any measurable anti-proliferative effect on the cell lines tested. AE106 exhibited weak inhibitory effects. In contrast, MAZ51 exhibited a strong inhibitory effect, and proliferation of most of the tested cell lines was inhibited by 50% even at a concentration of 2.5 μM. The notable exceptions are the G and MTLN3 cell lines, which were less sensitive to the action of MAZ51. Thus, MAZ51 is a potent inhibitor of tumour cell proliferation, in addition to blocking endothelial cell proliferation.
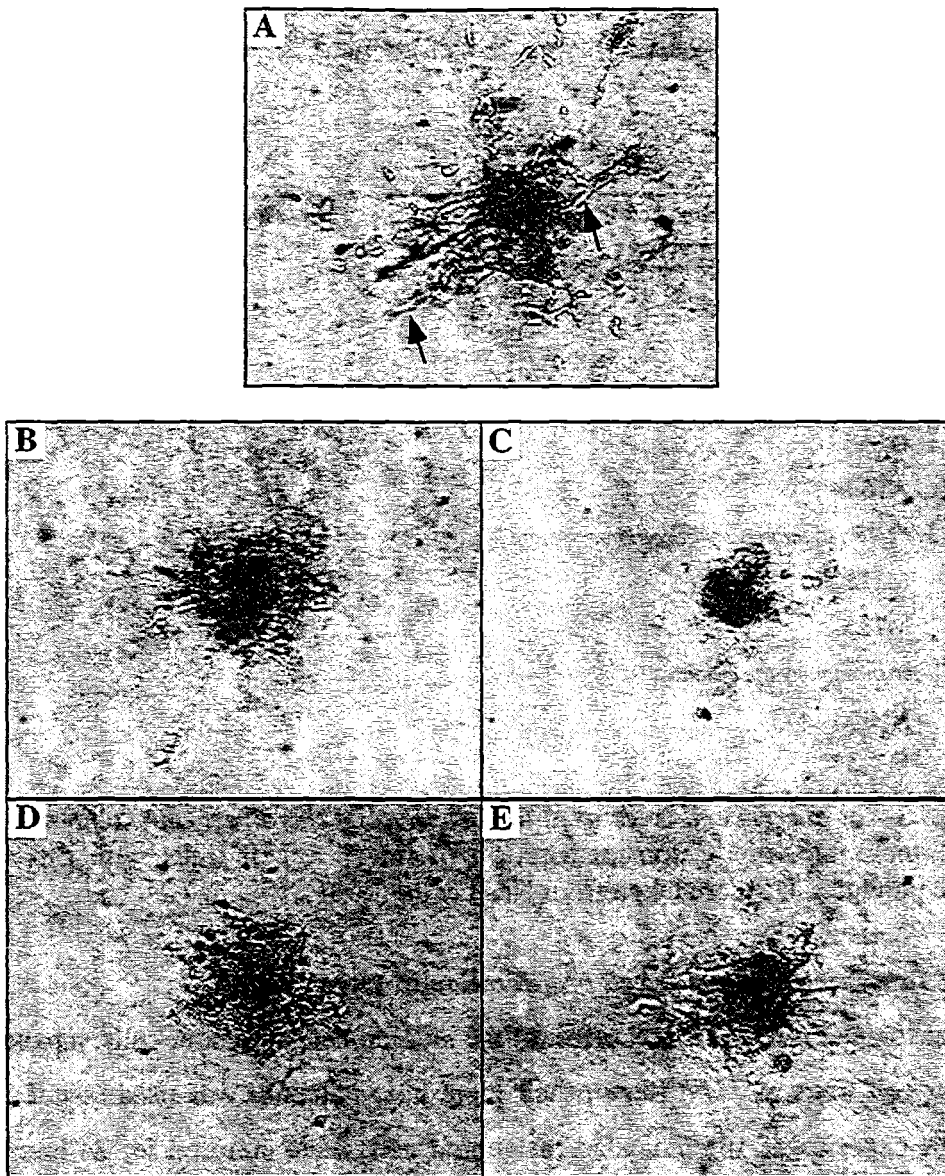
Figure 3.27 *Inhibition of sprouting formation by AE87, AE106, MAZ51 and MAZ51-2.* HUVEC spheroids were cultured in 50% collagen gel containing either 1% DMSO (A) or 10 μM one of the inhibitors: AE87 (B), AE106 (C), MAZ51 (D), MAZ51-2 (E). Disappearance of most sprouts from inhibitor-containing gels but present in DMSO control gel(arrows) is apparent.
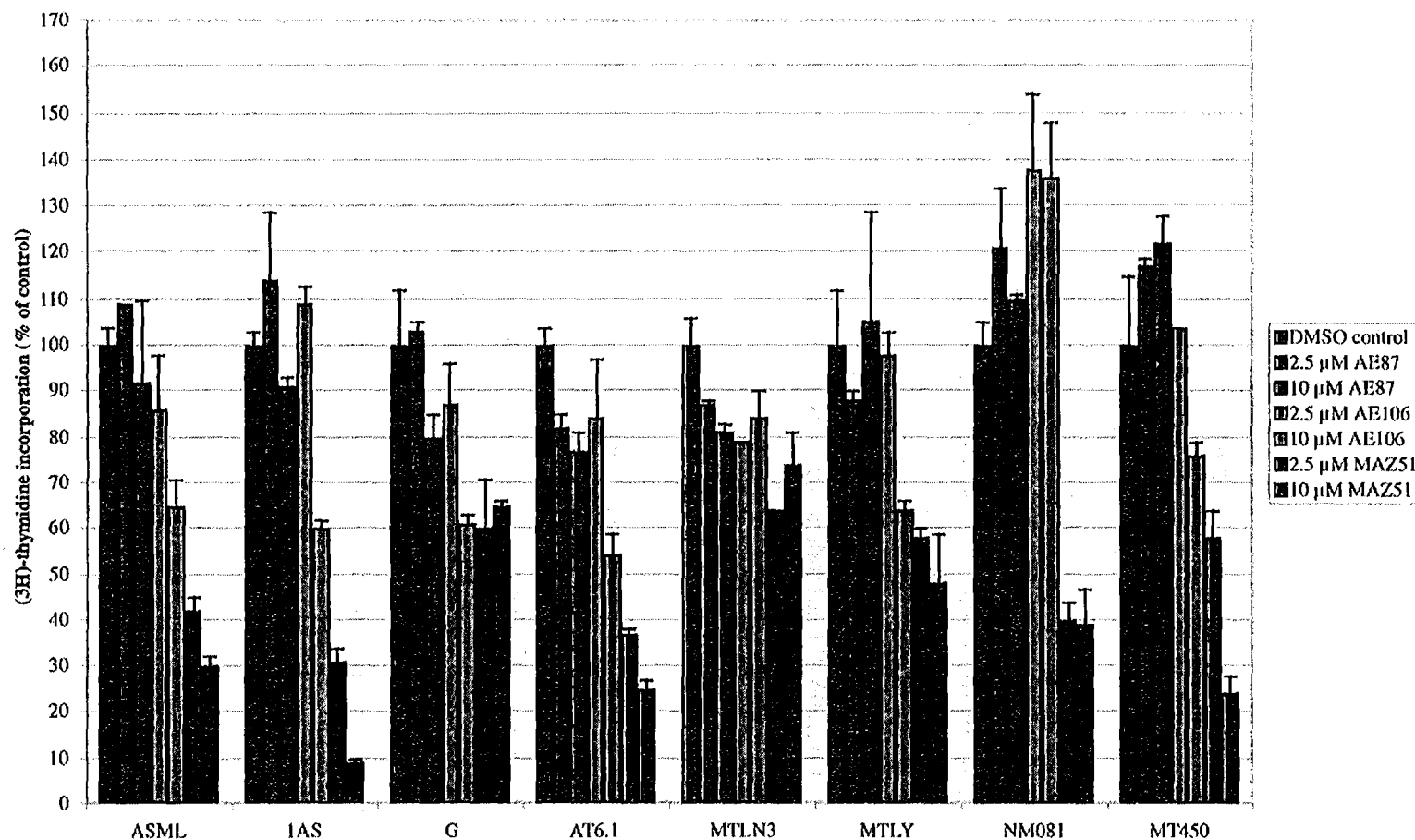
Figure 3.28 *Direct effect of AE87, AE106 and MAZ51 on rat tumour cells.* Cells from the indicated rat tumour cell lines were grown for 24 hours in the presence of 1% DMSO (solvent control), 2.5 μM or 10 μM of the indolinones. Tritiated thymidine was added to the medium for the last 4-6 hours of incubation. Cells were harvested and the amount of incorporated into DNA radioactivity was quantified. Data are expressed as percent of proliferation of cells treated with solvent control (% of control).

3.5.2.2 Induction of apoptosis in tumour cells in vitro

MAZ51 inactivates a range of RTKs, which is probably the cause of the observed suppression of tumour cell proliferation. In the next step, I tested whether this reduction in the proliferation rate is accompanied by an increase in tumour cell apoptosis. The same panel of rat tumour cells was used for this purpose as for the proliferation experiment. Rat tumour cells were cultured for 24 hours in standard growth media supplemented with 2.5 µM or 10 µM AE87, AE106, and MAZ51, respectively. Cells which were grown in medium containing 1% DMSO (solvent control) were used as a control. The amount of apoptosis in a given cell culture was quantified as described for HDMEC (see 3.5.1.3). The experiment (**Fig. 3.29**) demonstrated that in the majority of cell lines, MAZ51 had strong apoptotic effect, with the 1AS, AT6.1 and MT450 being the most susceptible cell lines. Interestingly, ASML and MTLY did not show much apoptosis in response to MAZ51. The anti-proliferative effect of MAZ51 on these cells may therefore be explained as a cell cycle arrest. Consistent with the weak anti-proliferative action seen in the previous experiment, AE87 and AE106 did not induce apoptosis in the given tumour cell lines. An exception is AT6.1, which seems to have a particularly high sensitivity to indolinone treatment.

3.5.2.3 Effect of indolinones on 1AS rat pancreatic carcinoma tumour growth in vivo

The previous experiments established that AE87 and AE106 have anti-angiogenic properties and that MAZ51 is a strong inhibitor of both angiogenesis and tumour cell growth in vitro. I therefore tested whether these indolinones can also affect in vivo tumour growth. The 1AS rat pancreatic carcinoma cell line was used for the initial experiment, because these cells form rapidly growing non-metastasising tumours in vivo. Four groups of male and female BD10 rats were injected subcutaneously with 1AS cells. Treatment with 100 µl of 10 mg/ml indolinones (AE87, eight per group; AE106, eight per group; MAZ51, eight per group) or the solvent (100% DMSO, eight per group) was commenced on the 2nd day after tumour cell injection and corresponded to approximately 4 mg indolinone per kg body weight per day. The injections were performed intraperitoneally until the tumours reached the maximal legal size (50 mm) in diameter, with the tumours measured every 5 days. As shown in **Figure 3.30**, tumours of the rats treated with MAZ51 were significantly smaller than
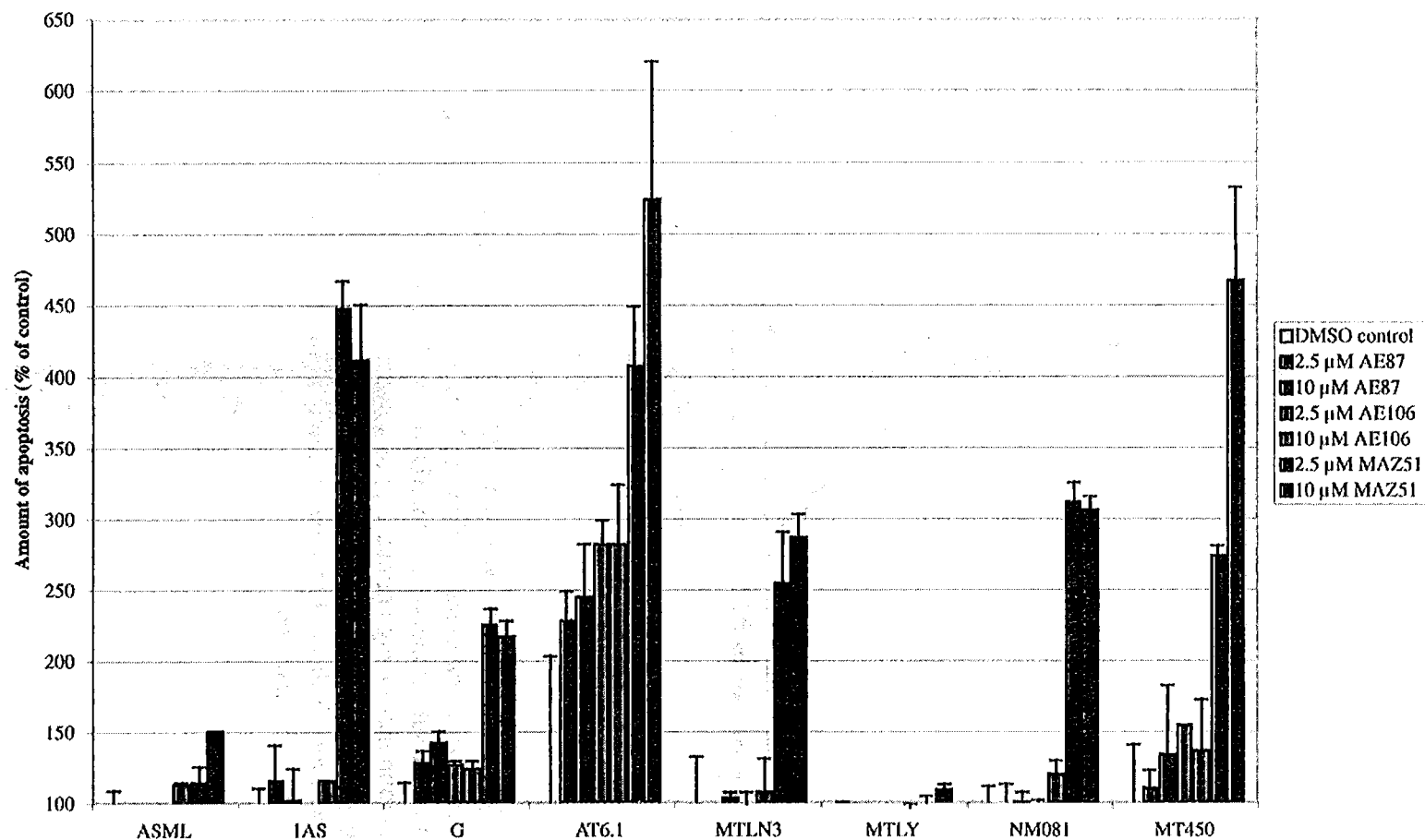
Figure 3.29 *Induction of apoptosis in rat tumour cells by AE87, AE106 and MAZ51.* Cells from the indicated rat tumour cell lines were grown for 24 hours in the presence of 1% DMSO (solvent control), 2.5 µM or 10 µM of the indolinones. Apoptosis was quantified using anti-DNA-peroxidase antibody. The amount of chromophoric substrate produced in a chromogenic reaction catalysed by peroxidase was measured photometrically at 405 nm and correlated with the amount of apoptosis-associated cytoplasmic mono- and oligonucleosomes. Data are expressed as percent of apoptosis in the 'solvent' control cells (% of control).
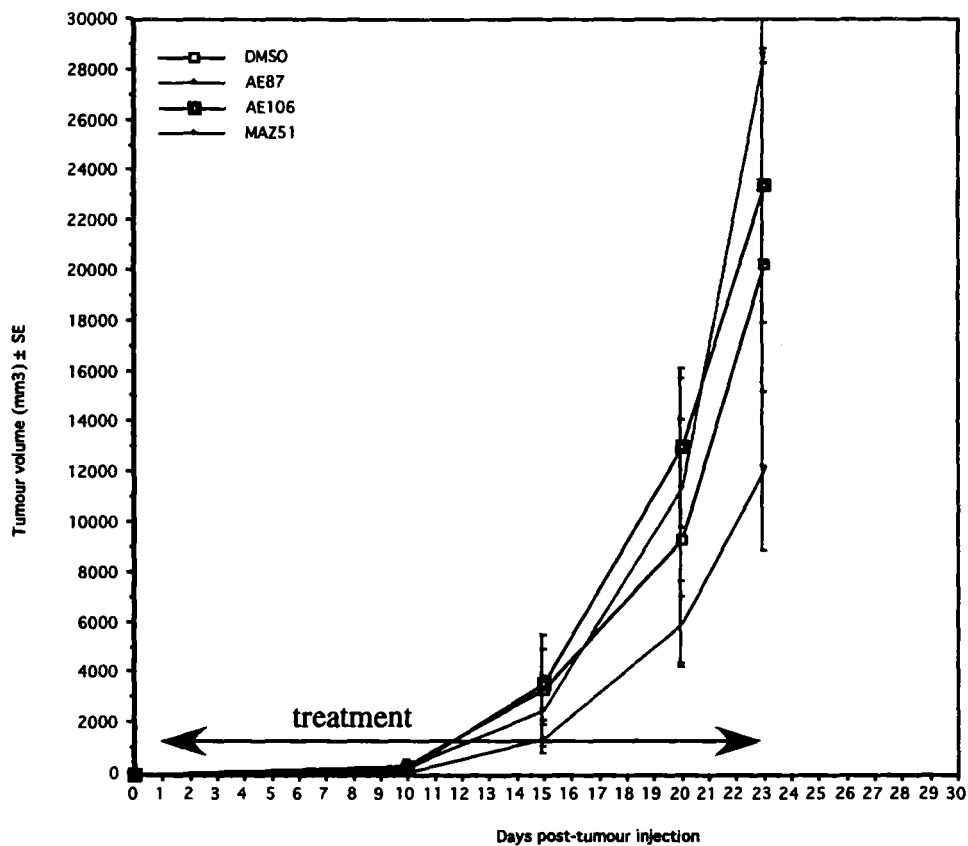
Figure 3.30 *Effect of AE87, AE106 and MAZ51 on the growth of 1AS rat pancreatic carcinoma tumours* in vivo. 1AS rat pancreatic carcinoma cells were injected subcutaneously into BD10 rats. Intraperitoneal injections of the indolinones (1 mg/animal/day) or solvent were initiated on the following day and lasted until the tumours reached legal size. The tumours were measured every 5 days. The mean tumour volumes measured in 8 animals per group are presented.

the control ones treated with DMSO. However, rats which received AE87 or AE106 developed tumours bigger than in the control group. Autopsies showed that a significant proportion of the injected substance in the AE106 and MAZ51 groups had precipitated and was deposited on the outer surface of the guts. This may explain why the anti-tumour effect of MAZ51 was comparatively modest compared to its effects in cell toxicity. During the course of injections, none of the manifestations of acute toxicity such as changes in body weight were noted.

3.5.2.4 Effect of MAZ51 on MT450 rat mammary carcinoma tumour growth in vivo

Although 1AS tumours are non-metastatic, the 1AS model has a major drawback in that the 1AS tumours exhibit considerable size variation, which results in relatively large standard error values. MAZ51, which showed an effect on 1AS tumour growth *in vivo*, was therefore used in an attempt to inhibit the growth of MT450 rat mammary carcinoma tumours, which are metastatic but show a consistent and uniform tumour growth *in vivo*. Two groups of male and female Wistar Furth rats were injected subcutaneously with MT450 cells. Due to the extensive precipitation of the indolinones noted in the earlier experiments, animals were treated with a higher dose of the inhibitor (200 µl of 10 mg/ml MAZ51, eight per group) or the solvent (200 µl of 100% DMSO, eight per group). Intraperitoneal injections were initiated on the 2nd day after tumour cell injection and corresponded to approximately 8 mg indolinone per kg body weight per day. The indolinone and control injections are on-going experiments and will be stopped when the tumours will have reached the maximal legal size (50 mm) in diameter. As shown in **Figure 3.31**, the interim results demonstrate that MT450 tumours are considerably smaller when the animals received MAZ51. Due to time constraints, the metastasis rate of the MAZ51-treated and control tumours have not yet been measured. It turned out that at 8 mg/kg/day MAZ51 had a noticeable toxic effect. Several animals within the MAZ51 group developed anaemia characterised by a pale colour of corneas and muscular weakness. Subsequently, approximately 1 week after the start of MAZ51 injections, one animal of the MAZ51 group died. The remaining animals, however, have tolerated MAZ51 at this concentration. So far it is not possible to determine whether the toxicity was due to MAZ51 or due to the extensive intraperitoneal precipitation of the substances in the
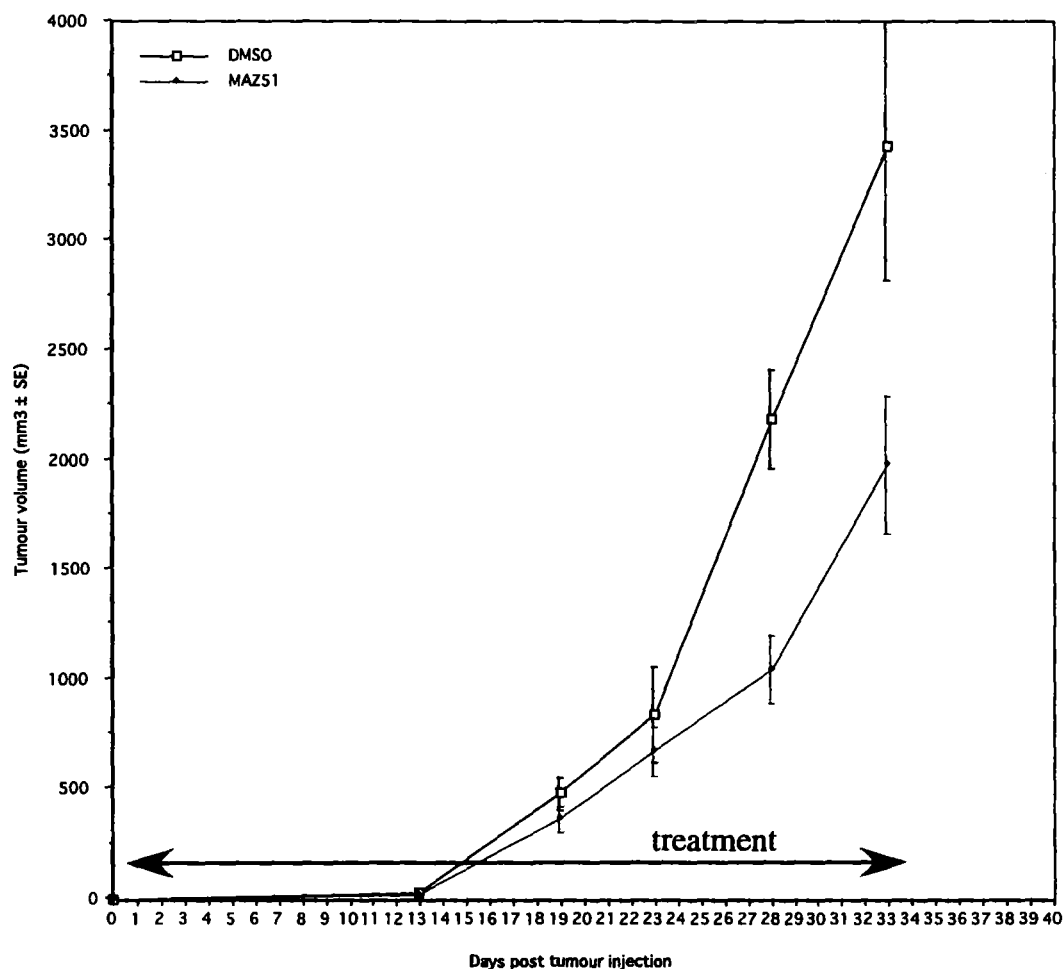
Figure 3.31 *Inhibitory effect of MAZ51 on the growth of MT450 rat mammary carcinoma tumours* in vivo. MT450 rat mammary carcinoma cells were injected subcutaneosly into Wistar Furth rats. Drug treatment with 8 mg/kg/day of MAZ51 in 100% DMSO or solvent control (100% DMSO) was commenced on the next day after tumour cell injection. Tumours were measured every 4-5 days. The mean tumour volumes measured in 8 animals per group are presented.

abdominal cavity of the injected rats.

3.5.2.5 Effect of MAZ51 on the growth of established MT450 rat mammary carcinoma tumours in vivo

In the final experiment, I wanted to verify that the *in vivo* effect of MAZ51 on MT450 tumours observed when the indolinone was injected starting from the first day post tumour injection can be reproduced in the case of already established tumours. In parallel with the animals used in the previous experiment, two groups of male and female Wistar Furth rats were injected subcutaneously with MT450 cells. Animals received 200 μl of 10 mg/ml MAZ51 (eight per group) or 200 μl 100% DMSO solvent (eight per group). This dose equates to approximately 8 mg MAZ51 per kg body weight per day. Intraperitoneal injections were initiated on the 15th day after tumour cell injection when the tumours were already of 1 cm in diameter. The experiment is still ongoing and will be stopped when the tumours have become 50 mm in diameter or the animals have become moribund. **Figure 3.32** demonstrates the interim result of this experiment. Tumours of the animals, which received intraperitoneal injections of MAZ51, again are smaller that those of the control group, suggesting that at the given concentration MAZ51 can also inhibit the growth of already established tumours. However, as before, MAZ51 exhibited general toxicity that led to the death of one animal in the experimental group. Due to time constraints, the metastasis rate of the MAZ51-treated and control tumours have not yet been measured.
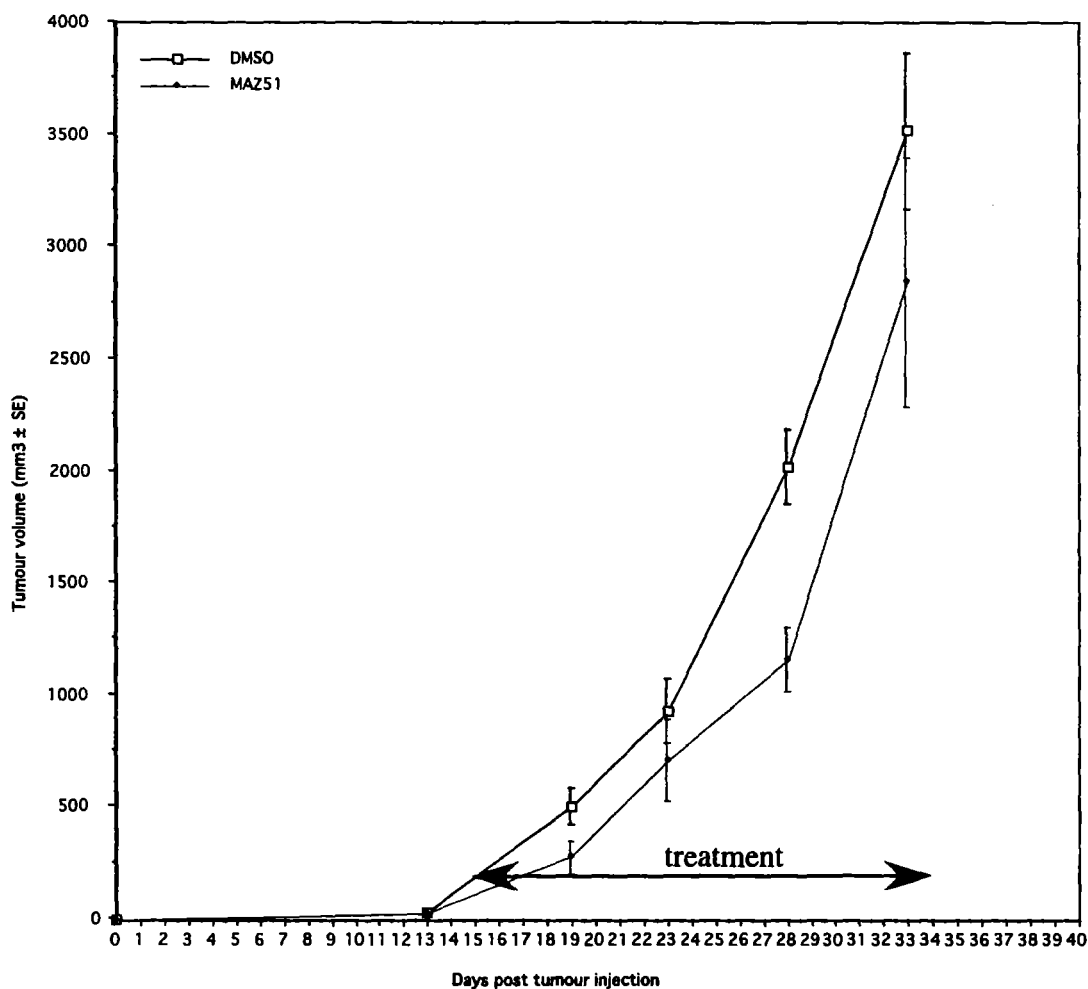
Figure 3.32 *Inhibitory effect of MAZ51 on the growth of established MT450 rat mammary carcinoma tumours* in vivo. MT450 rat mammary carcinoma cells were injected subcutaneosly into Wistar Furth rats. Drug treatment with 8 mg/kg/day of MAZ51 in 100% DMSO or solvent control (100% DMSO) was commenced 15 days after tumour cell injection. Tumours were measured every 4-5 days. The mean tumour volumes measured in 8 animals per group are presented.

3.6 Characterisation of anti-tumour properties of plant-derived hyperforin

Hyperforin is a major active constituent of St. John's Wort (*Hypericum perforatum* L.), which has long been known as a folk remedy for burns and skin injuries (Hänsel *et al.*, 1993) and lately gained a reputation as an effective anti-depressant (Linde *et al.*, 1996). An acylphloroglucinol-type compound that consists of a phloroglucinol skeleton substituted with lipophilic isoprene-chains (Bystrov *et al.*, 1975; Erdelmeier, 1998; **Fig. 3.33**), hyperforin is a natural antibiotic that inhibits the growth of several gram-positive bacteria (Gurevich *et al.*, 1971; Schempp *et al.*, 1999). Recent studies also implicate hyperforin as a potent antiproliferative agent for mammalian cells *in vitro* (Schempp *et al.*, 2000). This latter property prompted me, in collaboration with the group of Dr. Jan Simon, Department of Dermatology, University of Freiburg, to test the anti-tumour activity of hyperforin both *in vitro* and *in vivo*.
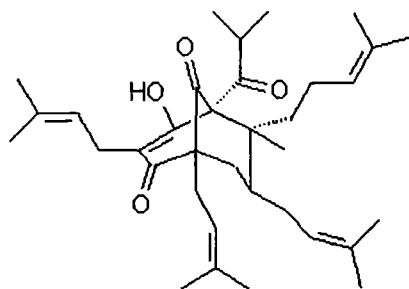
Figure 3.33 *Chemical structure of hyperforin, a major active constituent of St. John's Wort (Hypericum perforatum L.).*

3.6.1 Anti-proliferative action of hyperforin on tumour cells and vascular endothelial cells *in vitro*

Before undertaking *in vivo* tumour studies, I wanted to test whether hyperforin has any direct anti-tumour effect and whether it may also be anti-angiogenic *in vitro*. For this purpose, an in vitro proliferation assay using MT450 rat mammary carcinoma and HDMEC cells was performed. The two types of cells were grown for 24 hours in normal culture medium containing either 0-200 µg/ml hyperforin or the corresponding solvent, namely 10% DMSO. Tritiated thymidine was added to the medium for the last 4-6 hours of the incubation after which the cells were harvested and the incorporated radioactivity was counted. As demonstrated in **Figure 3.34**, hyperforin but not the solvent had a strong anti-proliferative effect on both tumour cells and endothelial cells. Interestingly, the HDMECs were two times more sensitive
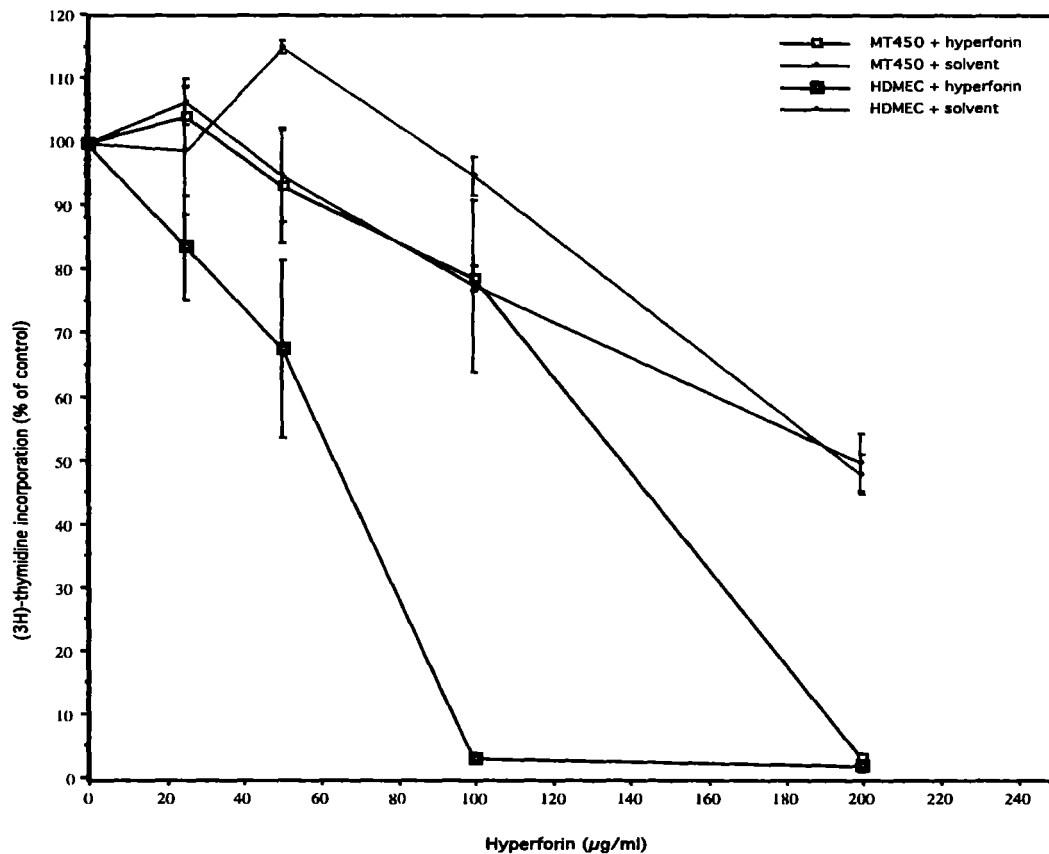
Figure 3.34 *Inhibition of tumour and endothelial cell proliferation by hyperforin.* MT450 and HDMEC were incubated with increasing concentrations of hyperforin or solvent control for 24 hours. A pulse of tritium was given to the cells for the last 4-6 hours of the incubation after which the cells were harvested and the amount of incorporated radioactivity was measured. Data are expressed as percent of non-treated cell proliferation (% of control).

to the action of hyperforin than the MT450 cells: IC_{50} 65 μg/ml (HDMEC) as opposed to IC_{50} 145 μg/ml (MT450). It seems therefore that hyperforin is a potent anti-tumour agent which may also target tumour-induced angiogenesis.

3.6.2 Induction of apoptosis by hyperforin *in vitro*

To determine whether the inhibitory action of hyperforin on tumour cells and endothelial cells is associated with the induction of apoptosis, an *in vitro* apoptosis assay was performed (**Fig. 3.35**). As described previously, cells were grown for 24 hours in normal culture medium containing either 0-200 μg/ml hyperforin or the corresponding solvent after which the amount of apoptosis was quantified. In this experiment, hyperforin specifically induced apoptosis in MT450 tumour cells. However, neither the hyperforin treatment nor the treatment with solvent induced apoptosis in the HDMECs. When the endothelial cells treated with 200 μg/ml hyperforin were observed under the microscope, overwhelming proportion of cells were found dead. These cells could be stained with 0.2% trypan blue, which is taken up mainly by necrotic cells and therefore is used to determine cellular viability. This experiment thus raises the interesting question of whether hyperforin is a cell-specific inhibitor acting in some cases as an apoptosis-inducing agent and in others such as endothelial cells by causing necrosis.

3.6.3 Anti-tumour activity of hyperforin *in vivo*

To assess the potential of hyperforin to inhibit tumour growth *in vivo*, female Wistar Furth rats were injected subcutaneously with rat mammary carcinoma MT450 cells. Drug treatment with 2 mM hyperforin (8 animals per group) was commenced 15 days after tumour cell injection when tumours were readily detectable and could easily be measured. The control group of eight animals received 10% DMSO (solvent control). To compare the effect of hyperforin with that of a clinically used anti-cancer drug, injections with an equimolar concentration of paclitaxel were administered to another group of 8 animals in parallel. Daily injections of the drug/control were administered subcutaneously at the site of the tumour cell injection for two weeks. As shown in **Figure 3.36**, hyperforin inhibited tumour growth to a similar extent as paclitaxel. These data suggest that the potential of hyperforin to inhibit tumour growth *in vivo* is commensurate with that of an established anti-cancer drug. The survival rate of the animals after the completion of the treatment was established (**Fig. 3.37**).
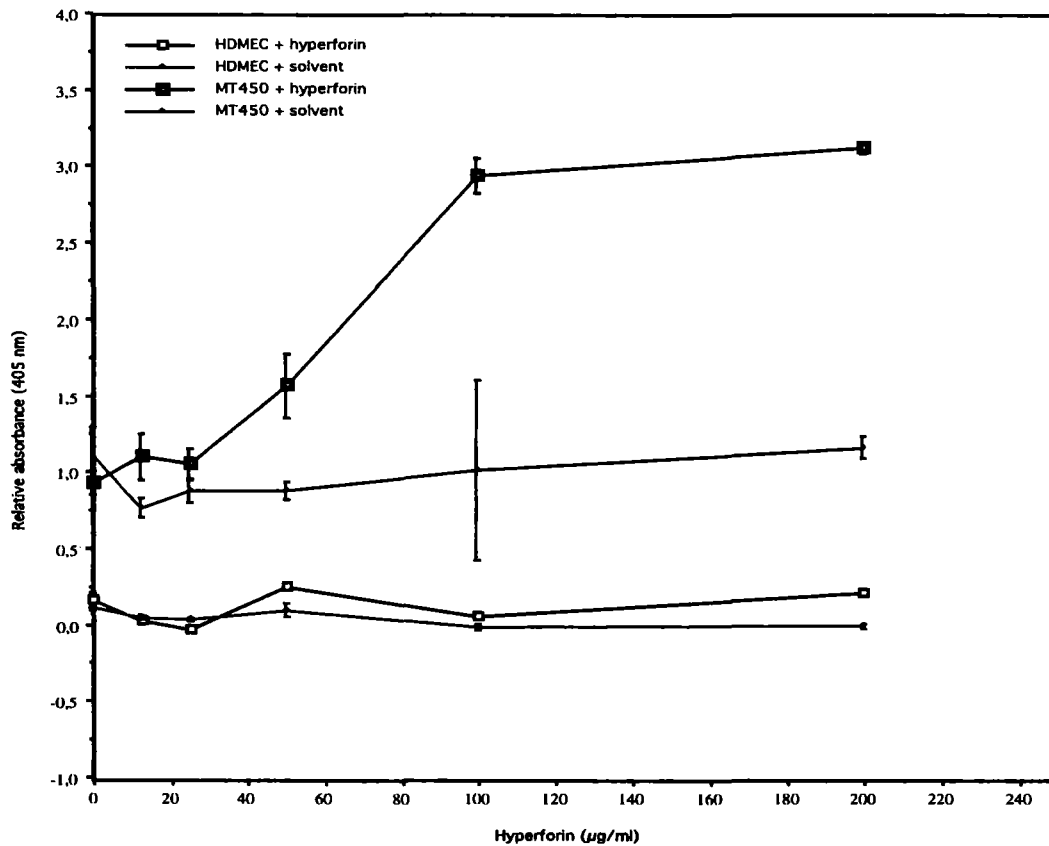
Figure 3.35 *Difference in the apoptogenic effects of hyperforin on MT-450 rat mammary carcinoma cells and HDMECs.* MT-450 and HDMECs were incubated for 24 hours with 0-200 µg/ml hyperforin or corresponding solvent control. Apoptosis was quantified using anti-DNA-peroxidase antibody. The amount of chromophoric substrate produced in a chromogenic reaction catalysed by peroxidase was measured photometrically at 405 nm and correlated with the amount of apoptosis-associated cytoplasmic mono- and oligonucleosomes present in the cell lysate. Data are expressed as relative absorbance at 405 nm, using non-treated cell sample as a blank.
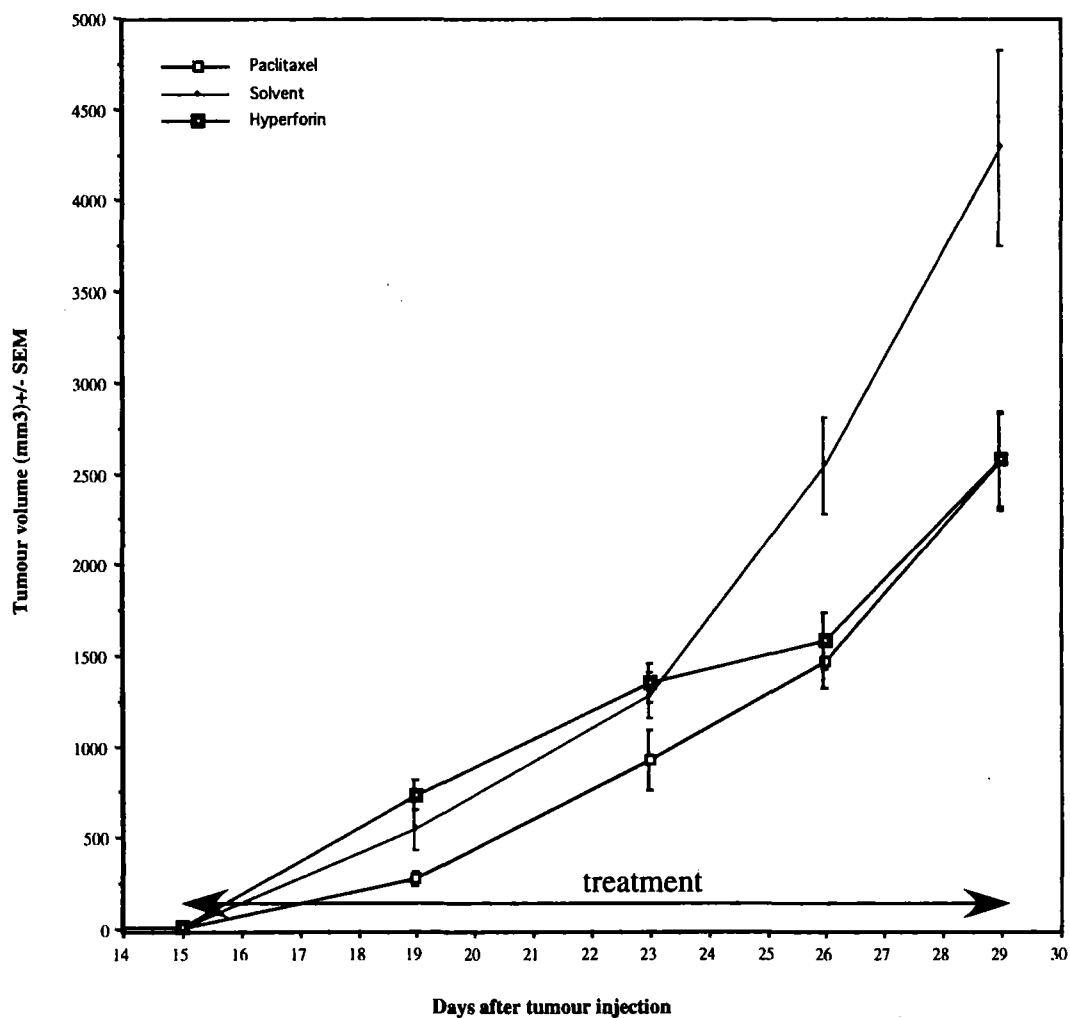
Figure 3.36 *Inhibition of MT450 rat mammary carcinoma tumour growth* in vivo *by hyperforin* MT450 rat mammary carcinoma cells were injected subcutaneosly into female Wistar Furth rats. Drug treatment with 100 µl of equimolar (2 mM) concentrations of hyperforin or paclitaxel (clinically-used drug) or solvent control was commenced 15 days after tumour cell injection and lasted for a period of two weeks. Tumours were measured every 4-5 days and the mean tumour volumes measured in 8 animals per group are presented.

121
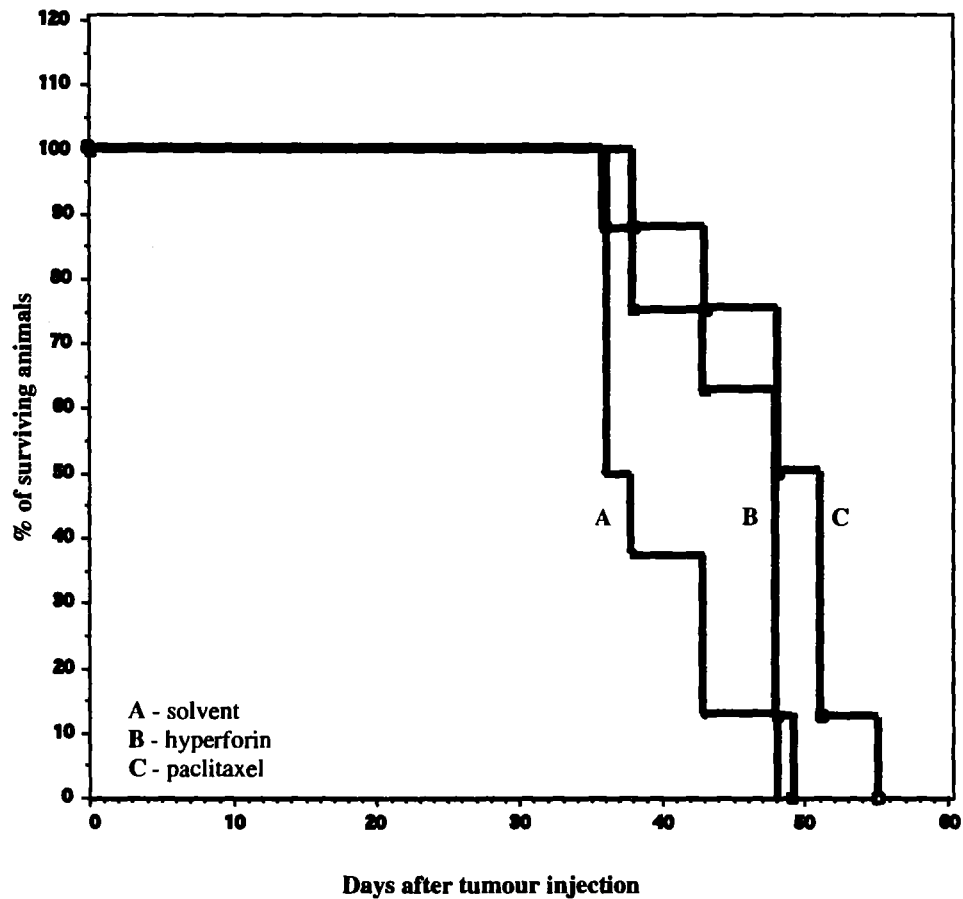
Figure 3.37 *Survival curve for the hyperforin-treated animals.* After the treatment had been completed the animals were observed and killed when the size of the primary tumour reached the legal limit (50 mm in diameter) or the animals became moribund.

Consistent with the tumour-inhibiting effect of hyperforin, animals that had received the hyperforin treatment survived for a longer time, as compared to the control group of animals. During the course of injections, none of the manifestations of acute toxicity such as changes in body weight were noted.

3.7 Publications and patents

The following publications have been the result of the work described in the thesis:

Patent application to the Forschungszentrum Karlsruhe (AE87, AE106 and MAZ51 kinase inhibitors). **Chirchin, V.,** Giannis, A., Mazitschek, R., Sleeman, J. (2001).

Kirkin, V., Mazitschek, R., Krishnan, J., Steffen, A., Mandriota, S., J., Waltenberger, J., Pepper, M., S., Giannis, A., and Sleeman, J., P. (2001) Characterisation of indolinones which specifically inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 but not VEGFR-2. *Eur. J. Biochem.* (submitted).

Schempp, C., M., **Kirkin, V.,** Simon-Haarhaus, B., Kersten, A., Termeer, C., C., Wissel, S., Sleeman, J., P., and Simon, J, C. (2001) Hyperforin is anovel anti-cancer drug that inhibits tumour cell growth by induction of apoptosis. *J. Natl. Cancer Inst.* (submitted).

Sleeman, J., Krishnan, J., **Kirkin, V.,** and Baumann, P. (2001) Markers for the lymphatic endothelium: in search of the Holy Grail? *Microscopy Research and Technique* (in press).

Kirkin, V. and Sleeman, J., P. (2000) Establishment of lymphatic endothelial cells in culture. In 24th Annual Meeting of the German Society for Cell Biology, Karlsruhe, March 26th-30th, 2000. *Eur. J. Cell Biol.* Supplement 50 (Volume 79).

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Chapter 4

Discussion

The primary goal of my work was to identify novel anti-cancer drugs that would inhibit either tumour growth directly or would affect tumour-associated angiogenesis and lymphangiogenesis. In the initial part of my thesis work, recombinant rat VEGF, VEGF-C and VEGF-D proteins were produced and characterised, and were found possess full biological activity. Together with *in vitro* kinase assays, these proteins allowed me to establish system for screening a panel of novel indolinones for their ability to inhibit the activity of a number of RTKs. Of 32 indolinones screened, I identified four potential inhibitors of tumour growth. One of them, namely MAZ51, could suppress tumour growth in rats, while the others could inhibit angiogenesis *in vitro*. An investigation into whether hyperforin has any anti-tumour properties was also performed. It was found to be able to reduce tumour growth directly by inducing tumour cells to apoptose and indirectly via inhibition of endothelial cell proliferation. Thus, the compounds identified in the course of this work may be of pharmaceutical value and should contribute to development of better cancer treatment.

4.1 Creation of cellular assays for RTK activity

4.1.1 Rat VEGF-C and VEGF-D

The recombinant rat VEGF-C and VEGF-D proteins I made in *Drosophila* cells are biologically active. This could be proven by the cellular phosphorylation assays in which both VEGFR-2 and VEGFR-3, the cognate receptors for VEGF-C and VEGF-D, became phosphorylated in response to activation by VEGF-C and VEGF-D present in the conditioned medium of the relevant transfectants. Furthermore, the purified mature VEGF-C protein induced DNA synthesis in primary vascular endothelial cells. These data are consistent with the fact that a biologically active VEGF protein has been produced in an insect system by others (Fiebich *et al.*, 1993).

Although biologically active in conditioned medium, purified rat $\Delta N\Delta C$/VEGF-D loses its ability to activate VEGFR-2 and VEGFR-3. One reason for this could be that VEGF-D is especially sensitive to the purification procedure which itself causes the loss of VEGF-D activity. Nevertheless, purified $\Delta N\Delta C$/VEGF-C and $\Delta N\Delta C$/VEGF-C($Cys_{152}Ser$) proteins retained their biological activity. There may therefore be an alternative explanation to the loss of activity of VEGF-D: rat VEGF-D may require some receptor-binding cofactors that are present in the medium and become lost upon purification. Consistent with this notion, when proteins present in the conditioned

medium of the appropriate S2 transfectants were analysed on non-reducing SDS-PAGE gels, ΔNΔC/VEGF-C and ΔNΔC/VEGF-D samples contained multiple bands (**Fig. 3.4**). Besides the ones corresponding in size to the monomeric forms of the proteins (13 kDa) and to covalent dimers (26 kDa), some intermediate bands of approximately 20 kDa were apparent in both the ΔNΔC/VEGF-C and ΔNΔC/VEGF-D samples. Preliminary data suggest that such intermediate bands are missing in the non-reducing samples of the inactive purified ΔNΔC/VEGF-D protein. The presence of these intermediate protein bands specifically recognised by anti-His antibody as well as by specific VEGF-C and VEGF-D anti-sera (data not shown) may therefore be attributed to the formation of covalently linked protein complexes in the transfectant medium. This possibility deserves further investigation.

Although human VEGF-C and VEGF-D have been described to be very homologous in their structure and properties (Achen *et al.*, 1998; Stacker *et al.*, 1999), their rodent counterparts seem to exhibit less similarity especially in their receptor specificity. Thus, it has very recently been shown that, unlike murine VEGF-C, murine VEGF-D cannot bind murine VEGFR-2 receptor despite its ability to bind murine VEGFR-3 and human VEGFR-2/VEGFR-3 (Baldwin *et al.*, 2001). Due to the unavailability of rat VEGFR-2 and VEGFR-3, I used human VEGFR-2 and murine VEGFR-3 receptors in this thesis. The binding of the rat ligands to these receptors was not affected by the species-specific differences and therefore it is obscure whether rat VEGF-C and VEGF-D bind to rat VEGFR-2 differently. Furthermore, the presence of the covalent dimers in the non-reducing rat recombinant ΔNΔC protein samples is at odds with the published observations that human ΔNΔC/VEGF-C and ΔNΔC/VEGF-D form strictly non-covalent dimers (Joukov *et al.*, 1997; Stacker *et al.*, 1999). Moreover, differences between rodent VEGF-C and VEGF-D also exist as the mutation of Cys_{141} completely destroyed biological activity of ΔNΔC/VEGF-D, whereas the analogous mutation in ΔNΔC/VEGF-C does not influence the ability of VEGF-C to bind to and activate VEGFR-3.

4.1.2 Attempts to establish LE cultures

The lack of availability of LE in culture is limiting for experiments aimed at understanding the processes of physiological and pathological lymphangiogenesis. Such cultures would also permit selection of potential inhibitors of lymphangiogenesis. LE cells from large lymphatic ducts, such as thoracic and

mesenteric ducts, have been successively grown in culture (Johnston and Walker, 1984; Gnepp and Chandler, 1985; Leak and Jones, 1993; Weber *et al.*, 1994; Yong and Jones, 1991; Borron and Hay, 1994). However, these LE cells are morphologically and functionally different from the LE derived from peripheral tissues. They would therefore not be appropriate for studying lymphangiogenesis. In my thesis work, I made attempts to establish LE cultures from peripheral tissues. One of them was to make use of the biologically active rat ΔNΔC/VEGF-C and ΔNΔC/VEGF-C(Cys$_{152}$Ser) I made to induce lymphangiogenesis in rats.

Matrigel has successfully been used in different experimental settings where angiogenic growth factors such as FGFs (Passaniti *et al.*, 1992) and SF/HGF (Grant *et al.*, 1993) were used to induce angiogenesis *in vivo*. I attempted to induce LE growth into matrigel using recombinant VEGF-C proteins, basing my experimental approach on a method shown to work for SF/HGF-induced angiogenesis (Grant *et al.*, 1993). Matrigel was mixed with ΔNΔC/VEGF-C and ΔNΔC/VEGF-C(Cys$_{152}$Ser) and injected subcutaneously into rats. Following the extraction and sectioning of the plugs, however, no specific induction of lymphangiogenesis occurred, based on the observation that the amount of the VEGFR-3-positive cellular structures found in all the analysed gels was similar in both VEGF-C-containing and control gels containing no recombinant growth factors. The failure of the ΔNΔC/VEGF-C to induce lymphangiogenesis may be due to the lack of the putative ECM-binding domains present in the full-length VEGFs, which might lead to quick release of the growth factor from matrigel. This presumption, however, is weakened by the inability of the recombinant rat VEGF to induce angiogenesis in the same experiment. The rat VEGF protein used corresponds to the described human VEGF$_{189}$ species known to be almost completely sequestered in the ECM and hence expected to bind to matrigel which resembles the ECM. Such bound VEGF should be gradually released from the gel to induce proliferation of vascular endothelial cells. Therefore, an unidentified technical problem probably accounts for the failure of this approach. Nevertheless, the VEGFR-3-positive structures also observed in the growth-factor deficient matrigel deserve further investigation. Two questions remain to be answered: a) Is matrigel on its own lymphangiogenic? and b) Is VEGFR-3 expression up-regulated on cell types other than LE which invade matrigel? If matrigel proves to be lymphangiogenic, it could be used as a model system to study lymphangiogenesis *in vivo*.

The conditional lymphangiomas seem to be a more reliable source of peripheral LE. The experiments with rats demonstrated high reproducibility of the method originally described for mice (Mancardi *et al.,* 1999). The lymphatic endothelial origin of the white adjuvant-induced tumours in rats was assumed on the basis of immunohistochemistry. Indeed, most of the cells forming lymphangiomas stained positively for the putative LE marker VEGFR-3 and the pan-endothelial marker CD31. However, the major obstacle in this approach was the short life span of the lymphangioma-derived cells in culture, which resulted in the inability to achieve large numbers of cells for their characterisation and further experiments. Additional work needs to be performed to establish culturing conditions that permit the propagation of LE in culture. Alternatively, or in addition, approaches could be made to immortalise the LE cells. In fact, I made attempts to immortalise the cells derived from the lymphangiomas by a) infecting them with a retrovirus whose genome encodes E6 and E7 proteins of human papillomavirus 16 and b) their transfection with a telomerase construct. Both the immortalisation protocols have been described to work in certain cell types (*e.g.* epithelial cells, Halbert *et al.,* 1991 and T cells, Hooijberg *et al.,* 2000). However, these measures did not lead to a delay in the onset of senescence as judged by morphological observations. Further, investigations are required to study the sequence of events necessary to render LE cells immortal. Recently, the ability to cause differentiation of embryonic stem (ES) cells into endothelial cell *in vitro* has been demonstrated (Hirashima *et al.,* 1999). This opens another perspective for LE culturing. It would be of great interest to check whether VEGFR-3$^+$ ES cells can be induced to differentiate into a LE lineage using the lymphangiogenic factors VEGF-C and VEGF-D.

4.2 Selection and characterisation of indolinones

4.2.1 Screening procedure

To get an initial indication of the possible RTK targets of the available indolinones a screening procedure was developed. As a first step, bacterial recombinant GST fusion proteins containing the catalytic domains of various RTKs were used in *in vitro* kinase assays. This allowed me to select among the 32 indolinones a group of 16 substances with a good inhibitory activity ($\geq$ 50% inhibition) towards potential RTK targets. Thus, the number of the candidates for the further studies was halved, which eliminated the necessity to use the large quantity of samples in further more sophisticated experiments. However, this screening method employed rather artificial conditions because the substrate phosphorylation reaction has a limited number of components and is performed out of the cellular context. Moreover, the catalytic domains of bacterial GST fusion proteins, due to the lack of other functional RTK domains and compromised post-translational modification, are likely to have a different conformation in comparison to the intact membrane-bound protein expressed in eukaryotic cells. Such a method therefore cannot reflect the real affinity of the indolinones for a particular RTK. Furthermore, the ability of the indolinones to permeate membrane in cellular assays is a critical factor for their cellular activity. Also, solubility tests demonstrated that the indolinones are poorly soluble in water (data not shown). For these reasons, the overwhelming majority of the indolinones selected on the basis of the *in vitro* kinase assays did not have any inhibitory effect on the activation of cellular VEGFR-2 and VEGFR-3.

The affinity of the same indolinone for different RTKs is largely dependent on the conformation of the reactive centre of the protein (Mohammadi *et al.*, 1997). Even such closely related RTKs as the members of the VEGFR family revealed different affinity for the same indolinones. Thus, in the cellular experiments, AE87, for instance, was weakly inhibitory for VEGFR-3 but did not affect VEGFR-2, and intriguingly, at a high concentration (50 μM) it seems to have stimulated VEGFR-1 phosphorylation. As the experiment was performed twice and every time yielded the same result, the possibility of an artefact was excluded. It is difficult to explain how indolinones might stimulate RTK phosphorylation because these molecules, unlike adenosine-phosphates whose structure they mimic, normally do not contain phosphate groups. One possibility may be that AE87 down-regulates some phosphatases, which

results in the prolonged retention of the phosphate in the VEGFR-1 kinase. The ability of AE87 to stimulate VEGFR-1 and not VEGFR-2 phosphorylation could potentially be useful, as VEGFR-1 biological function is poorly understood and seems to be involved in the negative regulation of angiogenesis (Karkkainen and Petrova, 2000).

Interestingly, while many of the indolinones could inhibit RTK phosphorylation in the *in vitro* kinase assay and not in the cellular assays some of them behaved in an opposite fashion. Thus, MAZ51 was weakly inhibiting for VEGFR-3 phosphorylation in the *in vitro* kinase assay (35% inhibition) but proved to be a potent inhibitor of VEGFR-3 in the subsequent experiments. It can therefore be concluded that in order to gain more objective data, use of more than one screening method should be used.

4.2.2 Properties of AE87, AE106, MAZ51 and MAZ51-2

The unambiguously active indolinones that could inhibit many RTKs including the VEGFRs both in the *in vitro* kinase assays and in the cellular phosphorylation assays are AE87, AE106, MAZ51 and MAZ51-2. The especially prominent, though differential, VEGFR-3-inhibiting potential makes them a useful tool to study the VEGFR-3 signalling. Unfortunately, due to the failure to establish LE cultures, it was impossible to verify whether the identified VEGFR-3 inhibitors could inhibit lymphangiogenesis in a LE model system. Currently the compounds are being tested by Dr. Wilting (Albert-Ludwigs-Universitat, Freiburg) for their ability to inhibit lymphangiogenesis in a chicken chorioallantoic membrane (CAM) assay. Once successful, such studies could help further elucidate the role of VEGFR-3 in pathophysiological lymphangiogenesis.

Another useful feature of these indolinones is their obvious anti-angiogenic potential that could be demonstrated in the *in vitro* experiments. In line with the differential VEGFR inhibitory potential of the four indolinones, their anti-angiogenic effect is also variable. Thus, AE87 is weak, AE106 – moderate, whereas MAZ51 and MAZ51-2 are the strongest anti-angiogenic substances. Interestingly, while being unable to inhibit VEGFR-2 phosphorylation in the cellular assay when present in medium AE106 could inhibit VEGF-stimulated proliferation of endothelial cells with IC_{50} about 1 μM (**Fig. 3.25, B**). One explanation may be based on the fact that AE106 inhibited VEGFR-1 phosphorylation in the cell as was shown in the cellular phosphorylation assay. Since VEGF activates two receptors VEGFR-1 and VEGFR-2 (Hanahan, 1997), it is likely that inhibition of VEGFR-1, which is known to form

functional heterodimers with VEGFR-2 (Huang *et al.*, 2001), or of some kinases downstream of the two VEGF receptors will cause suppression of endothelial cell proliferation.

MAZ51 and MAZ51-2 are quite distinct from AE87 and AE106 in their anti-angiogenic properties. For instance, they apparently do not block FGFR signalling, whereas AE87 and AE106 do have weak and moderate, respectively, inhibitory effects on FGFRs, as was assessed indirectly by measuring FGF-induced proliferation of HDMECs. In order to prove this directly it would be necessary to perform FGFR cellular phosphorylation studies analogous to the described VEGFR experiments. On the other hand, the MAZs and not the AEs killed endothelial cells by inducing them to undergo apoptosis. It also appears that MAZ51 and MAZ51-2 are very strong and probably broad-range kinase inhibitors. Thus, in addition to its anti-angiogenic properties, MAZ51 could strongly inhibit proliferation of a variety of tumour cell lines. In the most cases, this reduction in proliferation was again accompanied by an increase in cellular apoptosis. The induction of programmed cell death by the MAZs in endothelial and tumour cells might be explained by the blockade of some key kinases such as serine/threonine kinases PKA and PKB/Akt. The latter are found downstream of RTKs, where they play pivotal roles in cell survival (Cross *et al.*, 2000). In contrast, AE87 and AE106, which may inhibit only certain receptor tyrosine kinases, while not perturbing the balance of pro-apoptotic and survival signals inside the cell, might cause the depletion of mitogenic signals and the ensuing withdrawal of the cell from the cell cycle. In order to further study this hypothesis, it would be necessary to check the inhibitory potential of the indolinones on a range of 'survival-related' kinases in *in vitro* kinase assays.

The fact that not all the tumour cell lines tested became apoptotic after MAZ51 treatment may be due to cell type-specific differences existing in the cellular responses to indolinones as described for some other anti-cancer drugs such as betulinic acid (Pisha *et al.*, 1995). Thus, the independence of a particular tumour cell from survival factor signalling may render the cell insensitive to the action of RTK inhibitors. Nevertheless, the growth of tumours formed by cells with a good apoptotic response to MAZ51 such as 1AS and MT450 could be significantly retarded by MAZ51. It is still unclear though why AE87 and AE106 that inhibited proliferation in endothelial cells did not affect tumour growth *in vivo*. To address this question, the analysis of tumours is necessary. Staining of the tumour sections for markers of

apoptosis, angiogenesis and lymphangiogenesis shall also allow me to decipher whether the tumour cells or endothelial cells of blood and lymphatic vessels are affected by MAZ51. Due to the time limitations, this analysis could not be performed in the frames of the thesis.

Given its potency to inhibit tumour growth *in vivo* MAZ51 deserves further research. It is necessary to determine exactly what the other cellular targets for this indolinone are. This question could be addressed with help of *in vitro* kinase assays using a range of candidate RTKs. The broad spectrum of MAZ51 activity is potentially advantageous. Thus, inhibition of a range of kinases may ensure that the tumours will not be able to circumvent its activity. Moreover, a number of tumour-related processes such as uncontrolled cell proliferation, angiogenesis and lymphangiogenesis (the letter two also accompanied by hyperproliferation) are likely to be inhibited at the same time. A major disadvantage of such broad specificity, however, can also be envisaged. The animal experiments showed that at higher doses (8 mg/kg/day) the toxic effect of MAZ51 becomes noticeable. It will therefore be necessary to test whether other proliferating cells of the organism are also susceptible to the action of MAZ51. However, it should also be noted that at this point it is not possible to say whether the toxic effects are direct, or due to the precipitation of MAZ51 in the abdominal cavity.

As mentioned earlier, the solubility of indolinones seems to be the major problem in both their characterisation and their possible clinical applicability. For instance, the two most potent indolinones MAZ51 and MAZ51-2 have the poorest solubility among the four indolinone candidates for anti-cancer treatment. In the *in vitro* experiments 1 to 5% DMSO was used to keep the substances in solution. In the animal experiments, 100% DMSO solutions were used to allow higher concentrations of AE87, AE106 and MAZ51 to be delivered. Nevertheless, upon autopsy a great deal of precipitation of the substances was observed in the peritoneal cavity. Clumps of crystalline indolinones were detected on the surface of the intestinal tract and the peritoneum. While such clumps might serve as drug depots for slow release, their presence indicates overall reduction in bioavailability of the indolinones. A minor chemical modification such as the addition of hydrophilic groups to the 3-substituents of the core oxindole structure is likely to improve the solubility and hence bioavailability of the indolinones. Thus, other pre-clinical studies such as

improvement of the substance delivery methods as well as bioavailability are necessary.

MAZ51 may have the potential to enter clinical trials upon the completion of its characterisation. Although AE87 and AE106 do not seem to inhibit tumour growth *in vivo*, they are definitely worth further research in terms of their ability to block angiogenesis and lymphangiogenesis as they may prove to be useful for studying these processes.

4.3 Hyperforin: a novel anti-cancer drug

Given the potent anti-proliferative effect of hyperforin on mammalian cells *in vitro* (Schempp *et al.*, 2000), the hypothesis that hyperforin may inhibit tumour growth was tested. The *in vitro* experiments demonstrated that hyperforin is a potent anti-proliferative agent, which however may have a different mode of action in different cells. Thus, tumour cells were killed through induction of the programmed cell death, which is consistent with the mode of action of other cytotoxic drugs (Hickman, 1992; Debatin, 1999). Unlike the tumour cells, endothelial cell death did not exhibit obvious features of apoptosis. This observation is consistent with the notion that many other cytotoxic drugs are known to induce apoptosis with selectivity for certain tissues. For example, mentioned earlier betulinic acid selectively inhibits the growth of malignant melanoma (Pisha *et al.*, 1995).

The exact mechanism of how hyperforin induces apoptosis in tumour cells is not known, although there is an opinion that it activates the intrinsic apoptosis pathway (Schempp *et al.*, manuscript submitted). Consistent with its anti-tumour and angiogenic effects *in vitro*, when injected subcutaneously at the site of MT450 rat mammary carcinoma tumours in Wistar Furth rats, hyperforin inhibited growth of already established tumours (15 days post-implantation) to a similar extent as the cytotoxic drug paclitaxel. An important advantage of hyperforin seems to be its ability to block both tumour cell proliferation and angiogenesis. Remarkably, no signs of acute toxicity in rats were noticed, suggesting that hyperforin might be suitable for clinical use. In fact, hyperforin is an already established drug used to treat depression and therefore should be easily adopted for anti-cancer use.

Recent studies on hyperforin, however, indicate that it may also have some adverse properties for potential use in tumour therapy. Thus, it has been shown to activate the pregnane X receptor, which mediates hepatic cytochrome P450 gene expression. The

cytochrome P450 is an active player in drug metabolism and hyperforin may thereby actuate the metabolism of co-administered drugs (Moore *et al.*, 2000; Obach, 2000; Wentworth *et al.*, 2000). Furthermore, it is likely that hyperforin may suppress proliferation of cells other than tumour and endothelial cells. These aspects need to be analysed in more detail.

4.4 Conclusion

In the course of this thesis work several potential anti-cancer drugs have been identified. Four of them are small synthetic compounds that are capable of inhibiting angiogenesis and presumably lymphangiogenesis. MAZ51 is especially prominent as it can suppress the growth of established tumours *in vivo*. Since MAZ51 is likely to have a broad specificity, it has the potential to interfere with both tumour cell proliferation and tumour-host interactions – a desirable property of an anti-cancer drug. Hyperforin characterised here for its anti-tumour potential is another example of an anti-cancer agent with a broad spectrum of activity. Due to its established clinical use, this drug seems to be particularly suitable for treating cancer. Further studies are necessary to define the exact mechanism of action of the identified substances as well as to reduce their possible side effects.

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