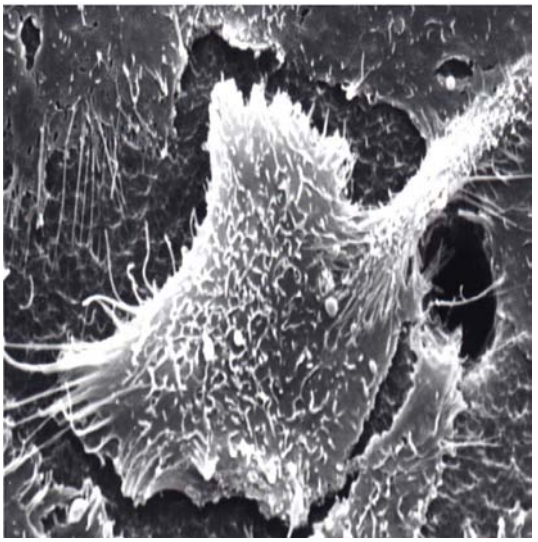
Haakon R. Skogseth

# Invasive properties of cancer - a treatment target?

In vitro studies in human prostate cancer cell lines



Thesis for the degree doctor philosophiae

Trondheim, November 2006

Norwegian University of Science and Technology
Faculty of Medicine
Department of Laboratory Medicine,
Children's and Women's Health

NTNU
Innovation and Creativity

Front cover: Scanning electron microscopic picture of a prostate adenocarcinoma cell (PC-3), after migration through a reconstituted basal lamina (Matrigel ®). The cell has moved through the pore shown to the right. Before migration, the cell must have been capable of degrading and destroying the Matrigel® protein matrix which covered the pore. Conditioned medium from a mouse fibroblast cell line (NIH-3T3) was used as chemo-attractant. The specimen was prefixed in glutaraldehyde, postfixed in osmiumtetroxid, critical-point dried and stained with gold-palladium.

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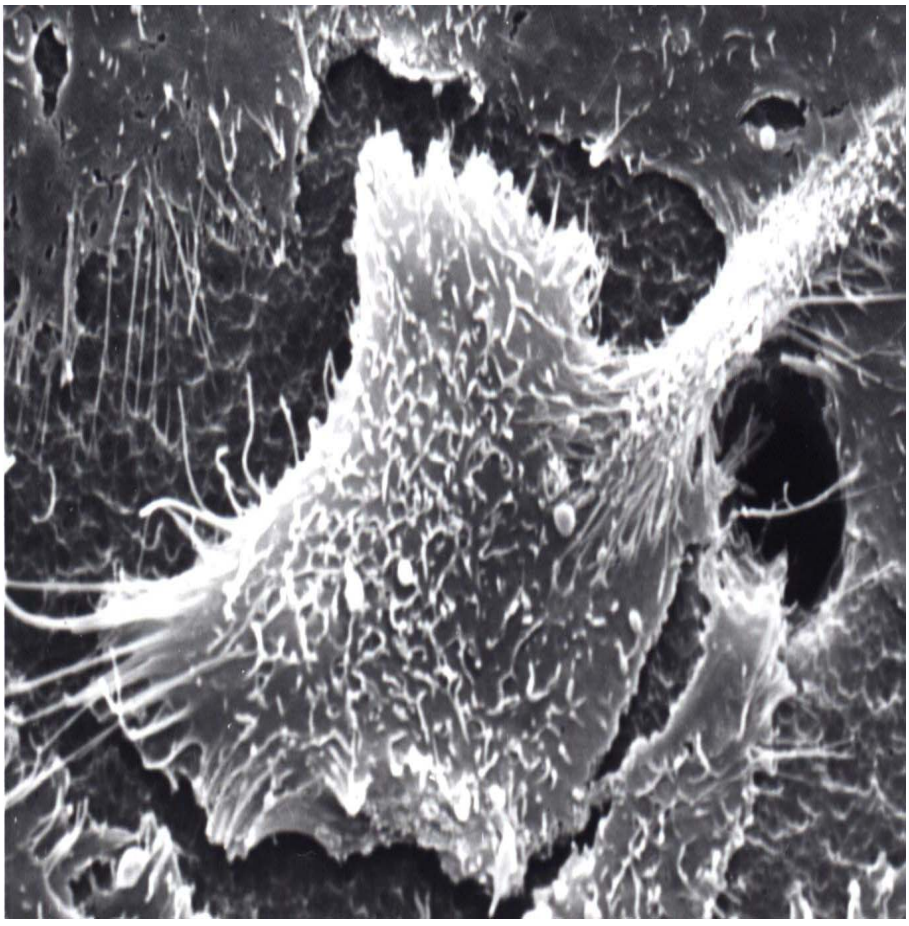

Department of laboratory medicine, Children's and Woman's Health,

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# Contents

# Acknowledgements

The present thesis is the result of studies carried out from 2001-2005, during my doctoral fellowship at the Department of Laboratory Medicine, Children's and Woman's Health, The Norwegian University of Science and Technology in Trondheim.

I am greatly indebted to my supervisor, Associate professor Jostein Halgunset, who let me harvest from his considerable tree of knowledge. I am likewise grateful to my co-supervisor Professor Erik Larsson, and Associate professor Kåre E. Tvedt. Together we constituted a research group with an excellent friendship. I would also like to thank Eli Helge, Borgny Ytterhus, Eli Johannesen, Linh Hoang, Hege Wiksen and Unn Granli, the technical staff at the Section of Morphology, for their assistance. I wish to thank Associate professor Anna Bofin for her help with my English. During the research period I have received substantial financial support from The Cancer Foundation at St. Olav's Hospital, which has been important for the realization of this work.

# Abbreviations

| | |
|---|---|
| uPA | urokinase plasminogen activator |
| uPAR | urokinase plasminogen activator receptor |
| MMP | matrix metalloproteinase |
| TKI | tyrosine kinase inhibitor |
| BL | basal lamina |
| ECM | extracellular matrix |
| TIMP | tissue inhibitor of metalloproteinase |
| PAI | plasminogen activator inhibitor |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| GPI | glycosyl phosphatidylinositol |
| PSA | prostate specific antigen |
| HRPCA | hormone refractory prostate cancer |
| TK(R) | tyrosine kinase (receptor) |
| TGF | transforming growth factor |
| HER-2 | human epidermal growth factor receptor 2 |
| SH2/3 | Rous' sarcoma virus homology 2 and 3 |

# Introduction

## *Prostate cancer: the clinical and scientific challenge*

Prostate carcinoma is the most frequent cancer among men in Norway with 3327 new cases in 2003 [1], and it is a leading cause of cancer deaths in men. More than any other, prostatic cancer is a disease of the elderly. In fact in developed countries 82 % of cases occur in men older than 65 years [2]. An expected increase of longevity will probably result in 40 % more prostate cancer cases in the future [3]. However, the patient cohort is heterogeneous especially regarding progression of the disease. In most patients the cancer remains slow-growing, but in a minority of cases the tumor is highly aggressive, leading to early metastasis with painful bone lesions, and unfortunately to death in the course of a few years [4]. An important challenge for the future will be to find a diagnostic method that can reliably identify such aggressive cases.

## Treatment options and shortcomings of existing therapy

Asymptomatic prostate cancer is usually discovered by high levels of prostate specific antigen (PSA) in the blood. Patients with increased PSA generally undergo ultrasound guided biopsies for histological diagnosis. The Gleason grading system provides visual markers for estimation of prognosis and the selection of therapy [5-7]. Today, the Gleason grade and serum level of PSA, combined with more sophisticated methods such as magnetic resonance imaging (MRI) and computer tomography (CT), provide the best available estimate of tumor stage and aggressiveness [8]. This information enables the oncologist to assess whether the patient may be cured or not. Radical perineal prostatectomy and radiation therapy are preferred treatments for

prostate cancers localized within the prostate and adjacent tissues [9]. In these cases the treatment aims at a complete cure. However, at the time of presentation, more than 50% of patients either have locally advanced disease or secondary lesions [10]. Although surgical resection of isolated metastases is beneficial for some patients, the overall efficacy of surgery is limited in such cases [11, 12]. Fortunately, and due to the fact that the level of testosterone is of crucial importance for prostate cell division and differentiation [13-16], approximately 80% of prostate cancers are initially sensitive to androgen hormone stimulation. Consequently, anti-androgens, like Casodex® or Soladex® [17, 18] can be used with good effect in this patient category. With time most prostate cancers become less androgen dependent, and will thus acquire resistance to anti-androgen therapy. These tumors are commonly called hormone refractory prostate cancers (HRPCA) [19]. However, cell clones that are hormone insensitive may respond to other therapeutic modalities such as chemotherapy [20-22], immunotherapy [23], or growth factor inhibitors [24, 25].

## *Future prospects*

In general, today's anti-cancer drugs are designed to inhibit the uncontrolled proliferation of tumor cells, even though some new drugs may also affect other aspects of malignant behaviour, e.g. by preventing formation of new blood vessels (neoangiogenesis). However, carcinogenesis and tumor progression are associated with abnormalities in various aspects of cell behavior, not only an increased proliferation rate [26]. There is also a reduced tendency to undergo apoptosis [27], altered cell adhesion [28, 29] and augmented motility [30, 31]. In recent years many investigators have focused on the development of drugs with the potential to influence the malignant phenotype. Clearly, our understanding of molecular and cellular processes behind such mechanisms has increased substantially through the past decades. This knowledge has led to an extensive search for treatment directed against the cancer cells' ability to infiltrate surrounding tissue and form metastases. A malignant epithelial cell population is primarily characterized by its ability to penetrate anatomical barriers such as basal laminas (BL) and interstitial stroma. These events are thought to be induced by the production of proteolytic enzymes secreted by the cancer cells or by adjacent tissue [32-34]. Extracellular proteolysis occurs widely in nature and serves many different purposes. In multicellular organisms proteases are involved in cell growth and tissue differentiation. Besides these important processes, such enzymes have a role in blood coagulation, blood pressure regulation [35-37] and in the digestion of food. However, it is well-documented that various proteases participate in invasive growth of numerous cells, especially the metalloproteinases and serine proteases [38]. Among them plasminogen activator (PA) has attracted most interest (Figure 1). A number of investigators have demonstrated a strong correlation between increased PA-activity and the capacity of malignant tumors to invade

surrounding tissue [39-42]. The production of PA may become a central target for novel drugs with inhibitory effect on tumor cell invasion, and in the development of diagnostic methods that can identify aggressive variants.

Different cancers show different patterns of metastasis. Bone, as well as lung and liver, are the most frequent metastatic target sites for metastases from primaries of the breast and prostate, although the precise molecular mechanisms underlying such preferences of tissues need to be further elucidated. Both homotypic and heterotypic cell-to-cell adhesion interactions, in addition to cell-matrix interplays, are thought to participate in the determination of organ-specific tumor localization. It appears that bone matrix possesses unique biological features which permit circulating prostate cancer cells to home, survive and proliferate [43] . Mechanisms involved in malignant cell adhesion have recently been reviewed [44]. Drugs affecting the cancer cells' ability to adhere to various tissue components may be of therapeutic importance, with possibilities to block the formation of metastases. Moreover, collaboration has been demonstrated between the receptor for urokinase (uPAR) and cell adhesion molecules such as integrins. Such interplay participates in reorganization of the cytoskeleton, e.g. by stimulating the production of membrane wrinkles and the creation of lamellopodia and uropodia, all of great importance in cell adhesion and cell motility [45-48]. Drugs modifying the expression or functional properties of cell adhesion molecules may conceivably be useful suppressors of the metastatic process.

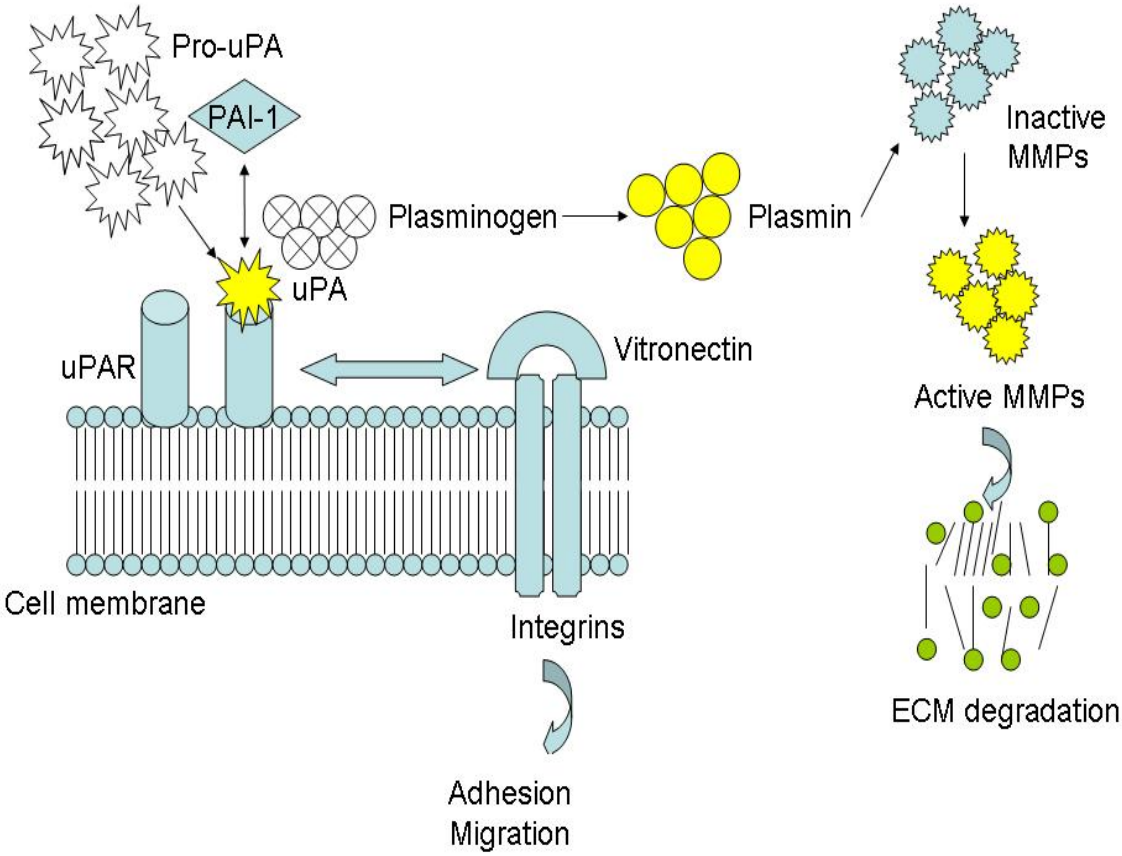


*Figure1. The proteolytic efficacy of uPA relies on its interactions with factors such as plasminogen activator inhibitor-1 (PAI-1), the receptor binding uPA (uPAR), and inactive metalloproteinases. Recent studies have implicated a role for uPAR in cytoskeletal rearrangement, possibly leading to altered cell migration.*

# *Tyrosine phosphorylation in malignant cell behavior*

## Regulatory principles

Protein tyrosine kinases (TKs) are enzymes that catalyze the transfer of phosphate groups from adenosine triphosphate (ATP) to tyrosine residues on specific protein substrates. Human cells contain more than 500 TKs [49], which play an important role in diverse cellular regulatory processes [50-53]. They work as mediators of signals leading to cell proliferation, differentiation, and migration, as well as to cell death [54]. There are two main classes of TKs, receptor bound and non-receptor bound.

Growth factor receptors, which are transmembrane molecules composed of an extracellular ligand site, a transmembrane adaptor and an intracellular domain with enzymatic activity, are activated by binding an extracellular signal molecule, such as a growth factor (Figure 2). For instance, the EGFR family consists of four related transmembrane receptors that are involved in regulation of cellular growth and differentiation. In the absence of a ligand, a receptor TK is unphosphorylated and monomeric. When a ligand binds to the extracellular domain of the receptor, an oligomerization of receptors takes place, which in turn leads to phosphorylation of regulatory tyrosines [55-57]. Multiple cytoplasmic signalling pathways, including the rat sarcomas (RAS) mitogen-activated protein kinase pathways, the phosphoinositol 3-kinase (PI3K) pathway and the protein kinase C pathway may then be activated. TK signaling is terminated through the action of tyrosine phosphatases or by other inhibitory intracellular molecules.

The non-receptor TKs are cytoplasmic proteins, exhibiting considerable structural variability. They are for instance known as mediators in Src homology-2 (SH2) and

Src homology-3 (SH3) signaling pathways, and are maintained inactive by intracellular inhibitor proteins. They are activated e.g. when these inhibitors are dissociated from the enzyme [58].

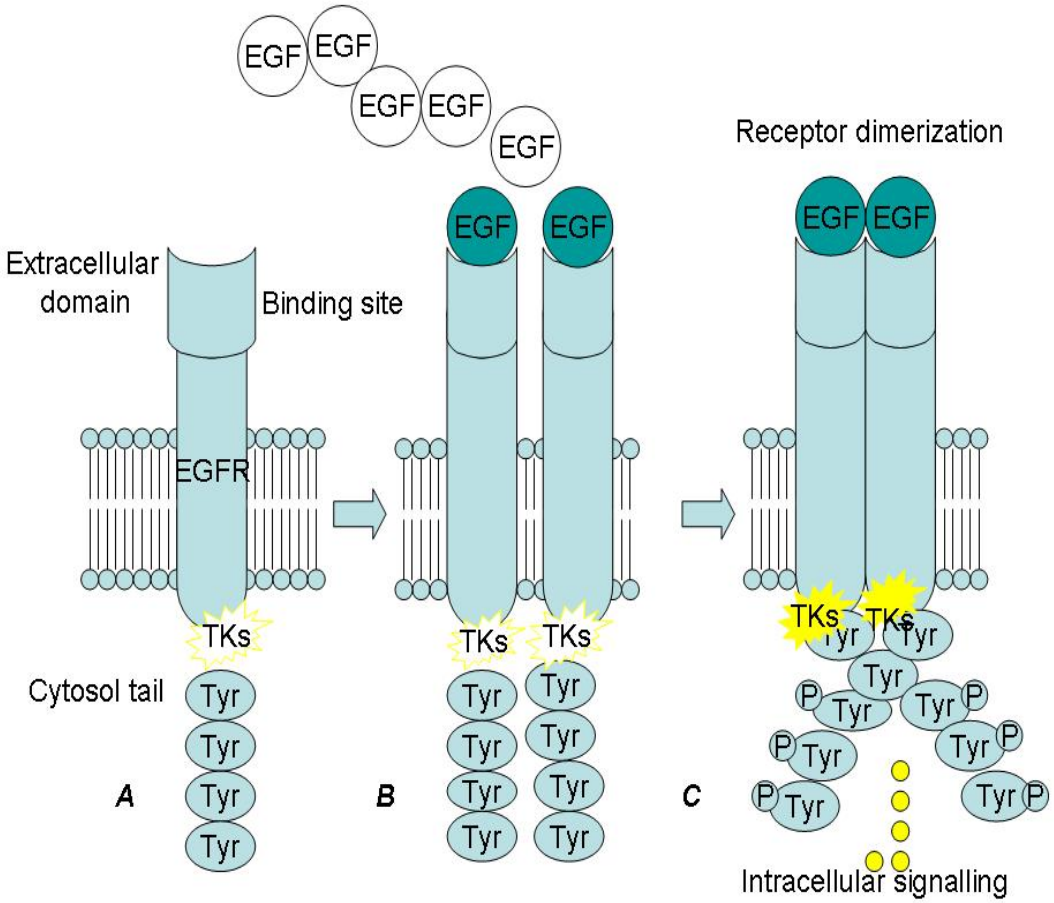


*Figure2. Structure (a) and activation (b) of a typical tyrosine kinase receptor, the receptor binding epidermal growth factor (EGF). These receptors have one transmembrane segment. The extracellular portion of the receptor binds the ligand (EGF in this case). Inside the cell, a portion of the receptor has tyrosine kinase activity. The remainder of the receptor contains a series of tyrosine residues that are substrates for the tyrosine kinase. The activation of receptor tyrosine kinases starts with the binding of a messenger, causing receptor aggregation or clustering. Once the receptors aggregate, they cross-phosphorylate each other at a number of tyrosine amino acid residues (c). The formation of tyrosine phosphate (Tyr-P) residues on the receptor creates binding sites for cytosolic SH2 domains.*

## Dysfunctions of TKs

Enhanced expression of EGFR or its ligands such as EGF and transforming growth factor (TGF) can increase signaling via receptor-mediated pathways that may lead to excessive proliferation and cellular transformation. Several studies have shown that EGF binding to EGFR on tumor cells blocks apoptosis, and consequently promotes tumor growth and viability. Moreover, EGFR and its ligands have an important role in regulating angiogenesis [59-62]. Recent studies have demonstrated that TKs are dysregulated in cancer cells in several ways. Dysfunctions in growth factor signal cascades probably represent a main characteristic in the progression of malignant cell behavior [63-66].

Normally, the level of intracellular protein phosphorylation is tightly controlled. However, in cancers, various TK dysregulations occur (table 1), such as uncontrolled expression of TK-receptors or their ligands [67]. An abnormality frequently seen is the fusion of TK-receptors with partner proteins, resulting in constitutive oligomerization in the absence of ligands. In this way autophosphorylation is promoted, resulting in uncontrolled activation of growth responses [68-71]. Another important mechanism in irregular activation of TKs involves mutations that disrupt the autoregulation of the kinase [72], Finally, increased TK activity may be due to a decrease of factors that limit TK activity, such as tyrosine phosphatases or other TK inhibitory proteins [73]. The network of regulatory pathways involving TKs is exceedingly complex, and its ramifications are not yet known in great detail. However, it seems beyond doubt that fundamental cellular processes, such as growth, survival, differentiation and motility, are largely determined by the phosphorylation status of key control proteins, and that these signal systems may be extensively interwoven. Thus, it is to be expected that many of the processes characteristic of the

malignant phenotype may be modified by inhibition of various TKs, and that meticulously selected tyrosine kinase inhibitors (TKIs) therefore may have a role to play as anti-cancer drugs.

*Table1. Examples of dysregulated tyrosine kinases in various cancer types.*

[74-76]

| Tyrosine kinase | Mechanism | Examples |
|---|---|---|
| EGFR (ErbB1) | Mutation (EGFR-vIII) | Gliomas |
| | Mutations in TK-domain | Non-small cell lung cancer |
| | Over-expression or growth factor mediated activation | Head- and neck, lung-, breast-, prostate- and colorectal cancer |
| HER2 (ErbB2) | Over-expression (e.g. amplification of the gene) | Breast-, ovary- and lung cancer |
| c-Kit (cellular homolog of the feline sarcoma viral oncogene) | Mutation (constitutive TK activity) | Gastrointestinal stromal tumors (GIST) |
| PDGFR (platelet-derived growth factor receptor) | Mutations | Fibrosarcoma, chronic myelomocytic leukaemia |
| | | |
| *Non-receptor tyrosine kinases* | | |
| Bcr-Abl (Breakpoint cluster region-Abelson) | Mutation (translocation) | Chronic myeloid leukemia |

## Inhibitory strategies

TK activity may be inhibited by antibodies against TKRs or their ligands, preventing the binding of ligands to the receptors or restraining the dimerization of the latter [77, 78]. However, the most obvious candidate drugs are those which consist of small molecules capable of traversing the cytoplasmic membrane and binding to the intracellular domain of the TK-receptor, thereby blocking its interaction with ATP or protein substrates [79, 80].

Recently, a number of small molecular TKIs have become available, and some are already used in the treatment of human tumors. The most successful so far are Gleevec® (imatinib) [81] and Iressa® (ZD1839) [82-84], which are often given in combination with cytostatic drugs [85].

# Objectives

So far, the rationale behind the use of TKIs in cancer chemotherapy is the idea that these substances will inhibit excessive cell proliferation. However, an effective inhibition of cell division will inevitably also cause damage to many normal cell populations. It is an interesting possibility that in malignant tumors the disrupted proliferation control and the unchecked invasive behavior may depend on similar mechanisms and related regulatory key points. Anti-cancer drugs specifically designed to counteract the tumor cells' invasive behavior could be less toxic with fewer side effects, since this phenotype, characterized by cell migration across tissue and organ limits, is only shown by very few normal cell types.

The main objective of the work carried out in preparation of this PhD thesis was to explore the possibility that TKI treatment may be used to modify cancer specific behavior in an *in vitro* model. In the included papers we focused on the production of proteolytic enzymes and the expression of cell adhesion molecules in cultured prostatic cancer cells, examining the possibility that these functions may be susceptible to TKI treatment. Even though the cell culture model used in these studies is highly artificial, we believe that the results obtained through such experiments may be useful in the search for new pharmaceutical principles in the treatment of human cancer.

# General materials and main methods

(Details in enclosed publications)

## *Cell lines*

The human prostatic carcinoma cell lines PC-3 [86] and DU-145 [87] and LNCaP [88] were obtained from the American Type Culture Collection (ATCC). The cells were maintained as monolayers in 75 $cm^2$ tissue culture flasks in Ham's F-12 medium supplemented with 10% foetal bovine serum, l-glutamine, penicillin and streptomycin. Cells were grown at 37° C in a humidified environment containing 5% $CO_2$. During the experimental period, the cells were repeatedly tested and found to be free of mycoplasma.

## *TKIs*

Genistein and the tyrphostins AG -1478, AG-490 and AG-1296 were obtained from Calbiochem, San Diego, California. Genistein, which is a broad range TKI, inhibits substrate phosphorylation by EGFR and p60 kinases [89]. The tyrphostins constitute a group of compounds which are inhibitors of various tyrosine kinases, thus, AG 490 is able to selectively inhibit JAK-2 [90], whereas AG 1296 is a selective inhibitor of PDGF-receptor kinase [91]. Tyrphostin AG- 1478 is reported to be a highly specific inhibitor of EGF induced tyrosine phosphorylation [92].

## *Cell toxicity measurement*

Effects on cell proliferation caused by the TKIs were examined by a standard (3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) (MTT) assay. Oxidation of MTT takes place only when mitochondrial reductase enzymes are active, leading to conversion of MTT to a purple formazan, and the amount of color thus produced is

directly related to the number of viable cells. Induction of apoptosis was examined by a caspase colorimetric assay, capable of detecting three different enzymes (caspase 1, 3 and 8). Cell viability was analysed by flow cytometry using annexin/propidium iodide and APOPTEST-FITC-kit. Non-cytotoxic doses of TKIs were chosen for the rest of this investigation.

## *Determination of cell invasion*

Falcon invasion chambers were used to determine cell invasion (figure 3). They consists of a 24-well plate with cylindrical cell culture inserts, whose lower opening is closed by an 8 μm pore size polyethylene terephthalate (PET) membrane covered with a thin layer of a protein mixture (Matrigel®) (Becton &Dickinson Labware, Sweden). The protein layer occludes the pores, thus preventing non-invasive cells from passing through the membrane. In contrast, invasive cells are capable of penetrating through the membrane pores, presumably by a process involving proteolytic degradation of the matrix.

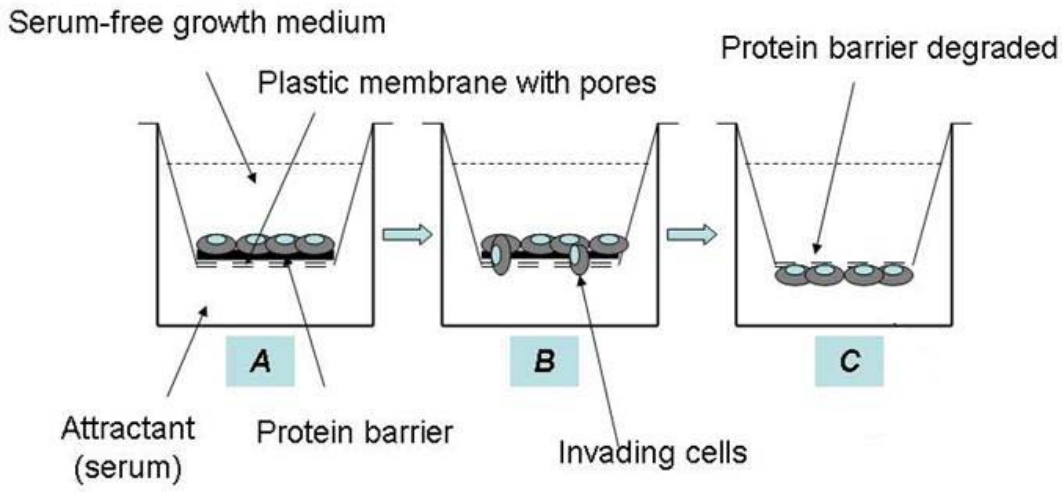


*Figure3. Falcon invasion chambers. The culture insert is occluded with a porous plastic (PET) membrane. The pores are covered with a thin layer of a protein mixture (Matrigel®) (A). Invading cells must destroy the protein layer before entering the attractive cell environment (B), and such cells will thus become localized on the lower side of the PET membrane (C), directly in contact with the medium of the main well.*

## *Quantification of proteolytic enzymes and their receptors*

The activity of uPA in the culture medium as well as in cell homogenates was measured with a colorimetric substrate, and an enzyme-linked immunosorbent assay (ELISA) was used to assess the amount of enzyme protein as well as that of enzyme receptors. Another colorimetric substrate was used to measure MMP activity in cell homogenate. Several MMPs are able to cleave the substrate used, among others MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13 and MMP-17.
Immunocytochemistry using an antibody against uPAR (Dia-Service, Sweden) was used to examine the receptor distribution in cells grown in Falcon invasion chambers.
Human Focus microarrays (Affymetrix, Santa Clara, CA) were used to examine the overall transcription pattern of genes related to proteolysis. More detailed mRNA quantification was done with real time reverse transcriptase polymerase chain reaction (qRT-PCR).

## *Cell adhesion measurement*

Adhesion to extracellular matrix was assayed by seeding fluorescence labelled cells on culture substrates covered with collagen type I, collagen type IV, fibronectin, laminin or vitronectin, followed by measurement of the fluorescence intensity after removal of non-adherent cells.
The expression levels of integrin β1, α2, α3 and α5 subunits were assessed by flow cytometry of cells labelled with monoclonal murine antibodies. Human Focus microarrays (Affymetrix, Santa Clara, CA) were used to measure the levels of various adhesion molecules' mRNA transcripts.

# Summary of results

The thesis represents a systematic investigation of effects caused by treatment of prostate adenocarcinoma cells PC-3 and DU-145 with TKIs. In order to assess the toxic effects caused by the TKIs, various parameters such as induction of apoptosis, altered proliferation rate and reduced cell viability were measured. After an initial mapping of the TKIs' cellular toxicity, the dose range was chosen so as to cause minimal damage to the cells. Thus, the drug treatment was non-toxic under the conditions used, except that genistein at the highest dose produced 10-15% decreased viability in both cell lines.

### Cell invasion (Paper II)

Cell invasion as measured in an artificial BL model was increased by the addition of plasminogen to the culture medium. This plasminogen effect was decreased by at least 60 % in both cell lines when α-2 anti-plasmin was added to the medium. The increased invasion induced by plasminogen was also counteracted by treatment with either of the two TKIs genistein or AG-1478. In the absence of plasminogen TKI had little or no effect on the invasive capability of the cells. Moreover, external uPA added to the medium failed to regenerate the decreased cell invasion caused by TKIs.

### Production of proteolytic enzymes (Paper I and IV)

Genistein treatment led to a dramatic reduction of uPA secretion in both cell lines, and a decreased expression was also demonstrated at the mRNA level. AG-1478 inhibited the production of uPA in PC-3, whereas DU-145 showed a slight increase of uPA secretion. However, no significant alteration in uPA mRNA expression was found after AG-1478 treatment. Moreover, treatment with TKIs led to approximately

a 50 % reduction of MMPs in PC-3 and DU-145 cell lysates (Paper II). However, only minor alterations of MMP mRNA were demonstrated.

**Expression of cell-membrane associated molecules (Paper III and Paper V)**

By immunohistochemistry an intense uPAR immunostaining was shown in actively invading cells, particularly at the leading edge membrane. Moreover, our results point out interesting differences in treatment response between the two cell lines, inasmuch as both TKIs induced a decreased level of uPAR proteins in DU-145, while PC-3 remained unaffected. Thus**,** TKI treatment was actually leading to an equalization of uPAR expression in the to cell lines. A reduction of uPAR gene expression was found in TKI treated DU-145 cells, while no significant change was demonstrated in PC-3 (Paper IV).

Examination of the cells' ability to adhere to various ECM proteins revealed that TKI treatment led to an overall reduced adhesion to the proteins tested. This observation was supported by the demonstration of a reduced expression of various integrin subunits in cells treated with TKIs, a pattern which was partly verified at the mRNA level (Paper IV).

# The original papers

I. Haakon Skogseth, MSc, Erik Larsson, MD, PhD, Jostein Halgunset, MD. Inhibitors of tyrosine kinase inhibit the production of urokinase plasminogen activator in human prostatic cancer cells. APMIS. 2005; 113: 332-9.

II. Haakon Skogseth, MSc, Erik Larsson MD, PhD, Jostein Halgunset MD. The invasive behavior of prostatic cancer cells is suppressed by inhibitors of tyrosine kinase. APMIS. 2006; 114: 61-6.

III. Haakon Skogseth, MSc, Erik Larsson, MD, PhD, Jostein Halgunset, MD. Urokinase plasminogen activator receptor (uPAR) expression is reduced by tyrosine kinase inhibitors. APMIS. 2006; 114: 307-13.

IV. Haakon Skogseth, MSc, Turid Follestad, PhD, Erik Larsson, MD, PhD, Jostein Halgunset, MD. The transcription levels of invasion related genes in prostate cancer cells are modified by inhibitors of tyrosine kinase. APMIS. 2006; 114: 364-71.

V. Haakon Skogseth, MSc, Randi Utne Holt, MSc, Erik Larsson, MD, PhD, Jostein Halgunset, MD. Tyrosine kinase inhibitors alter adhesivity of prostatic cancer cells to extracellular matrix components. APMIS. 2006; 114: 225-33.

# General discussion

### *In vitro* models- limitations and possibilities

Although malignant tumors of the prostate are a major contributor to cancer related morbidity and death in Western countries, the study of prostate carcinogenesis and tumor progression has not improved the treatment of this common disease to any degree. Partly this might be explained by a lack of representative *in vitro* models. So far, the limited number of available prostate cancer models, compared to the number for other neoplasms, is conspicuous. For a long time, only three cell lines, namely PC-3, DU-145 and LNCaP, were routinely used to study the biology of prostate cancer *in vitro*. This situation may be explained by the low success rate, which actually is in the 1% range, in efforts to establish cell lines from prostatic tumor tissue. Currently, no more than 10 prostate cancer cell lines are available worldwide, and many of them do not reproduce typical features of prostatic epithelium, such as the expression of androgen receptors and/or secretion of prostate specific antigen (PSA). Moreover, several of these cell lines only grow *in vivo* as xenografts [93], and their usefulness is therefore limited.

One distinct advantage with the use of cell lines is their ability to proliferate at constant pace through many passages, and therefore to give possibilities for long series of experiments without concern about shortage of tissue. However, the critical issue regarding the use of established cell lines is how representative they are of the corresponding cell type within the organism. Without doubt, a weak point in the use of simple cell culture systems is the fact that they fail to address the important and complex interaction between diferent cell types within a tumor. For example, in most cancer tissues, the interactions between malignant cells and their surrounding stroma

is of great importance. Thus, carcinoma cells seem to induce the stromal component to produce proteolytic enzymes, which contribute to the invasive capability of the malignant cells. This is in turn believed to be triggered by the release of chemical factors produced by the cancer cells, probably leading to increased stromal expression of cytokines and/or growth factors [94, 95]. Thus, proteolytic enzymes are often seen localized in the interface between the tumor cells and the stroma, while no such activity appears in the inner part of the tumor [96]. Hopefully, the development of *in vitro* co-culture models of epithelial and stromal cells may produce suitable tools to explore this kind of interaction.

Despite the shortcomings of cell culture models, their use in cancer research has not decreased. It is well-documented that we can learn much about dysregulated cellular functions from studies of living cultured cells. Clearly, the behaviour of single cells in an organism is continuously adjusted according to the messages received as part of an extensive communication with the rest of the body. Yet established cell lines will retain several distinctive traits from the cells of origin. However, the complexity of multicellular organisms represents in itself a problem for the demonstration of direct cause-and-effect relationships. This has led to the need of simpler experimental systems, in which single factors can be more easily manipulated. The usefulness of cell culture studies must therefore be evaluated in such a context. Moreover, cell lines can be established from tumor tissue obtained from different metastatic localizations, giving the investigator the opportunities to explore factors favouring the spread of cancer and the tendency to metastasize to a particular organ. For instance, PC-3 cells, which have been isolated from a skeletal metastasis, appear to differ in a numerous of ways, e.g. regarding the production of proteolytic enzymes and their receptors and inhibitors, from DU-145 cells, which originate from a metastatic brain lesion. Not

surprisingly, the effects of TKI treatment were not identical in the two cell lines. The differences between androgen- independent and androgen-dependent metastatic lesions from prostate primaries are even more conspicuous. For instance, PC-3 and DU-145, both androgen-independent cell lines, exhibited higher levels of EGFR expression and autocrine induced tyrosine phosphorylation than normal prostatic epithelial cells or the androgen-responsive prostate cancer cell line LNCaP [97]. Moreover, an aberrant expression of EGFR or its ligand TGF have been demonstrated with strikingly high frequency in aggressive variants of prostate cancer, thus implying the presence of feedback loop for the hormone-independent growth [98]. The demonstration of such phenomena has guided investigators' attention to a possible therapeutic potential for the TKI genistein, and its effects on cultured cells have been reviewed thoroughly. Together, these results suggest that regulators of the cell cycle may represent a potential molecular target for this soy isoflavone [99, 100].

Since the occurrence of prostatic intra-epithelial neoplasia and carcinoma *in situ* are associated with progression towards an invasive phenotype, the establishment of cell lines from early stages will carry important prospects for future research. However, today such possibilities appear far from realistic, since the establishment of cell cultures from those tissues seems difficult. More realistic is the development of co-culture systems, providing the ability to observe prostate cancer cells in interaction with bone cells, which may provide insight into the mechanisms underlying prostate cancers' tendency to spread to bone.

## Cancer specific treatment

Cancer is commonly understood to be the result of dysregulation of cell growth, and anti-cancer drugs have mainly been sought among chemical substances directly

inhibiting the cell-cycle or interfering with signal systems promoting cell proliferation. The development of growth factor receptor inhibitors has recently become a central field of research. However, despite the development of several chemicals with such properties, usually TKIs, the presently available drugs serve as second or third line therapy rather than constituting a primary choice.

Various TKIs have been used in clinical trials, and their effects have been thoroughly reviewed. In summary, treatment with TKIs has shown positive effects in patients with non-small cell lung cancer, breast cancer and in patients with leukemia, especially when given in combination with conventional cytostatics or radiation therapy [101-103]. Not surprisingly, the best results are obtained when the TKs are mutated and permanently active, for instance in chronic myeloid leukaemia. There are also promising results with TKIs in the treatment of HER-2-positive breast cancers [101]. Moreover, TKIs have been shown to counteract other aspects of malignant disease, such as neoangiogenesis [104].

Despite extensive research into the biology of prostate cancer, which has led to ever increasing knowledge about its nature, a major breakthrough in the treatment of the disease has not yet appeared. Advances in clinical treatment of patients have up to now been achieved by optimization of available conventional therapies which have been used for many years. Considering the number of deaths caused by prostate cancer, it is obvious that today's treatment is inadequate, and that the development of more efficient drugs is greatly needed. Hopefully, the increased knowledge about malignant epithelial cells' behaviour will open doors for new treatment strategies against the cancer's primary characteristic, namely its ability to invade into neighbouring tissue and secondarily its ability to form distant metastasis. A therapy effectively preventing prostate cancer spread would undoubtedly be a revolution in

the treatment of HRPCs. A number of investigators have suggested that TKIs have the potential to modulate the invasive capacity of human cancer cells, especially in cases with dysregulated growth factor receptor pathways [105, 106]. Our own results, achieved by the study of *in vitro* invasion, support this view by demonstrating that the invasive property of prostatic cancer cells can be modulated by TKIs. Interestingly, the PC-3 cell line's invasive capacity is influenced much more by TKIs than that of DU-145. Moreover, TKs appear to be especially effective inhibitors of over-expressed proteins, for example uPA in PC-3 cells. On the other hand, uPAR is more than twice as much expressed in DU-145 than PC-3 cells, and for this protein the DU-145 cell line is most readily affected by TKIs. These observations suggest that the expression of uPA and its receptor are activated through common signalling pathways induced by growth factors. In addition, our results indicate that the inhibition of extracellular proteolysis and of invasive growth may be achieved at substantially lower doses of TKIs than those needed for reduction of the tumor growth rate, which suggests that TKIs may be specifically designed to counteract the cancer cells' ability to destroy surrounding tissues and to form metastases.

A huge challenge in the search for drugs directed against prostate cancer invasion is to understand the complex interplay between the various mechanisms involved in cell migration, how these mechanisms are regulated, and how they may be modified. For instance, in order to metastasise to bone, prostate cancer cells must both detach from the primary tumor site and attach to bone matrix, whereupon they must survive and continue to proliferate and extend into the available space in the new environment. This illustrates that the process of invasion and metastasis consists of repeated detachment and attachment, thereby pointing out a central role for adhesion molecules as contributors to the invasive phenotype. Thus, loss of E-cadherin has been shown to

corrrelate with the invasive capacity of several tumors [107], while it is also widely held that prostate carcinoma cells depend on increased levels of integrins in order to attach to bone [108]. Drugs with suppressive effects on integrins, while stimulating the production of cadherins, may therefore play a role in invasion-inhibitory therapy. The results as shown in paper IV and V suggest that TKIs may play such a role.

## Further development

Forthcoming studies on clinical handling of prostate cancer will without doubt focus on the identification of aggressive cases with an increased risk for progressive disease and formation of metastases. Based on such information patients will be selected for an individualized therapy, and unnecessary therapy can be avoided in many cases. The identification of suitable therapy will mainly be performed by evaluation of biopsies. This field has been reviewed several times, for example by Huges and collaborators [109]. One observation that could be informative regarding the identification of aggressive cases, is the observed over-expression of insulin-like growth factor binding protein 2 (IGBFB2) in malignant prostate epithelium [110]. For future development of new therapy, it should be kept in mind that the biological machinery is fundamentally the same in normal and transformed cells. This means that conventional anti-cancer drugs, which are essentially anti-proliferation drugs, will inevitably produce deleterious effects also in actively proliferating normal cell populations. Thus the risk of unwanted side effects is usually the factor limiting the extent of the treatment. TKIs which have been specially designed and selected to suppress various aspects of the cells' invasive behaviour, might be expected to show less general toxicity than ordinary cytostatics, because of their relative specificity for malignant cells. At the present time, available TKIs are not the result of a systematic

search in this direction, and therefore the major challenge is still to find new drugs with specific anti-tumour effects and minimal general toxicity.

There is abundant evidence that the PA-system, with the key component uPA, its cell surface receptor uPAR and their inhibitors (PAIs), plays a key role in tumour invasion and metastasis. Thus, the PA-system seems well suited as a therapeutic target for patients with solid malignant tumours. However, this system provides proteolytic activity in many biological processes involving tissue remodelling, wound healing, ovulation and angiogenesis [111]. Activation of the PA-system is initiated by the release of PAs from specific cells in response to external signals, which via plasminogen results in a broad of spectrum protease activity. Because of the high concentration of plasminogen in virtually all tissues, altered occurrence of PAs may produce undesirable effects. For instance it is reasonable to assume that immune-response cells may be affected. This problem must be further investigated, preferentially using relevant animal models to explore and validate possible side-effects caused by drugs with the ability to inhibit the PA system. However, similar dilemmas are generally raised in most systemic treatment strategies. Targeted drug delivery supposes effective, precise and safe distribution of drugs, producing less systemic adverse effects. The controlled delivery of drugs is still a great challenge, and the success of TKIs affecting the PA-system may probably depend largely on improving their pharmacokinetics in terms of plasma stability and precise cellular uptake. Strategies for successful systemic delivery of PA-influencing TKIs may therefore be important in the future.

It is well known that genistein treatment of cultured cells may cause inhibition of cell proliferation and induce apoptosis [100]. However, when we measured caspase-enzymes (Paper I)) in TKI-treated PC-3 and DU-145 as an indication of programmed

cell death, no effect was demonstrated even at doses which produced a dramatic reduction of the cells' invasive capacity (Paper II) . This supports the notion that the observed effects, e.g. the reduced production of uPA, are not secondary to non-specific cytotoxicity. In clinical trials, the drugs still seem to be well-tolerated with fewer side effects than conventional cancer therapy. The most frequent side effects reported are a mild skin rash and brief diarrhea [112]. However, if new substances are systematically sought with the invasion-aspect in mind, one may hope to see drugs which can achieve good pharmaceutical effect at lower dosage, and with reduced risk of side effects. Targeted invasion-inhibitory therapy may therefore have the potential to reduce some of the problems presently seen in the field of cancer chemotherapy.

The high rate of mutations in many cancer cells creates an additional problem which must be considered, namely the ability of cancer cells to acquire drug resistance. As TKIs gradually have been introduced in cancer therapy, this has become a great challenge [113].

# Conclusion

The results presented in this thesis demonstrate that two TKIs, genistein and tyrphostin AG-1478, in an *in vitro* model system influenced the expression of several proteins thought to play important roles in cancer invasion and metastasis. Moreover, the observations highlight the heterogeneity present in different cell lines and surely also can be transferred to the *in vivo* situation. A challenge for the future will be to further analyse the frequency of, and mechanisms behind, dysregulated TKs in different human tumors, aiming at obtaining a tailored treatment. Thus the development of TKIs with effects which are better than or at least similar to those shown by genistein and AG-1478, with tolerable side effects, will be given high priority. Only through such efforts may TKI treatment be fully developed and find its place in the treatment of malignant tumors, solely or in combination with other drugs.

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# Paper I

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Paper I is not included due to copyright.

# Paper II

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# Paper III

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# Paper IV

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# Paper V

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