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Explorations of combinational therapy in cancer : targeting the tumor and its microenvironment by combining chemotherapy with chemopreventive approaches

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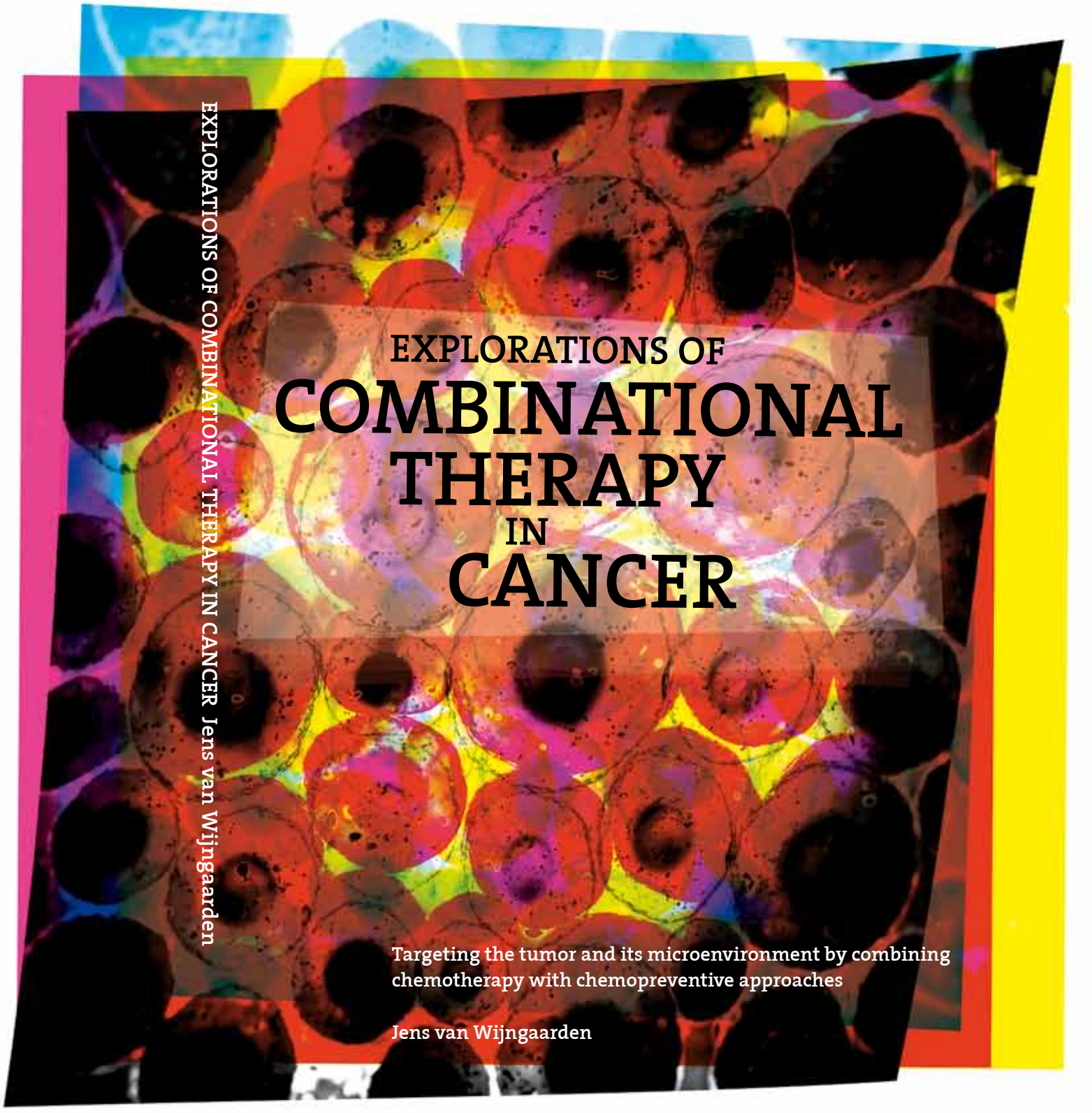

EXPLORATIONS OF COMBINATIONAL THERAPY IN CANCER

Targeting the tumor and its microenvironment by combining chemotherapy with chemopreventive approaches

Jens van Wijngaarden

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Targeting the tumor and its microenvironment

by combining chemotherapy with chemopreventive approaches

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Chapter 1

General introduction

1. Tumorigenesis

Cancer is fundamentally a disease of imbalance in the regulation of cell proliferation. Cellular proliferation is a tightly regulated process. Several check mechanisms prevent the uncontrolled proliferation of cells. Cancer cells have defects in the regulatory circuits that govern normal cell proliferation and homeostasis. The defects in these regulatory circuits are caused by genetic changes.

The genetic changes through which cancer develops can be divided in two broad categories: changes in oncogenes and changes in tumor suppressor genes. Where oncogenes promote the malignant phenotype, tumor suppressor genes are genes which inhibit cell division, regulate survival or other properties of cancer cells. Typically, changes in many genes are required to transform a normal cell into a cancer cell[1,2], which may take many years to accumulate.

While there are many distinct types of cancer, there are believed to be six essential differences that set human malignancies apart form normal cell physiology[1], self-sufficiency in growth signals; insensitivity to growth-inhibitory signals, evasion of programmed cell

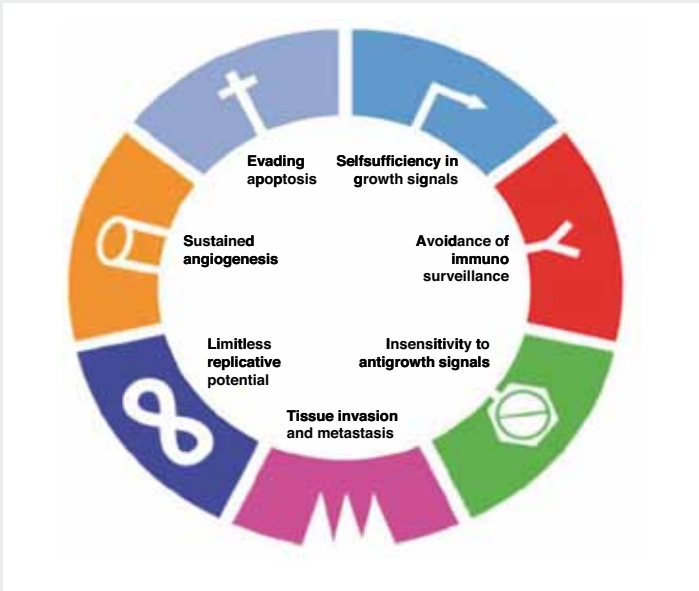

Figure 1

death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (figure 1, adopted from Keereweer et al.). Each of these so-called hallmarks is an acquired trait by genetic alterations in cancer cells and represents the successful ability of a tumor to evade the anti-cancer mechanisms present in cells and tissues.

The great majority of cancers occur in epithelial tissues (>80%), yielding carcinomas[3,4]. In the development of epithelial cancer, roughly, several phases can be distinguished (figure 2, adapted from the National Cancer Institute). Hyperplasia is a reversible proliferation of cells beyond that which is ordinarily seen. Hyperplastic cells remain subject to normal regulatory control mechanisms. Dysplasia is the earliest form of pre-cancerous lesion and can be divided into low –or high-grade dysplasia, where high-grade dysplasia represents a more advanced progression towards malignant transformation. A carcinoma in situ is a localized form of cancer which has not invaded past the basement membrane into surrounding tissues. The cells in a carcinoma in situ grow rapidly and without regulation. The final step is an invasive carcinoma which has the potential to metastasize.

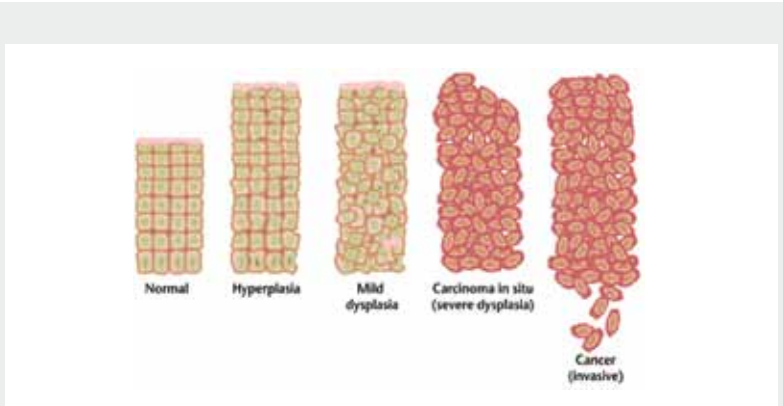

Figure 2

2. Tumor progression

2.1 ROLE OF THE TUMOR MICROENVIRONMENT

Cancer has long been regarded a disease consisting of a group of transformed cells which have acquired proliferative and invasive capacities. Accordingly, therapeutic anti-cancer therapies have been concentrated on and limited to targeting tumor cells alone (see also chapter 3). In order for cancer to be effectively controlled, carcinogenesis and tumor progression needs to be viewed involving complex interactions with its environment; the tumor microenvironment. Currently, more and more data indicate that we need to revise our ideas on carcinogenesis and carcinomas and regard these as phenomena that occur in tissues, not just in cancer cells.

The development of a tumor takes place in an environment that consists of a complex system containing many different cell types. The tumor microenvironment contains endothelial cells and their precursors, pericytes, smooth muscle cells, fibroblasts of various phenotypes, myofibroblasts, neutrophils and other granulocytes (eosinophils and basophils), mast cells (MCs), T, B, and natural killer lymphocytes and antigen presenting cells such as macrophages and dendritic cells. All these cells can in one way or another participate in tumor progression.

For example, experiments in mouse models have shown that fibroblasts in the stromal microenvironment play an important role in tumor formation[5-7]. Next to this, cancer-associated fibroblasts (CAFs) have been shown to induce tumorigenesis in prostatic epithelial cells[8]. Moreover, CAFs have been shown to be able to promote tumor growth and increase angiogenesis[9-10]. Furthermore, myofibroblasts, also known as activated fibroblasts, constitute an important niche for tumor development through the promotion of angiogenesis[11-13].

The presence of leucocytes in tumor tissues was until late thought to be an attempt of the immune system to eradicate the tumor. This idea needs to be revised. It has been shown that leukocyte cells such as macrophages, granulocytes and mast cells all have been associated in one way or another with promotion of malignancy. Tumor-associated leucocytes are variably loaded with an assorted array of cytokines, cytotoxic mediators as well as proteolytic enzymes that promote all the steps associated with malignancy within tumors[14-16].

The role of granulocytes has been extensively studied with contradictory results. For example, it has been shown that circulating neutrophilic polymorphonuclear cells (PMNs) isolated from tumor bearing animals reduce the number of metastatic foci in the lungs[17]. On the other hand, *in vitro* studies reveal that PMNs stimulate tumor cell attachment to endothelial monolayers, a relevant step for tumor migration[18, 19]. Next to this, neutrophylic granulocytes have also been shown to promote the migratory capacity in breast cancer cells[20]. Other authors have shown that tumor-associated PMNs were involved in tumor angiogenesis by the production of vascular endothelial growth factor (VEGF) and Interleukin (IL)-8 and in tumor invasion by the release of matrix metalloproteinases (MMPs) and elastase[21-23].

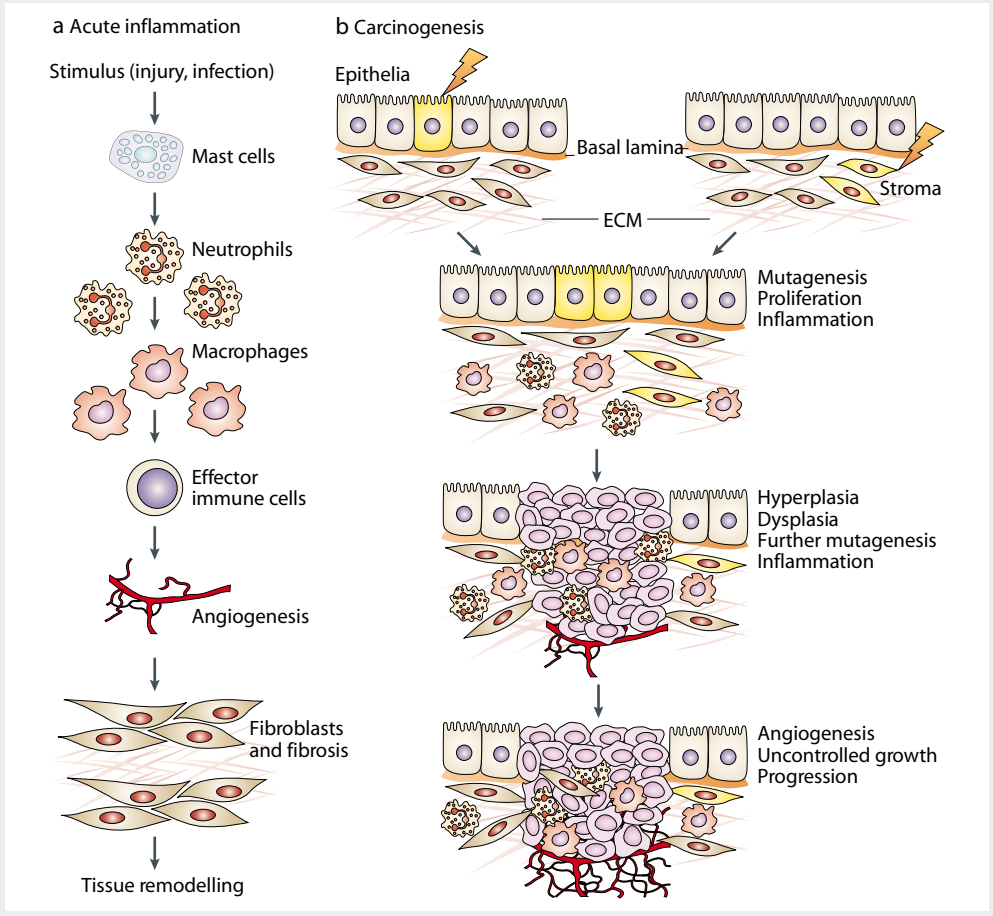

Figure 3

Figure 3 (adapted from Albini et al.) illustrates the sequence of events and involvement of the tumor microenvironment during carcinogenesis[24]. A current concept of tumor progression and interaction with the microenvironment is that it roughly resembles an inflammatory process. The transformation taking place during tumorigenesis may lead to disrupted proliferation. This disruption is regarded as cell damage and causes an inflammatory reaction, in order to repair and reconstruct the damaged lesion. This inflammatory reaction includes leukocyte infiltration and stromal and endothelial cell activation. This alteration of tissue homeostasis further promotes tumor progression, which in turn further activates the surrounding stroma, eventually also leading to neovacularization, or tumor angiogenesis, which is a critical step in the further progression, invasion and metastasis of a tumor. As such, in fact, a reaction to restore the damage caused by the tissue transformation, paradoxically results in further promoting the progression, survival and replication of the dysfunctional epithelial cells.

2.2 TUMOR ANGIOGENESIS

Angiogenesis is an essential and critical step in the further progression, invasion and metastasis of a tumor. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is dependent on a balance between pro- and anti-angiogenic factors. Anti-angiogenic factors predominate in tissues where the vasculature is quiescent. In contrast, when the balance is in favor of pro-angiogenic factors, angiogenesis is promoted. For a tumor to grow beyond a certain size there is a need for nutrients, oxygen and the efficient removal of waste product by acquiring its own vasculature through angiogenesis. This onset of angiogenesis during tumor progression is called the 'angiogenic switch' as first postulated by Folkman[25]. This requirement may vary, however, among tumor types and change over the course of tumor progression[26, 27], but gaining access to the host vascular system and the generation of a tumor blood supply are rate-limiting steps in tumor progression.

In normal physiological angiogenesis there is a tightly regulated balance of pro- and anti-angiogenic signals, which results in rapid maturation and stabilization of newly formed microvasculature if the balance is in favor of pro-angiogenic factors. In tumor angiogenesis, this process is incomplete and cut short, leading to formation of tumor vessels that are structurally different from their normal counterpart. Tumor vasculature is distinctly

disorganized, tortuous and spatial distribution overall is heterogeneous. Also, tumor vasculature spreads without any organization, is irregularly shaped, dilated, leaky, and poorly differentiated. Tumor vessels may have thin walls, with only partial endothelial linings, loss of adherence between endothelial junctions, loosely attached or absent perivascular cells and a discontinuous basement membrane[27-31]. Further to this, it has even been observed that tumor cells in certain circumstances may undergo a dedifferentiation program, leading them to act as endothelial cells and line the tumor vasculature themselves[32-34]. The concept of selectively targeting tumor vasculature in anti-angiogenesis therapy without affecting the quiescent organ vasculature is based on the phenotype of immature angiogenic blood vessels being distinctly different from that of normal and resting blood vessels.

As described above, the tumor microenvironment plays an important role in the onset and activation of tumor angiogenesis because of the alteration of surrounding tissue homeostasis. Likewise, tumor cells acquire certain specific genetic traits allowing the onset and progression of tumor angiogenesis. During tumor angiogenesis, pro-angiogenic factors are upregulated, and anti-angiogenic factors downregulated by both the tumor cells themselves as in the tumor microenvironment. Numerous of such endogenous pro- and anti-angiogenic factors have been identified, such as the pro-angiogenic factor VEGF-A, member of a gene family that further includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. Other pro-angiogenic factors include fibroblast growth factors (FGFs), angiopoetins, platelet-derived growth factors (PDGFs), transforming growth factor-beta (TGF-β) and many others. Anti-angiogenic factors include, among many others, thrombospondins[35], endostatin (a proteolytic cleavage product of collagen XVIII)[36], and soluble factors like interferon-α and -β (IFN-α and -β) and angiostatin[37].

Both pro- as anti-angiogenic factors can be regulated and derived from both the tumorcells themselves as from tumor-infiltrating inflammatory cells. As such, the angiogenic switch is an intrinsic event of multistage tumorigenesis where genetic and epigenetic events within tumor cells cooperate with inflammatory responses and cells of the tumor stroma to define the ultimate cocktail of pro- and anti-angiogenic factors. This results in a direct or indirect shift of the balance in favor of an onset of tumor angiogenesis[37].

The importance of tumor-stroma interactions with concurrent angiogenesis and tumorigenesis and tumor progression has been widely recognized. The question that remains is which comes first: the dysfunction of epithelial cells or the changes in their microenvironment? For example, it has been shown that transformed stroma can induce malignancy in lung and mammary epithelia[5, 6]. Moreover, taken the above into account one has to ask whether tumorigenesis and progression can occur because, instead of despite, of the tumor microenvironment. One way or another, because the role of the different players in the tumor microenvironment is now beginning to become known, they need to be and are now coming into the picture as targets for anti-tumor therapy.

2.3 TUMOR METASTASIS

Further along the way in tumor development and progression, tumor cells may acquire invasive properties and further on acquire the ability to metastasize. In order for a tumor cell to metastasize, it needs to acquire the ability to migrate and invade. Migratory cancer cells undergo dramatic molecular and cellular changes by remodeling their cell-cell and cell-matrix adhesion and their actin cytoskeleton, molecular processes that involve the activity of various signaling networks. Metastasis formation is driven by genetic alteration of many genes, such as the activation of oncogenes like RAS and MYC[38, 39] and inactivation of metastasis-suppressor genes such as p53 and NM23[40, 41]. Further to this, the metastasizing tumor cells need to be able to adapt to and survive and grow in a new environment.

Next to this, the metastasis site itself needs to have the right environmental properties for the tumor cell to thrive. The theory that metastasis is not just dependent on the acquired traits of the tumor cell at stake, but also dependent on the properties of the metastatic niche is called the 'seed and soil' hypothesis and was postulated by Stephan Paget more than a century ago[42].

The 'seed and soil' hypothesis was based on the observation that certain tumors metastasize to certain sites, i.e. tumors do not metastasize randomly. For example, breast cancer and prostate cancer is most likely to metastasize to bone tissue, malignant melanoma has a tendency to metastasize to the brain, whereas colon cancer has the liver as a preferred secondary site. As well as during primary tumor development, the microenvironment of the secondary site plays a major role in metastasis, survival and progression.

It is thought that the specific secondary sites contain the microenvironment with specific local molecular mediators as to support the suitable type of cancer cells. This concept still holds ground to this day. Breast cancer for example, frequently metastasizes to

the skeleton. It is estimated that 85% of individuals with advanced breast cancer disease have bone metastases[43]. When metastasizing to bone, breast cancer cells first come into contact with the bone marrow inside the bone, through circulation in blood (hematogenous spread). It has been shown that the bone marrow is particularly favorable for the retention and extravasation of circulating cancer cells[44]. Moreover, the bone itself is an abundant storage of growth factors. Mineralized bone contains insulin growth factors (IGFs), TGF-β, FGFs, PDGFs and bone morphogenetic proteins (BMPs), which are constantly released in the bone marrow through osteoclastic bone resorption, further positively affecting the growth of the local bone metastases[45, 46].

Further major components of mineralized bone further are osteopontin (OPN), bone sialoprotein (BSP) and type I collagen, which all help mediate local adhesion, motility, survival and growth by interactions with matrix molecules such as integrins, of which αvβ3 and αIIβ3 integrins seem to participate in determining the osteotropism of breast cancer metastases[47, 48]. It has been shown for example, that the breast cancer cell line MDA-MB-231-Bo2, a subclone of cell line MDA-MB231, which constitutively overexpresses αvβ3 integrin only metastasizes to bone. Similarly, de novo expression of αvβ3 integrin in a breast cancer cell line that would normally metastasize to the lungs, showed to promote its dissemination to bone[49, 50].

With regard to the intrinsic properties of breast cancer cells, it has been shown that breast cancer cells at one point in time express genes that are normally considered bone or bone-related and as such preferentially metastasize to bone. In expressing these genes, the breast cancer cells are well equipped to home, adhere, survive and proliferate in the bone microenvironment. This acquisition of bone cell-like properties by tumor cells is called osteomimicry[51]. Osteomimetic factors for example include OPN, osteocalcin, osteonectin, BSP, receptor activator of nuclear factor kappa B ligand (RANKL) and parathyroid hormone-related protein (PTHrP). Several of these molecules are related to the recruitment and differentiation of osteoclasts. For example, OPN is produced by many breast cancer cells and has a strong clinical correlation with poor prognosis and decreased survival[51-53]. It can contribute to tumor cell survival, proliferation, adhesion, and migration. In the bone, OPN is involved in the differentiation and activity of osteoclasts, and inhibition of mineral deposition in the osteoid[52].

So, taken together, as well as during tumorigenesis and tumor progression, in metastasis both the tumor microenvironment as the cancer cells themselves are well

equipped, suited even, for the event. It has been hypothesized that cancer cells, which are metastatic to bone after an initial growth phase that depends on their interaction with the local stroma, become independent of microenvironment's growth support and further progress autonomously[54]. This was postulated after the observation that decrease of bone turnover by bisphosphonates (bone resorption decreasing agents) before colonization of bone by breast cancer cells, inhibits to a great extent the formation of bone metastases, but when bisphosphonate treatment was given after the establishment of bone metastases, it was shown to have a minimal effect on the progression of cancer growth[54, 55].

2.4 THE PRINCIPLES OF DARWINIAN EVOLUTION IN CANCER

It has not been for long that it is recognized that tumor progression actually follows the principles of Darwinian evolution. This process is called somatic evolution. A tumor is a large genetically heterogeneous population of individual cells. Cells that acquire traits through genetic changes that enhance their survival or reproduction continue to multiply, and come to dominate the growing tumor, thereby promoting tumorigenesis. This process leads to the clonal expansion of the cells with favorable properties. Clonal expansion and genetic heterogeneity has been observed within many different types of neoplasms and the idea of Darwinian evolution within cancer is now an accepted concept[56].

The process of somatic evolution is not just important during tumorigenesis. It also allows the tumor to react to its environment in an adaptive manner. This has large consequences for the ability of the tumor to progress and metastasize, but also for its ability to react to cancer therapy. For the ability of a tumor to metastasize, the metastatic phenotype needs to be acquired by genetic alteration, but the tumor also needs to be able to survive and adapt in a new environment. This asks for a highly adaptive phenotype, one which can only be acquired by genetic favorable changes. This adaptive phenotype is not only largely important in the start, progression and metastasis of a tumor, but also has large implications in the phenomenon of 'acquired drug resistance' in anti-cancer therapy as further described below.

This principle of somatic evolution in cancer fits the current explorations on the concept of cancer stem cells. Even though still debated upon, some evidence indicates the existence of self-renewing stem/progenitor-like tumor cells, so-called cancer stem cells

(CSC's), which are critical for initiation and maintenance of the primary tumor and eventually metastasis. It is believed that both the tumor cells themselves as environmental factors induce some cancer cells to dedifferentiate to acquire stem cell like properties. In carcinomas it has been shown that cancer cells can lose their epithelial characteristics via a dedifferentiation process called the epithelial mesenchymal transition (EMT), which causes them to functionally transition to migratory matrix molecules producing mesenchymal cells. Functional dedifferentiation gives these cells the ability to adapt to all sorts of different situations and surroundings and have a major role in the promotion of tumor invasion, angiogenesis, intravasation and different processes during metastasis such as dissemination, colonization and formation[57]. Further to this, it has been shown that chemotherapy generally target cells with a more differentiated phenotype and thus indirectly selecting tumor cells with adaptive stem/progenitor phenotypes[58].

3. Tumor therapy

3.1 CONVENTIONAL TUMOR THERAPY: CHEMO MONOTHERAPY AND DRUG RESISTANCE

Cancer is one of the leading causes of death worldwide (data from the World Health Organization). Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. For this reason, the development of new and improved anti-cancer therapies is of large social importance, with in the future ideally resulting in cancer to be a chronic disease at most.

To date, one of the most commonly used therapies still remains chemotherapy. The era of chemotherapy began in the 1940s, with the first use of nitrogen mustard as an anti-cancer treatment by Louis Goodman and Alfred Gilman in 1942. A patient with non-Hodgkin's lymphoma was treated with this toxin, based on autopsy findings in soldiers dying of exposure to sulphur mustard gas during the First World War. These victims showed pronounced lymphoid hypoplasia and myelosuppression, leading to the proposal that these reagents may be used to counteract lymphoid tumors. The treated patient showed regression of the disease for a few weeks, establishing the principle of systemic drug administration to induce tumor regression. Follow-up drugs soon came into the picture, such as alkylating agents (e.g. cyclophosphamide) and antifolates (e.g. methotrexate). It was soon noted that tumors quickly became resistant to these drugs, which was an observation that predicted clinical experience with these agents up to the present[59, 60].

Therapeutic resistance can either be caused by intrinsic resistance, or by acquired drug resistance, which is the ability of the tumor cells to adapt to the given therapy by an evolutionary process. Depending on the sort of therapy, the type of cancer, and its stage, one or several genetic alterations are necessary to confer resistance to treatment. Some mechanisms of resistance require two genetic alterations, either because of haplosufficiency of a gene such that one recessive mutation cannot confer resistance, or because of the use of combination therapy that targets two different positions in the cancer genome. One of the first discovered genetic alterations leading to acquired therapy resistance was in methotrexate. This chemotherapeutic agent inhibits the dihydrofolate reductase (DHFR) gene. However, methotrexate therapy appeared to select for cells with extra copies of the DHFR gene, which are resistant to methotrexate[61-65]. The observation of chemotherapeutic drug resistance soon led to the idea to use combinations of drugs, each with a different mechanism of action.

3.2 CONVENTIONAL TUMOR THERAPY: COMBINATION CHEMOTHERAPY AND MULTI-DRUG RESISTANCE

Because of the observed drug resistance, it was hypothesized that cancer cells could conceivably mutate to become resistant to a single agent, but that by using different drugs concurrently it would be more difficult for the tumor to develop resistance. This approach was first successfully applied by Holland, Freireich, and Frei, who simultaneously administered methotrexate (an antifolate), vincristine (a vinca alkaloid), 6-mercaptopurine (6-MP) and prednisone in children with acute lymphoblastic leukaemia (ALL), thereby inducing longtime remission[59, 60]. With incremental refinements of original regimens, ALL in children has now become a largely curable disease. Currently, nearly all successful cancer chemotherapy regimens use this paradigm of multiple drugs given simultaneously.

Combination chemotherapy was devised to overcome resistance, by treating with agents that exert their effects by different mechanisms and/or are very different chemically. Unfortunately, it was soon observed that cancer cell populations can respond by becoming multi-drug resistant (MDR) to a panel of mechanistically and structurally diverse drugs. Resistant and MDR cell variants within tumors which are either inherently present or

generated may be selected, in a Darwinian fashion, during multiple cycles of chemotherapy. Of course, many chemotherapeutic agents are mutagenic, thus also increasing the frequency of resistant mutants in the cancer cell population.

Molecular mechanisms of drug resistance include overexpression of drug efflux pumps (generally ATP-binding cassette [ABC] transporter family members), such as the mdr-1 gene product P-glycoprotein (P-gp)/P-170 (ABCB1), multi-drug resistant associated protein-1 (MRP-1; ABCC1) and related proteins, and breast cancer resistance protein (BCRP; ABCG2). The three key mammalian transporters involved in transport of anti-cancer agents, such as the anthracyclines, are P-gp, MRP-1 and BCRP[66, 67].

In recent years, much effort has been made to identify agents that are able to overcome MDR, in order to improve chemotherapeutic treatment. These agents, called chemosensitizers, belong to a variety of structural classes, such as calcium channel blockers, drug analogs, cyclic peptides and steroids[68, 69]. Next to that, other therapeutic options in order to improve treatment benefit and overcoming the tumor's ability to escape therapy are being explored as further discussed below.

3.3 CHEMOPREVENTIVE TUMOR THERAPY; ANTI-ANGIOGENESIS THERAPY

The limitations of chemotherapy have led to the exploration of therapies with improved efficacy, amongst others chemopreventive approaches. Cancer chemoprevention, as first defined by Sporn in 1976, is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer[70]. It is based on the concepts of multistep carcinogenesis. Arresting one or several of these steps before cells are developing in tumorigenesis may then interfere with the disease's progression. Definitions of chemopreventive agents have become blurred a bit over time, because it has become clear that many chemopreventive agents not just have preventive effects by interfering with different stages during a tumor's development, but also have direct inhibitory effects on already established tumors or its microenvironment. One way or another, this field is extensively being explored and in some occasions used with positive effects in the clinical setting. Cancer chemoprevention may comprise of different approaches, of which some are shown in figure 4 (adapted from Soria J.C. et al.)[71].

One chemopreventive approach in anti-cancer therapy which is and has extensively

been explored is anti-angiogenesis therapy. As stated above, in general, for a tumor to grow beyond a certain size there is a need for nutrients, oxygen and the efficient removal of waste product by acquiring its own vasculature through angiogenesis. This process is essential for the growth of solid tumors and facilitates metastasis, thereby providing a rationale for anti-angiogenesis therapy in cancer[72, 73]. Tumor angiogenesis can be inhibited by endogenous anti-angiogenic factors, which results in inhibition or even regression of tumor growth and metastasis[74].

One of the first endogenous anti-angiogenic factors to be explored was endostatin[75]. Endostatin is a cleavage product of collagen XVIII that has shown to inhibit tumor-angiogenesis in experimental tumor models. Several studies have shown the inhibition of endothelial cell proliferation and migration and endothelial cell apoptosis *in vitro*[76-80], and growth of tumors and metastases *in vivo*[81-83]. In animals, endostatin causes tumor vessels to collapse, which leads to the deprivation of oxygen and nutrients and results in apoptosis

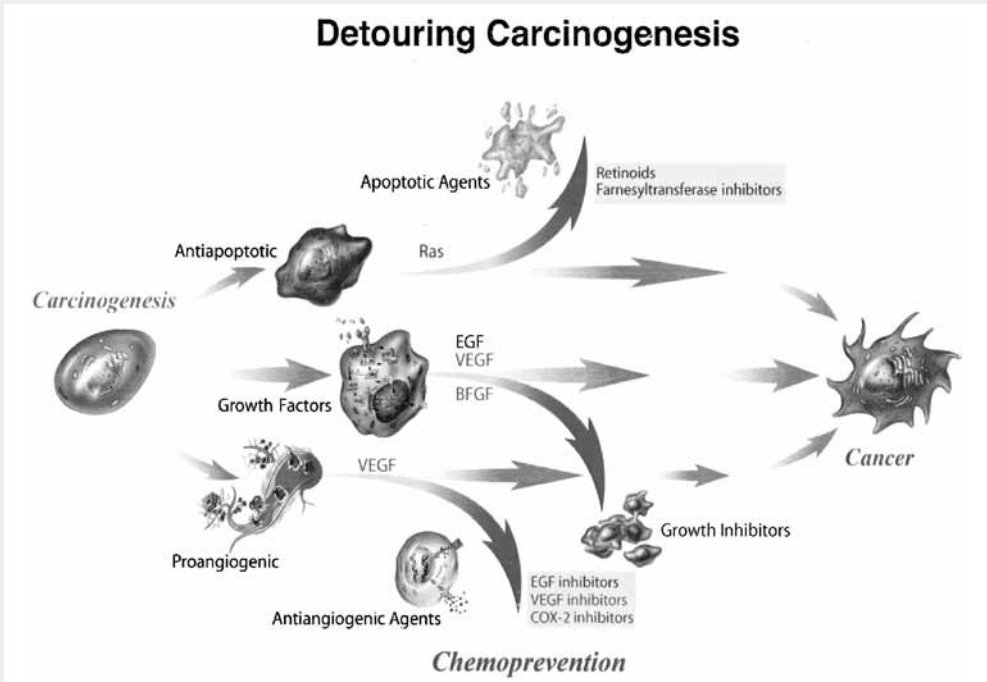

Figure 4

and necrosis of the tumor cells[84, 85]. No toxic side effects have been observed[76, 79] and systemic therapy has not shown to be associated with acquired resistance[86]. Where results of preclinical studies on endostatin were promising, however, the first phase I clinical trials were disappointing[87-89]. Although endostatin showed no treatment-related toxicity, no significant anti-tumor effect was observed. In one study a reduction in tumor blood flow and metabolism and an increase in apoptosis in tumor and endothelial cells was observed. However, no significant relationship between these biological markers and clinical outcome could be established[90]. Clinical trials with endostatin are however currently ongoing (data from clinicaltrials.gov).

As recently in the news, other examples of chemopreventive agents are non-steroidal anti-inflammatory drugs (NSAIDs) and specific cyclo-oxygenase (COX)-2 inhibitors, which are widely used in the treatment of pain and rheumatoid arthritis. A recent meta-analysis showed that daily use of the NSAID aspirin significantly reduced deaths due to several common cancers during and after the trials[91]. NSAIDs and COX-2 inhibitors both have shown promising results in the treatment of cancer in experimental and clinical studies[92, 93]. COX-2 is overexpressed in many malignancies and is involved in tumor development and growth. The effects of NSAIDs and specific COX-2 inhibitors on tumor cells include inhibition of cell proliferation, induction of apoptosis and reduction of cell motility and adhesion[93-101]. Furthermore, both non-specific and specific COX-2 inhibitors have shown to significantly inhibit tumor angiogenesis[102-105]. Moreover, combining NSAIDs and specific COX-2 inhibitors with chemotherapeutics has been shown to improve treatment outcome in several preclinical and clinical studies. For example, in experimental and clinical studies, the COX-2 inhibitor celecoxib, has shown to enhance the anti-tumor efficacy of several cytostatics, such as that of irinotecan, doxorubicin, bleomycin and 5-fluorouracil[106-109].

Overall, despite promising results in preclinical studies in which anti-angiogenic therapy translates into potent anti-tumor effects[110-119], implementation of these therapies in clinical settings has learned that beneficial effects in the patient are less pronounced. One of the explanations for this may be that tumors grow in well vascularized tissues and they may progress via increased reliance on vessel co-option from the microenvironment. As anti-angiogenic compounds do not affect incorporated pre-existent, or matured tumor vasculature, targeting of the existing tumor vessels may be an attractive adjuvant approach to accomplish tumor regression via disruption of the tumor's blood supply.

3.4 VASCULAR TARGETING

Next to targeting angiogenesis, another approach to target the tumor's blood vessel network exists, aiming to affect the already established tumor vasculature[120]. The preferential targeting of the already established tumor vascular network and makes use of so-called vascular-disruptive agents (VDAs)[121-123]. All VDAs currently examined draw on the differences between tumor and healthy vasculature to allow for highly selective targeting of tumor blood vessels[124, 125]. The VDAs can be divided into two categories: biologic and small-molecule agents. Where the first includes peptides and antibodies that deliver effectors to the tumor endothelium, the latter includes compounds that exploit the differences between healthy and tumor vasculature to induce selective vascular dysfunction[126-128].

Because of the difference between targeting angiogenesis and the existing vascular network, both could have their role in anti-cancer therapy. Where anti-angiogenesis treatment is thought to be well-suited for treating micrometastatic disease and early-stage cancer, disrupting established tumor vasculature leads to rapid vascular collapse, vessel congestion and tumor necrosis and is therefore more efficacious against large, already established tumors. Both approaches have shown promising results in ongoing preclinical studies, but treatments either targeting tumor-angiogenesis or established tumor vasculature alone has not yet shown to be fully effective[124, 129-133].

Effective targeting of tumor endothelium requires the availability of tumor-vessel specific targeting agents or VDAs with high enough specificity for existing tumor vasculature. Few candidate VDAs have been identified so far. This is, however, also due to the lack of adequate screening methods which are able to identify efficacy of candidate drugs and also discriminate between the different vascular targets.

3.5 EXPLORING THE TUMOR MICROENVIRONMENT AS ANTI-CANCER TARGET

Because of the remaining therapeutic gaps in the treatment of cancer and limitations of clinical therapies, research is ongoing to identify suitable drugs and targets in anti-cancer treatment. As stated above, the tumor microenvironment plays a large role in tumorigenesis, tumor progression, migration, invasion and eventually metastasis. As such, this has led researchers currently exploring the microenvironment as an anti-cancer target. It is im-

portant to recognize that therapeutic targets can be sought in both the environment of the developing tumor, as in the secondary microenvironment site in case of metastasis.

As the microenvironment has such a crucial role in carcinogenesis and metastasis, it represents a crucial target not only for cancer therapy but also for chemopreventive strategies as further elaborated on above. There is already a large amount of information about specific cells and molecules in the tumor microenvironment that are targets for cancer therapy at present[134, 135]. The supporting players in the tumor microenvironment include stromal fibroblasts, infiltrating immune cells, the blood and lymphatic vascular networks, and the extracellular matrix. Figure 5, (as adapted from Mueller M.M. et al)[133] shows the different players in the stromal compartment of a developing primary tumor. There is abundant evidence that an abnormal stromal context contributes to, or is even required for, tumor formation and progression. 'Normalization' of the stromal environment should therefore be able to slow or even reverse tumor progression[136-138].

The potential of a normal context to suppress a tumorigenic phenotype has been shown in different experimental settings. For example it has been demonstrated that the presence of a reconstituted physiological basement membrane induces pre-malignant breast epithelial cells to undergo growth arrest and form polarized alveolar structures, as normal epithelial cells would[139]. This normalization is in part mediated by integrins, as blockade of signaling by β1-integrin reverted tumorigenesis despite maintained genetic abnormalities in the epithelial cells[140].

Potential therapeutic target components of the tumor microenvironment include stromal cells such as endothelial cells, tumor associated fibroblasts, macrophages, extracellular matrix (ECM) molecules such as thrombospondin and fibronectin (FN), matrix-degrading proteases and inhibitors such as matrix metalloproteinases (MMPs) and tissue metalloproteinase inhibitors (TIMPs) and regulatory molecules such as integrins, growth factors and chemokines[134]. These agents may provide an interesting alternative to traditional tumor cell-directed therapy[141]. Because of the complexity of the tumor milieu, the most beneficial therapy will likely involve the combination of one or more agents directed at this new target. Advantages to targeting the stroma include the fact that these cells are not as genetically unstable as cancer cells, and are therefore less likely to develop drug resistance[142, 143]. Several success stories of drugs that target the tumor microenvironment have entered the clinic[134, 135].

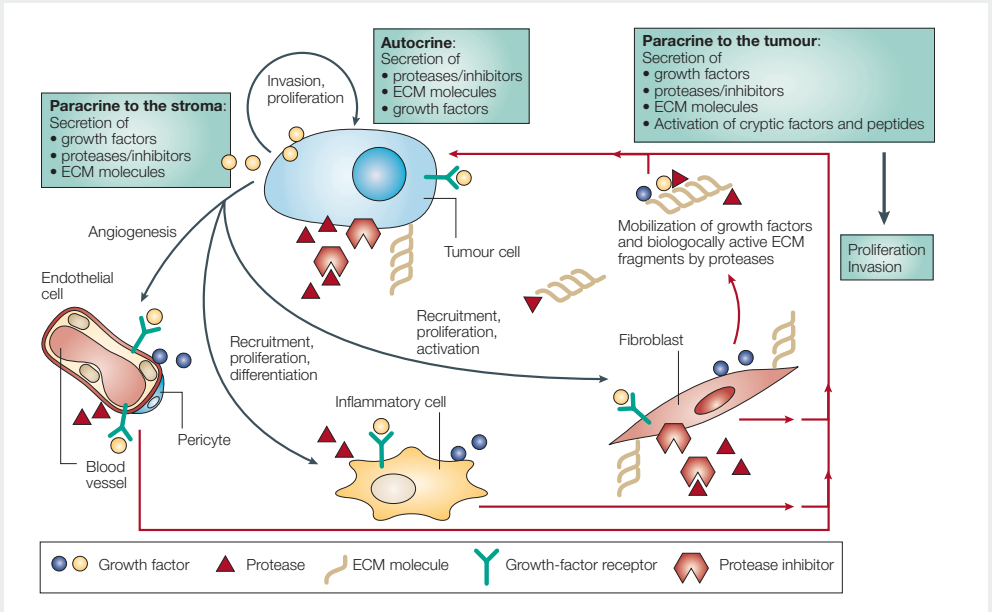

Figure 5

In discussing targeting the tumor microenvironment, we should also consider the environmental conditions in which metastatic tumors develop. As discussed above, metastatic cells need an appropriate microenvironment in which they can survive and proliferate. While experimental systems have shown that tumor cells arrive at secondary sites at relatively high rates, they only thrive in certain, stereotypical sites[42, 144, 145]. As such, next to targeting the microenvironment of the developing tumor, the molecular microenvironment of successful metastasis sites is a promising target for interfering with either the homing or the survival of metastatic cells.

One example of an important advance in this direction came from the discovery of two highly expressed chemokine receptors (CXCR4 and CCR7) on metastatic breast cancer cells. Their respective ligands (CXCL12 and CCL21) were preferentially expressed in the lung and regional lymph nodes, two important metastasis sites. When the interaction between one of these pairs (CXCL12/CXCR4) was blocked *in vivo* using neutralizing antibodies, there was a significant reduction in breast cancer metastases to both the lung and lymph nodes[146]. Inhibitors of chemokines and their receptors are in preclinical development and may offer a

means to interfere with the homing of tumor cells to secondary organs[147]. Another approach is targeting the progression or establishment of metastases by preventing the growth in the secondary site. This has been explored by administration of bisphosphonates (bone resorption decreasing agents) before colonization of bone by breast cancer cells, which was shown to inhibit to a great extent the formation of bone metastases[47].

4. Outline of this thesis

Because of existing therapeutic gaps in the treatment of cancer and cancer remaining one of the leading causes of death worldwide, the development of new and improved anti-cancer therapies is of large importance. In doing so, it is of vital importance, not just to enhance and improve existing therapies, but also to explore new therapeutic options such as chemopreventive agents, vascular disruptive agents and approaches interfering with the tumor microenvironment. Furthermore, it is important to understand the effect of these treatments on a genetic level and in doing so identify new possible therapeutic options in aforementioned targets.

In **chapter 2** of this thesis we studied the differential gene expression in a human renal cell carcinoma model after treatment with the chemopreventive anti-angiogenic agent endostatin. **Chapter 3** describes the setup and validation of a new screening model which is able to identify and discriminate between possible new anti-angiogenic drugs and the currently developed and investigated vascular disruptive agents. **Chapter 4** and **5** describe the application of two different combination approaches in anti-cancer therapy which are currently extensively explored. Where **chapter 4** describes the combinational therapy of the chemotherapeutic agent doxorubicin and the chemopreventive COX-2 inhibitor celecoxib, **chapter 5** studies the effects of the combinational therapy of the chemotherapeutic agent docetaxel with the bisphosphonate risedronate on breast cancer bone metastases. Finally, general conclusions and discussions are described in **chapter 6**.

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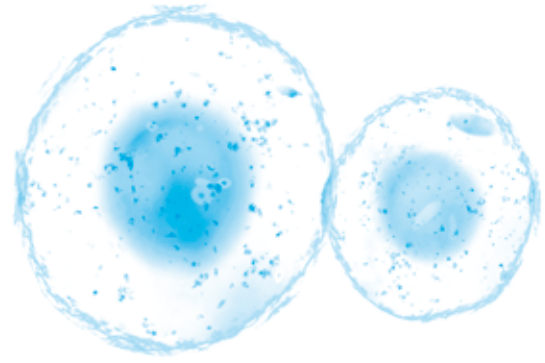
Chapter 2

Identification of differentially expressed genes in a renal cell carinoma tumor model after endostatin-treatment



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Abstract

Endostatin is a cleavage product of collagen XVIII that has shown to inhibit tumor-angiogenesis in experimental tumor models. At present, the exact molecular mechanism of action of endostatin is not completely elucidated. In this study, we wanted to identify specific target genes of endostatin. For this purpose, the human renal cell carcinoma RC-9 was subcutaneously implanted in nude mice and treated with endostatin. Tumor growth was inhibited by endostatin after 4 days of treatment. Using immunohistochemistry and the hypoxia marker pimonidazole, we demonstrate disintegration of blood vessels and hypoxia and anoxia as a result of the treatment. Hereafter, we applied the polymerase chain reaction (PCR)-based subtractive suppression hybridization (SSH) method, together with the mirror orientation selection (MOS) technique to identify specifically induced and suppressed genes after endostatin-treatment. We found eight genes to be specifically induced and 11 to be suppressed by the endostatin-treatment. Among other genes, core binding factor a-1/osteoblast-specific factor-2 (cbfa1/osf2) was found to be specifically suppressed by endostatin. Unexpectedly, cbfa1/osf2 was found to be specifically expressed in granulocytes in the tumor, not only in the experimental RC- 9 tumor model, but in sections of human breast cancer as well. Since an effect of anti-angiogenic therapy on granulocytes has been reported before, this might lead to new insights in the role of granulocytes in anti-angiogenic therapy in general. In conclusion, the SSH-PCR implemented with the MOS-technique is a powerful tool to identify differentially expressed genes. Using these techniques, we have identified several target genes of endostatin, of which cbfa1/osf2 was found to be specifically expressed in granulocytes in the tumor.

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is dependent on a balance between pro- and anti-angiogenic factors. Anti-angiogenic factors predominate in tissues where the vasculature is quiescent. In contrast, when the balance is in favor of proangiogenic factors, angiogenesis is promoted. This process is essential for the growth of solid tumors and facilitates metastasis, thereby providing a rationale for anti-angiogenesis therapy in cancer[1,2] Tumor angiogenesis can be inhibited by endogenous anti-angiogenic factors, which results in inhibition or even regression of tumor growth and metastasis in animals.[3] One of these factors is endostatin.[4]

Endostatin is a cleavage product of collagen XVIII that has shown to inhibit tumor-angiogenesis in experimental tumor models. Several studies have shown the inhibition of endothelial cell proliferation and migration and endothelial cell apoptosis *in vitro*,[4-8] and growth of tumors and metastases *in vivo*.[9-11] In animals, endostatin causes tumor vessels to collapse, which leads to the deprivation of oxygen and nutrients and results in apoptosis and necrosis of the tumor cells.[12,13] No toxic side effects have been observed[4,7] and systemic therapy has not shown to be associated with acquired resistance.[14]

The results of preclinical studies on endostatin have been promising; however, the first phase I clinical trials have been disappointing.[15-17] Although endostatin showed no treatment-related toxicity, no significant anti-tumor effect was observed. In one study, a reduction in tumor blood flow and metabolism and an increase in apoptosis in tumor and endothelial cells was observed. However, no significant relationship between these biological markers and clinical outcome could be established. [18] Further preclinical studies on endostatin may lead to a better understanding of its mechanism of action and may lead to other, possibly more effective therapeutic approaches.

At present, the exact molecular mechanism of action of endostatin is not completely elucidated. Endostatin can bind to $\alpha 5$- and αv-integrins on the surface of human endothelial cells, thereby inhibiting endothelial cell function.[19] Also, endostatin can bind to glypican[20] and heparin and heparan sulfate.[21] It has been shown that the binding of endostatin to $\alpha 5\beta 1$-integrin on the endothelial cell surface, results in a simultaneous or subsequent interaction with a heparan sulfate proteoglycan and caveolin-1. This interaction leads to an intracellular signaling cascade, which causes a reduced migratory capacity of the endothelial cell.[22]

Furthermore, endostatin has also shown to modulate VEGF and Wnt signaling pathways and matrix metalloprotease- 2 activation,[23-26] which all play an important role in angiogenesis.

In a previous study we have shown strong inhibitory effects of endostatin on VEGF-induced migration of human umbilical vein endothelial cells (HUVECs) *in vitro* and on the growth of the human renal cell carcinoma RC-9 *in vivo*.[6] After subcutaneous implantation of the RC-9 tumor in nude mice, we showed regression and inhibition of tumor growth after daily administration of endostatin. This effect was most pronounced after 4 days of treatment, after which the tumor volume stabilized.

In order to study the effect and mechanism of action of endostatin, we wanted to identify which genes were specifically affected by the treatment. For this purpose, the human renal cell carcinoma RC-9 was implanted in nude mice and treated with endostatin for 4 days, after which we applied the polymerase chain reaction (PCR)-based complementary DNA (cDNA) suppression subtractive hybridization (SSH) technique.[27] The SSH-technique compares two messenger RNA (mRNA) populations and identifies differentially expressed genes in one population. Furthermore, we implemented the mirror orientation selection (MOS)-technique, which significantly reduces the amount of false-positive genes.[28]

In the current work, we show that the endostatin treatment results in an effective inhibition of tumor growth, by causing disintegration of blood vessels, thereby generating hypoxia and anoxia, leading to tumor cell death. Hereafter, applying the SSH-technique implemented with the MOS-technique, we found several genes to be specifically induced and several to be suppressed after the endostatin treatment. In order to verify differential expression, we studied the histological expression of calpain-2, found to be induced and core binding factor a-1/ osteoblast-specific factor-2 (cbfa1/osf2), found to be suppressed after endostatin treatment.

Materials and methods

CELL LINES, MICE AND INTRATUMORAL INJECTION

The cell line RC-9 is derived from a patient with renal cell carcinoma in an advanced stage, which forms a solid tumor when transplanted in nude mice. Selected RC-9 tumor pieces of 1mm^3 were subcutaneous implanted into the right flank of halothane-anesthetized 6-week-old BALBc nu/nu mice. The human mammary carcinoma cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Biochrom, Basel, Switzerland), 10% fetal bovine serum, and penicillin/streptomycin (p/s, Life Technologies, Breda, the Netherlands) in a humidified incubator at 37°C at 5% CO2. For *in vivo* injections, cells were grown until 90% confluency and dissociated using 0.125% w/v trypsin, 0.05% w/v ethylenediaminetetraacetic acid (EDTA) solution in PBS (pH 7.2). Hereafter, 2·10^6 cells/100 ml PBS/ 10% FCS were injected subcutaneously in the right flank of halothane-anesthetized 6-week-old BALBc nu/nu mice. After approximately 3 weeks, mice having tumors with a volume of 100mm^3 were selected and divided into two groups. Tumor volume was assessed by measuring the two major diameters with a caliper and using the formula: tumor volume=/4π/6(d1·d2)$^{1/2}$. The treatment started when the tumor volume was approximately 250mm^3. Recombinant human endostatin was obtained from B Olsen (Harvard, Boston, USA) and was purified for *in vivo* use in mice as described earlier.[6] Mice bearing RC-9 tumors received daily injections around the tumor of 2 μg human endostatin in phosphate-buffered saline (PBS) during 4 days. The control group received injections of PBS alone. Mice bearing MDA-MB231 tumors received daily injections around the tumor of 200 μg endostatin as previously described.[29] After treatment, tumors were surgically removed and animals were killed. Tumors were cut in half, where one half was used for RNA isolation and one for immunohistochemistry. For RNA isolation, tumors were suspended in 2ml 4M guanidinium isothiocyanate lysis buffer and stored at -80°C until further use. For immunohistochemistry, the tumors were fixed in zinc–Macrodex formalin (ZnMF) fixative (0.1M Tris acetate (pH 4.5) containing 0.5% zinc-acetate, 5% dextran, and 10% formalin) overnight at room temperature.

IMMUNOHISTOCHEMISTRY

After overnight fixation, tissue was washed three times with PBS and subsequently processed for paraffin embedding. Human bone sections were decalcified in 5% sodium EDTA for 5 days at 4°C prior to paraffin embedding, as previously described. [30] Immunohistochemistry was performed on 5 µm of ZnMF-fixed paraffin-embedded sections. Sections were rehydrated and washed with PBS, followed by incubation with 40% methanol/1% H_2O_2 in PBS for a half an hour at room temperature to block endogenous peroxidase activity. After washing with PBS, the sections were incubated with 5 µg/ml proteinase K in 100mM Tris pH 8.0/ 50mM EDTA pH 8.0 for antigen-retrieval. Thereafter, sections were washed twice with PBS, once with 0.1M Tris-buffered saline (pH 7.4) containing 0.02% Tween 20 (TNT) and blocked with 0.5% Boehringer milk protein (BMP) (Boehringer, Mannheim, Germany) in TNT for 1 h at 37°C. This was followed by overnight incubation at 4°C with the primary antibody, diluted in BMP/TNT. The mousespecific rat monoclonal antibody ER-MP12/CD31 that binds to PECAM-1 was kindly provided by Dr P Leenen (Erasmus University of Rotterdam, The Netherlands). The mouse-specific rat monoclonal macrophage antibody F4/80 was a kind gift from Dr P Nijweide (Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands). The mouse-specific rat monoclonal granulocyte antibody RB6-8C5 that reacts with Ly- 6G, (also designated GR-1), was purchased from Pharmingen (Alphen aan den Rijn, The Netherlands). The goat polyclonal antibody calpain-2 that binds the large subunit of calpain-2 and the rabbit polyclonal antibody PEBP2αA (M-70) that binds to PEBP2αA, (also designated Cbfa1, Osf2 and AML3), were purchased from Santa Cruz Biotechnology (Heerhugowaard, The Netherlands). After three washes with TNT, the sections were then incubated with biotinylated secondary antibody diluted in BMP/TNT for 45 min at 37°C, followed by incubation with horseradish (HRP)-conjugated streptavidin (Amersham Pharmacia Biotech) diluted in BMP/ TNT for 30 min at 37°C. The signal was then amplified using biotinylated tyramids as described, [31] followed by incubation with streptavidin– HRP and final detection by the chromogen 3- amino-9-ethyl-carbazole (AEC), (Sigma Chemicals, Zwijndrecht, The Netherlands). The sections were counterstained with Mayer's Haematoxylin for 1 min and mounted under glass coverslips with aquamount. Representative pictures were taken with a Nikon DXM 1200 digital camera. Granulocytes were differentiated on the basis of morphology.

THE HYPOXIA MARKER PIMONIDAZOLE

Hypoxia in the endostatin-treated tumors was determined as previously described.[32] Briefly, mice were intravenously injected via one of the lateral tail veins with 0.1 ml of a solution of saline containing 2mg of the hypoxia marker pimonidazole-hydrochloride. This marker was given 30 min before the animals were killed. For further analysis, four to five frozen sections (5 µm thick) through central and peripheral tumor areas were prepared. Hereafter, sections were fixed in acetone and incubated overnight with rabbit antisera to pimonidazole adducts. The sections were then incubated with a fluorescent antibody and analyzed for the hypoxia signal using fluorescence microscopy.

RNA AND MRNA ISOLATION

RNA was isolated from a control and endostatin treated RC-9 tumor according to the method described by Chomczynski and Sacchi.[33] In brief, RC-9 tumors were homogenized in 2ml lysis buffer, extracted with phenol and chloroform, precipitated at -20°C with 100% isopropanol, resuspended in autoclaved denatured water, and stored at -80°C. RNA concentration was determined spectrophotometrically assuming 40 µg/ml per optical density at a wavelength of 260nm (1cm path length). mRNA was isolated from control and endostatin-treated RC-9 tumor RNA samples by using the Dynabeads mRNA Purification Kit in accordance with the manufacturer's instructions (Dynal AS, Oslo, Norway).

SSH AND THE MOS TECHNIQUE

SSH was performed using the PCR-Selectt cDNA Subtraction Kit (Clontech, Heidelberg, Germany). In this experiment, a forward and a reverse subtraction was performed. In the forward subtraction, mRNA of a control RC-9 tumor was referred to as tester and mRNA of an endostatin-treated RC-9 tumor as driver, and *vice versa* in the reverse subtraction. In addition, a control provided in the kit was included. cDNA was synthesized from both RC-9 mRNA samples and the control from the kit (human skeletal muscle mRNA) in accordance with the manufacturer's instructions. The cDNA of the RC-9 tumor samples and the control skeletal muscle cDNA were digested with *Rsa*I for 1.5 h at 37°C. Hereafter, digested RC-9 tumor

samples were divided in a tester and a driver sample. To produce a control tester, 0.2% HaeIII digested φX174 DNA was mixed with 1 µl of the skeletal muscle cDNA. The tester samples were ligated with two cDNA adaptors. To verify a ligation efficiency of at least 25%, a ligation efficiency test was performed, according to the manufacturer's instructions. Subsequently, two hybridizations were performed. In the first hybridization, an excess of driver was added to each tester sample. The samples were heat denatured for 1.5 min at 98°C and allowed to hybridize for 8 h at 68°C. The second hybridization consisted of adding freshly denatured driver cDNA to the two first hybridization samples. The samples were incubated overnight at 68°C. From hereon, the SSH-protocol was implemented with the MOS-technique as described by Rebrikov et al.[28] Briefly, each sample was divided into 10 independent samples and a 30-cycle primary PCR was performed. The 10 independent samples were then combined and a 12-cycle secondary PCR was performed using the same primer and conditions as the primary PCR. A nested PCR was performed with primers as described. The samples were then phenol/chloroform extracted and ethanol precipitated and adaptors were removed by *Xma*I digestion. Another hybridization was performed using freshly denatured driver and a final PCR was performed.

DNA CLONING

The differentially expressed sequences of the forward and reverse subtraction were cloned into a pCR®II vector using the TA cloning kit (Invitrogen, Groningen, the Netherlands). Two different concentrations of the PCR products, 0.5 and 1.5 µl, and 50 ng vector were used in the ligation reaction. Subsequently, 2 µl of each reaction was added to TOP10F' cells and incubated on ice for 30 min. The samples were subjected to a heat shock of 30 s at 42°C, 250 µl of SOC medium was added and the samples were incubated at 37°C for 1 h. Of each sample 10 and 25 µl were plated on Luria Bertani (LB) plates containing 50 mg/ml ampicillin (Roche Diagnostics GmbH, Mannheim, Germany), 42 µl IPTG (20 mg/ml) (Gibco/BRL Life Technologies), and 80 µl X-Gal (20 mg/ml) (Gibco/BRL Life Technologies). The plates were incubated overnight at 37°C. The white colonies were picked and the inserts were amplified in a PCR using M13 primers (Gibco/BRL Life Technologies). The PCR products were purified using Microspin S-300 HR columns (Amersham Pharmacia Biotech). The purified PCR products were analyzed for differential expression by Southern blotting. Differentially expressed PCR products were sequenced by the Leiden Genome Technology Center (LGTC). The sequences were compared

to available sequence databases, using Basic Local Alignment Search Tool (BLAST).

SOUTHERN BLOTTING

For probe synthesis, a nested PCR was performed with the unsubtracted controls of the forward and reverse subtraction. The products were digested with *Rsa*I for 2 h at 37°C and the PCR products were purified using Microspin S-300 HR columns (Amersham Pharmacia Biotech). Thirty ng of each product was denatured for 3 min at 100°C and added to Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech). Thereafter, 5 µl $\alpha[^{32}P]$dCTP was added and samples were incubated at 37°C for 30 min. Unincorporated nucleotides were removed by using a ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech). Before hybridization, the probes were denatured for 5 min at 95°C and put on ice for 5 min. For Southern blotting, 50 ng M13 PCR product of each clone was loaded on a 1% agarose gel containing 0.5 µg ethidium bromide. After running, the gels were incubated in 0.4M NaOH twice for 15 min. Hybond-N^+ membranes were incubated for 10 min in H_2O and for 15 min in 0.4mM NaOH. DNA was transferred onto the membrane overnight. Hereafter, the blots were neutralized in 2 x SSC for 5 min and incubated at 80°C for 30 min. The blots were prewetted in H_2O and for prehybridization, an equal volume of 2 x Denhardt's hybridization mix (6 x SSC, 5 x Denhardt's, 0.25% SDS, 50 µg/ml denatured fragmented salmon sperm DNA) was added, supplemented with 0.5 µg/ml denatured KS cell (a mouse osteoblast cell line) DNA. The blots were prehybridized for 2.5 h at 65°C. This was followed by hybridization of the blots overnight at 65°C with the $a[^{32}P]$dCTP labeled probes of the unsubtracted control of the forward or the reverse subtraction. Thereafter, the blots were washed in 1 x SSC/0.1% SDS for 20 min, then in 0.3 x SSC/0.1% SDS twice for 10 min and in 0.1 x SSC/0.1% SDS for 10 min at 65°C. The blots were exposed to a Phosphor- Imager (Molecular Dynamics, Sunnyvale, CA, USA) screen and analyzed.

STATISTICAL ANALYSIS

The data are expressed as mean ± s.d. For comparing endostatin-treated RC-9 tumor volumes with control tumor volumes, a Student's t-test was used. A P-value of <0.05 was considered significant.

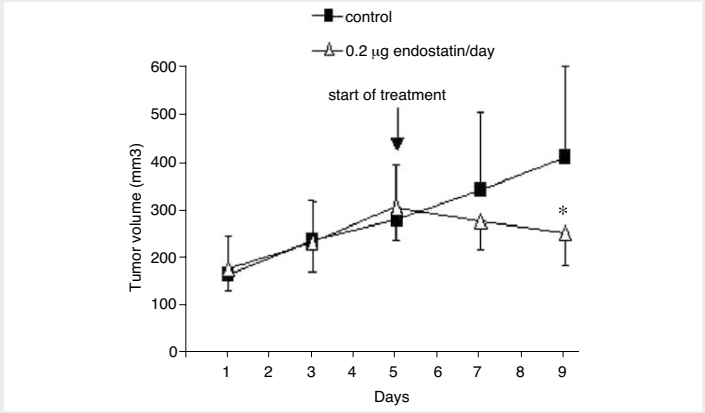

Figure 1 Effect of endostatin-treatment on RC-9 tumor growth *in vivo*. Effect of endostatin (0.2 μg/day, open triangles) or PBS (filled squares) treatment on the growth of RC-9 xenografts in nude mice. Treatment was started when a tumor had reached a size of 250 ± 20 mm^3. Tumor volume (mm^3) was measured as indicated in materials and methods. Values represent mean ± s.d., n = 7. *P<0.05 (endostatin vs control).

Results

EFFECT OF ENDOSTATIN ON RC-9 TUMOR VOLUME *IN VIVO*

In order to characterize the effect of endostatin treatment and to investigate which genes are specifically induced and suppressed after the treatment by using the SSH-PCR, RC-9 tumors were treated with endostatin or PBS. The human renal cell carcinoma RC-9 was subcutaneous implanted in BALBc nu/nu mice. When a tumor volume of approximately 250mm^3 was reached, daily injections of PBS or 0.2 μg recombinant human endostatin were administered around the tumor during 4 days. This dose and duration have proven to give optimal regression in previous experiments.[6] Figure 1 shows the effect of endostatin-treatment on tumor volume. Immediately after starting the treatment, the average tumor volume regresses compared to the control tumor volume. After 4 days of treatment, the difference in average tumor volume is significant (P<0.05). At day 5, tumors were surgically removed and prepared for immunohistochemical analysis and RNA isolation.

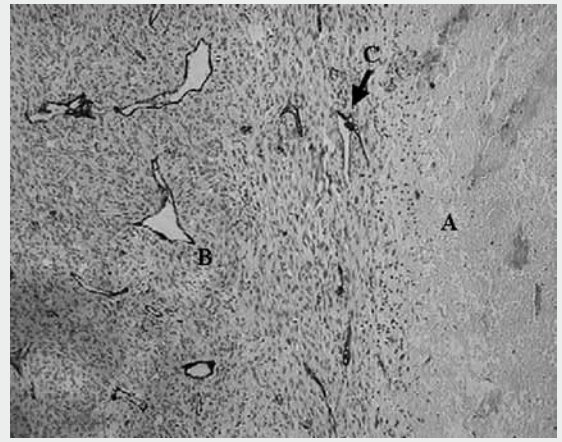

Figure 2 (a) CD31-stained histological section of an endostatin-treated RC-9 tumor obtained after 4 days of treatment. (A) center of the tumor; (B) outer rim of the tumor, containing blood vessels that stain positive for CD31; (C) area around the center of the tumor containing disintegrating blood vessels.

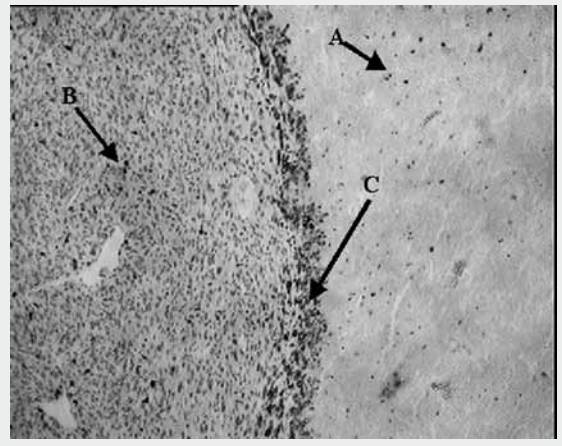

Figure 2 (b) F4/80-stained histological section of an endostatin-treated RC-9 tumor: (A) center of the tumor; (B) outer rim of the tumor; (C) F4/80-positive macrophages located as a ring around the center of the tumor (magnification: x 100).

IMMUNOHISTOCHEMICAL ANALYSIS OF THE EFFECT OF ENDOSTATIN ON RC-9

To study the effect of endostatin-treatment, we stained RC-9 tumors treated for 4 days with MP12 (PECAM-1/CD31), which recognizes endothelial cells and F4/80, which recognizes mature macrophages. Figure 2a shows an MP12 staining of an endostatin-treated tumor, in which MP12 stains the anatomical vessels. Figure 2b shows a F4/80 staining of an endostatin-treated tumor, in which F4/80 specifically stains tissue-macrophages. As can be seen in Figure 2a, 4-day endostatintreatment results in a necrotic center of the tumor without any vessel present (A), whereas the vessels in the outer rim of the tumor remain present after the

treatment (B). Between the necrotic center and outer rim of the tumor, the vessels show a loss of integrity as indicated by discontinuation in MP12 staining (C). Figure 2b shows that the necrotic center of the tumor (A) is separated from the viable outer rim of the tumor (B) by a small and distinct border containing a large quantity of tissue-macrophages (C).

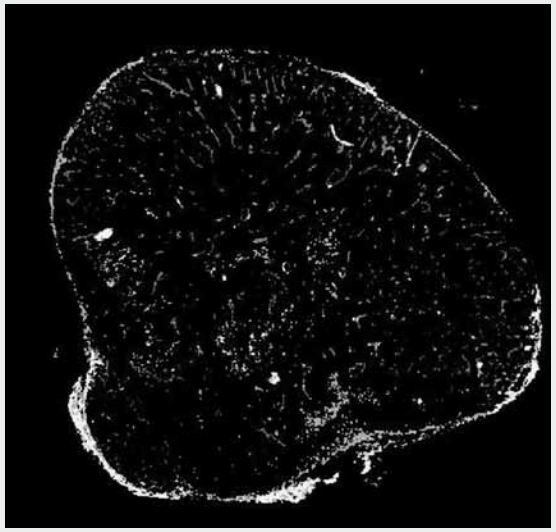

Figure 3 (a) Pimonidazole binding in a histological section of a control RC-9 tumor, as visualized by fluorescence microscopy. Hypoxic areas are indicated by green fluorescence light (magnification: x 40).

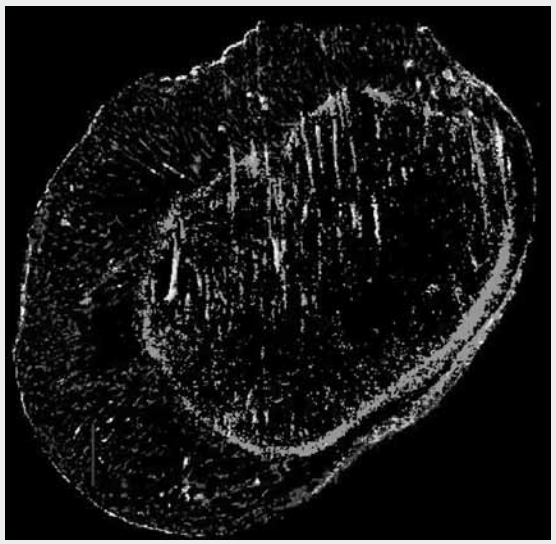

Figure 3 (b) Pimonidazole binding in a histological section of an endostatin-treated RC-9 tumor, as visualized by fluorescence microscopy. Hypoxic areas are indicated by green fluorescence light, (magnification: x 40).

CHARACTERIZATION OF HYPOXIA IN ENDOSTATIN-TREATED RC-9 TUMORS

In order to further characterize the effect of the endostatin-treatment on RC-9 tumors after 4 days, we used pimonidazole as a hypoxia marker. As can be seen in Figure 3a, the control RC-9 tumor shows little or no hypoxia, confirming a well-vascularized tumor. Figure 3b shows the endostatin-treated tumor. The center of the tumor has no functional vasculature and therefore, no pimonidazole-positive hypoxic area is observed. The distinct ring around the necrotic center, which contains macrophages and collapsing vessels, however, is strongly hypoxic. The outer ring of the tumor, with unaffected vessels, shows no hypoxia as visualized by the pimonidazole staining.

DIFFERENTIAL GENE EXPRESSION IN RC-9 AFTER ENDOSTATIN-TREATMENT

In order to examine as to which genes are specifically induced and suppressed after the endostatin treatment, we performed an SSH using mRNA from an endostatin-treated RC-9 tumor and from a control RC-9 tumor. After performing the SSH, the obtained sequences of the forward and reverse subtraction were checked for differential expression using Southern blotting and subsequential hybridization with tester cDNA (data not shown), before sequencing. Since RC-9 is a tumor of human origin, sequences found to be differentially expressed may be human or mouse, with human sequences being exclusively expressed by tumor cells, and mouse sequences by mouse derived tissue. Table 1a shows the genes found to be specifically induced and Table 1b shows the genes being specifically suppressed after endostatin-treatment in RC-9. In order to confirm differential expression, we examined the expression of an apoptosis-related gene, (calpain-2), which we found to be upregulated and a bone specific gene (cbfa1/osf2), which was unexpectedly found to be specifically suppressed after endostatin treatment. The expression of both genes was examined at protein level, by using immunohistochemical analysis.

Table 1 (a) Induced genes and (b) suppressed genes 4 days after endostatin-treatment of RC-9

Definition [a]	Frequency [b]	Organism [c]	E-value [d]	NCBI accession number [e]
(a) Induced genes				
Expressed only in placental villi, clone SMAP83	3	Homo sapiens	0	AB019568
Insulin-like growth factor binding protein-3	2	Homo sapiens	3E-65	X64875
Similar to glyceraldehyde-3-phosphate dehydrogenase	2	Homo sapiens	E-134	BC013310
Similar to deleted in polyposis 1, clone MGC:2267	1	Homo sapiens	0	BC000232
H2A histone family, member Z (H2AFZ)	1	Homo sapiens	E-174	XM_052419
Calpain 2, (m/II) large subunit (CAPN2)	1	Homo sapiens	0	XM_010682
a 6.2 kDa protein (LOC54543)	1	Homo sapiens	E-133	NM_019059
Stearoyl-coenzyme A desaturase 1	1	Mus musculus	8E-55	AK012370
(b) Suppressed genes				
Fibronectin	13	Homo sapiens	0	X02761
Chromosome 21q, section 76/105	2	Homo sapiens	E-149	AP001732
Chromosome 14 DNA sequence	2	Homo sapiens	2E-58	AL137230
BAC R-944C7 ofl ibrary RPCI-11 px19-like protein, clone MGC:15370	1	Homo sapiens	0	BC008866
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	1	Homo sapiens	E-139	BC003623
Calmodulin 2 (phosphorylase kinase, delta), clone MGC:2168	1	Homo sapiens	0	BC006464
Tubulin alpha 1, clone MGC:4760	1	Homo sapiens	E-157	BC009513
Guanine nucleotide binding protein (G protein), beta	1	Homo sapiens	0	BC000366
Clathrin assembly lymphoid-myeloid leukemia gene	1	Mus musculus	0	BC011470
Osteoblast-specific factor 2 (fasciclin I-like)	1	Mus musculus	0	NM_015784
10 days embryo cDNA, RIKEN full-length enriched library, clone:2610016K11	1	Mus musculus	E-117	AK011423

[a] Definition of the sequences, as found by comparing sequenced PCR products to available sequence databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).
[b] The frequency refers to the number of clones that showed homology with the identical gene found in the sequence database.
[c] As human xenografts were used in nude mice, sequences could be reduced to human or mouse origin.
[d] The value for the probability that the sequence match is found due to chance.
[e] NCBI accession numbers of the defined sequences, which can be used to retrieve gene definitions at http://www.ncbi.nih.gov in the nucleotidesection. All sequences shown in this table were tested for specificity using Southern blotting.

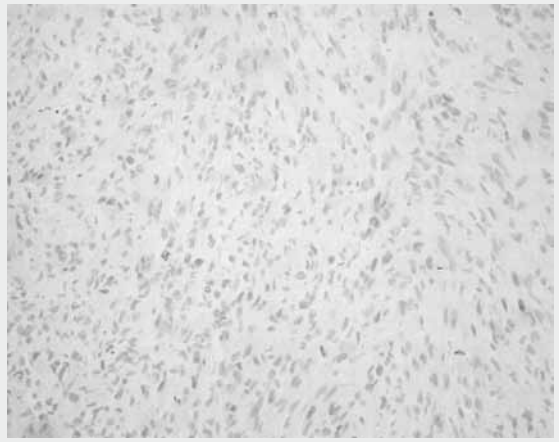

Figure 4 (a) Calpain-2-stained histological section of a control RC-9 tumor (magnification: x 200).

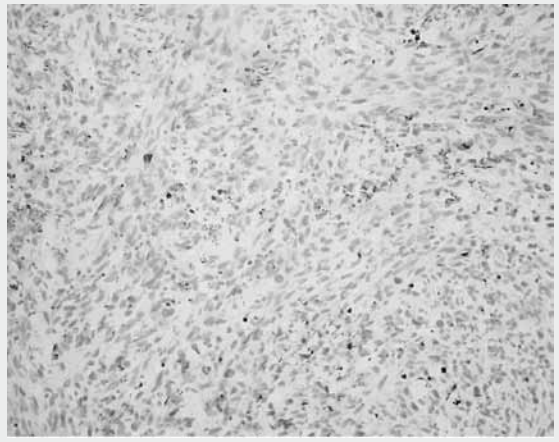

Figure 4 (b) Calpain-2-stained histological section of an endostatin-treated RC-9 tumor (magnification: x 200).

SCREENING OF DIFFERENTIAL EXPRESSION OF CALPAIN-2 IN RC-9

In order to verify the differential expression of calpain-2, we used several different control and endostatin-treated sections for immunohistochemical analysis. As can be seen in Figure 4a, control RC-9 tumors were negative for calpain-2, whereas sections of endostatin-treated RC-9 tumors stained positive for calpain-2 (Figure 4b). The positive expression was located to tumor cells.

SCREENING OF DIFFERENTIAL EXPRESSION OF CBFA1/OSF2 IN RC-9

In order to determine the expression pattern of cbfa1/osf2, which we found to be suppressed after endostatin-treatment, sections of different control RC-9 tumors and endostatin-treated tumors were used for immunohistochemical analysis. Human bone sections were used as a positive control (Figure 5a). In these sections, osteocytes (A) and bone-lining osteoblasts (B) show a positive nuclear staining, in agreement with previous reports.[34,35] The bone marrow (C) shows no staining. The expression pattern of cbfa1/osf2 was unexpected. Sections of control RC-9 tumors showed a positive nuclear staining of granulocytes (Figure 5b), whereas endostatin-treated tumors showed no staining (Figure 5c). Endostatin-treated RC-9 tumors did show to contain granulocytes, when sections were stained with a granulocyte-specific antibody (Figure 5d). As shown in Figure 6, the granulocytes we found to stain positive for cbfa1/osf2 included neutrophylic granulocytes (a), eosinophylic granulocytes (b) and basinophylic granulocytes (c).

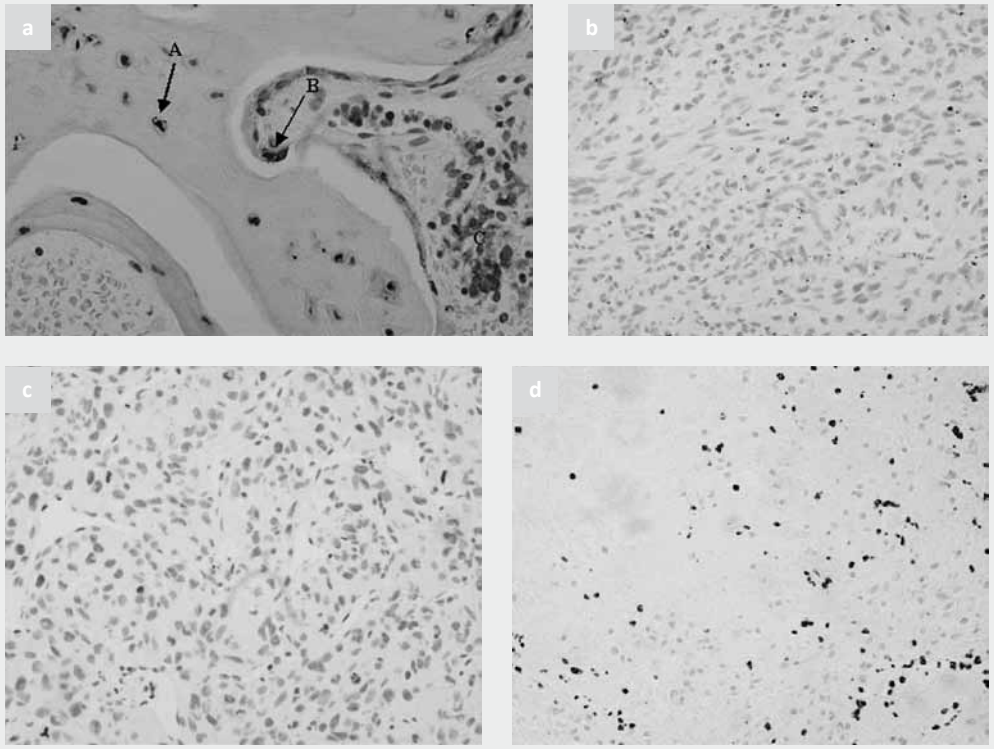

Figure 5 (a) cbfa1/osf-2-stained histological section of human bone tissue, with osteocytes (A) and bone-lining cells (B) staining positive and bone marrow (C), which is negative (magnification: x 400). **(b)** cbfa1/osf-2 stained histological sections of a control RC-9 tumor, with granulocytes staining positive (magnification: x 200). **(c)** cbfa1/osf-2-stained histological sections of an endostatin-treated RC-9 tumor staining negative (magnification: x 200). **(d)** Granulocytes stained with RB6-8C5 in an endostatin-treated tumor. Histological section of an endostatin-treated tumor stained with the RB6-8C5 antibody, which specifically recognizes granulocytes. Granulocytes stain positive, (magnification: x 200).

SCREENING OF DIFFERENTIAL EXPRESSION OF CBFA1/OSF2 IN MDA-MB231

In order to determine whether loss of cbfa1/osf2- positive granulocytes was restricted to endostatin treatment in the RC-9 tumor model, we stained histological sections of endostatin-treated MDA-MB231 tumors, which we found to be sensitive to endostatin-treatment as well.[29] As shown in Figure 7, cbfa1/osf2-positive granulocytes were present in control MDA-MB231 tumors (a) and were absent after endostatin-treatment (b).

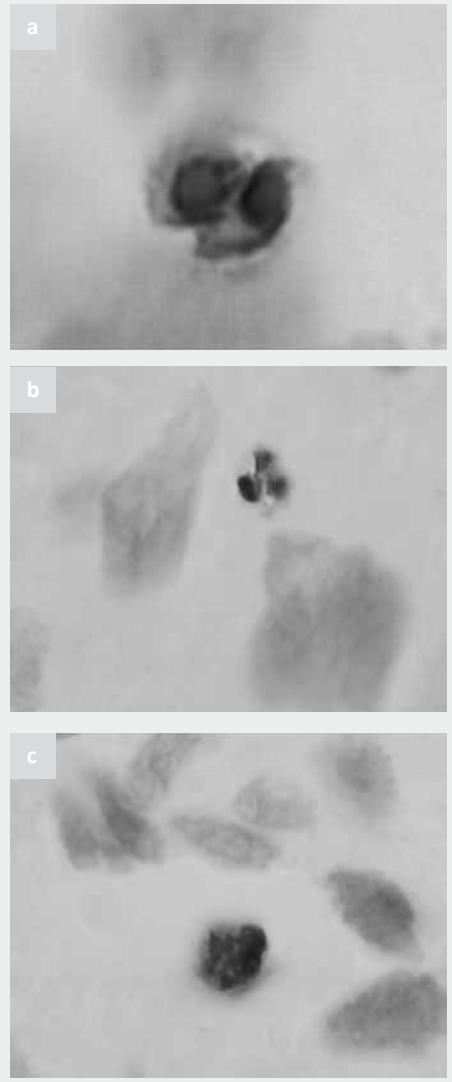

Figure 6 cbfa1/osf-2-stained histological section of a control tumor. Magnification of **(a)** a neutrophilic granulocyte **(b)** an eosinophylic granulocyte and **(c)** a basinophylic granulocyte (magnification: x 1000).

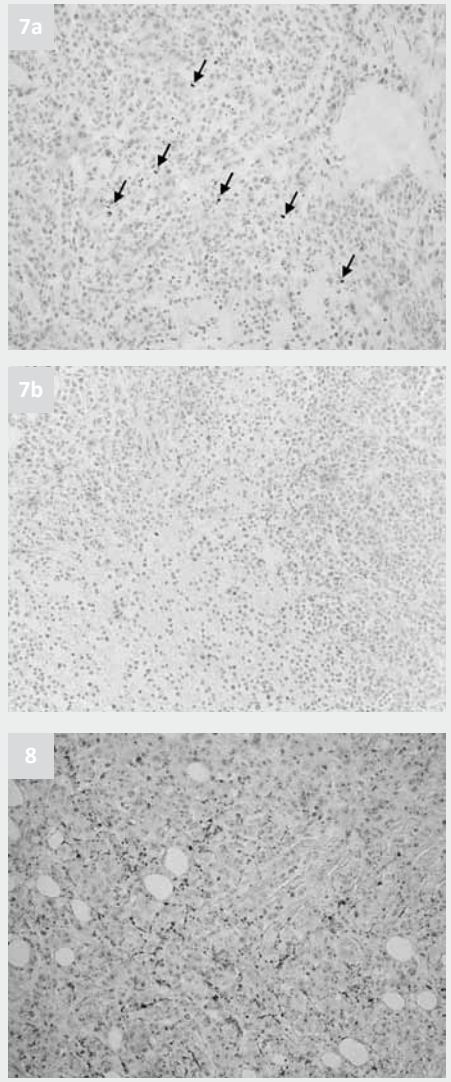

Figure 7 (a) cbfa1/osf-2-stained histological sections of a control MDA-MB231 tumor, with granulocytes staining positive (arrows indicate cbfa1/osf2-positive granulocytes) (magnification: x 200). **(b)** Cbfa1/osf-2 staining of an endostatin-treated MDA-MB231 tumor. Endostatin-treated MDA-MB231 tumor, stainingnegative **Figure 8** cbfa1/osf-2-stained histological section of human primary breast cancer (magnification: x 200).

EXPRESSION OF CBFA1/OSF2 IN SECTIONS OF HUMAN BREAST CANCER

In order to determine whether cbfa1/osf2-expression was limited to granulocytes found in the experimental RC-9 tumor model, we stained several sections of primary tumors and lymph node metastases of several patients suffering from breast cancer. We found expression of cbfa1/osf2 in granulocytes in both primary tumors (Figure 8), as well as in lymph node metastases. Positive granulocytes were found in five of the nine primary tumors and in four of the eleven lymph node metastases stained for cbfa1/osf2. Preliminary data show no significant correlation between the expression of cbfa1/osf2 by granulocytes in primary tumors or lymph node metastases and prognosis (data not shown).

Discussion

In this study, we have shown that endostatin treatment of human RC-9 xenografts in nude mice causes disintegration of blood vessels and subsequent tumor necrosis. Using the SSH-PCR combined with the MOS-technique, we found several genes to be specifically upregulated or suppressed by the endostatin-treatment. Among others, one gene, that was found to be specifically suppressed by endostatin, was cbfa1/osf2. Cbfa1/osf2 was specifically expressed in tumor-associated granulocytes.

Previous studies show that endostatin is an effective inhibitor of tumor growth in experimental tumor models.[6,7,9–14] In line with these studies, we have shown that endostatin inhibits the growth of human renal cell carcinoma RC-9 xenografts in nude mice. We show that the endostatin-treatment results in disintegrating blood vessels and hypoxia and anoxia, leading to tumor necrosis. This was indicated by a loss of nuclear staining of tumor cells in the center of the tumor. Furthermore, the center area of the tumor was surrounded by a distinct rim of tissue-macrophages, most likely present to remove dead tissue. These observations are in concordance with previous studies, which show that endostatin can selectively induce apoptosis of tumor endothelial cells *in vitro* and in animals, depriving the tumor of oxygen and nutrients and resulting in tumor cell death.[5,8,12,13]

Applying the SSH-PCR technique combined with the MOS-technique, we found eight genes to be specifically induced and 11 to be suppressed by endostatin-treatment. Calpain-2 (large subunit) was among the genes that were specifically induced by the endostatin-

treatment. Calpain-2 (or m-calpain) upregulation has been shown in response to a variety of apoptotic stimuli, in various cell types.[36–39] It has been shown, among others, that calpain-2 mediates the cleavage of Bax, a proapoptotic protein, during drug-induced apoptosis of HL-60 cells.[40] It has also been shown that calpain activation occurs after cleavage of caspase substrates and DNA fragmentation.[41] In our experimental conditions, the induction of calpain-2 is likely to be the result of the tumor cell apoptosis and death induced by the endostatin treatment. Fibronectin is an example of a gene that was specifically suppressed after the endostatin treatment.

Fibronectin is the ligand of $\alpha5\beta1$-integrin, which plays an important role in the anti-angiogenic effect of endostatin.[22] It has been shown that endostatin binds to $\alpha5\beta1$-integrin in an RGD motif dependent manner and competes for the RGD binding sites within fibronectin.[42] The mRNA levels of fibronectin have also shown to be significantly reduced in wounds of endostatin-treated mice[43] and in endostatin-treated endothelial cells.[44] This is in agreement with the specifically suppressed mRNA levels of fibronectin in our model. Taken together, these data are in favor of a reduced $\alpha5\beta1$-integrin interaction with fibronectin as a result of the endostatin-treatment, which may affect the migration and survival of endothelial cells and possibly the tumor cells as well.

A second gene that was specifically suppressed after endostatin-treatment, was cbfa1/osf2. cbfa1/osf2 (or Runx-2, AML-3, PEBP2α-A, or NMP-2) is known as a required transcriptional regulator of osteoblast differentiation and bone formation.[45–47] Therefore, our finding of cbfa1/osf2 expression in granulocytes in control RC-9 tumors was unexpected. Cbfa1/osf2 belongs to the family of mammalian Runt-domain containing factors, consisting of three characterized factors.[48] Like the two other family members, cbfa1/osf2 was first thought to play an important role in hematopoiesis. Surprizingly, few hematopoietic defects were seen in cbfa1/osf2 knockout mice. Mice embryos lacking cbfa1/osf2 had more granulocytes and fewer B cells than wildtype embryos.45,46 Cbfa1/osf2 knockout mice also demonstrated extramedullary hematopoiesis in the spleen and liver due to the congenital absence of bone marrow.[49] To our knowledge, this is the first report on expression of cbfa1/osf2 in granulocytes.

After endostatin-treatment, cbfa1/osf2-expressing granulocytes were no longer present in the tumor. Neutrophilic granulocytes have reported to be a target for the anti-angiogenic effect of angiostatin, another naturally occurring inhibitor of tumor angiogenesis.[50] Furthermore, it has been shown that neutrophilic granulocytes express $\alpha5\beta1$-integrins,

which can functionally bind to fibronectin.[51,52] It has been reported that endostatin can bind to α5β1-integrins[22] and we show here that fibronectin is specifically suppressed. Taken together, it is conceivable that the endostatin-treatment has an effect on granulocytes.

It is important to note that, although cbfa1/osf2- positive granulocytes were absent in the tumor after endostatin-treatment, granulocytes were still present. In addition, cbfa1/osf2 expressing granulocytes were not found in bone marrow of nude mice. Whether cbfa1/osf2 expression by granulocytes is restricted to a specific subset of tumor-associated granulocytes or granulocytes that are, for example, activated by certain inflammatory stimuli warrants further investigation.

In line with our findings in the RC-9 tumor model, cbfa1/osf2-positive granulocytes were present in non-treated MDA-MB231 tumors and were absent after endostatin treatment. This tumor model has previously been shown to be sensitive to endostatin treatment as well.[29] Taken together, this indicates that the loss of cbfa1/osf2-positive granulocytes might be a more common phenomenon in response to endostatin-treatment. Whether or not the absence of cbfa1/osf2-positive granulocytes is related to the efficacy of endostatin to inhibit tumor growth needs further investigation.

Also, we found expression of cbfa1/osf2 by granulocytes in histological sections of tumors of patients suffering from breast cancer. Cbfa1/osf2-positive granulocytes were both found in primary tumors as well as in lymph node metastases; however, preliminary data did not show a statistical significant correlation between cbfa1/osf2 expression in granulocytes and disease-free survival or prognosis. The finding of cbfa1/osf2-positive granulocytes in sections of patients suffering from mammary caricinoma shows that the results obtained are not restricted to experimental tumor models alone.

In summary, we have demonstrated that endostatin causes hypoxia and anoxia and tumor cell death in the mouse. Endostatin specifically induced and suppressed several genes. Among other genes, cbfa1/osf2 was specifically suppressed by endostatin. Unexpectedly, cbfa1/osf2 was expressed in granulocytes in the tumor. Since an effect of different anti-angiogenic therapy on granulocytes has been reported before, this might lead to new insights in the role of granulocytes in anti-angiogenic therapy in general. The mechanistic and functional role of cbfa1/osf2 in granulocytes warrants further investigation.

ACKNOWLEDGEMENTS

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Chapter 3

An *in vitro* model that can distinguish between effects on angiogenesis and on established vasculature: actions of TNP-470, marimastat and the tubulin-binding agent Ang-510

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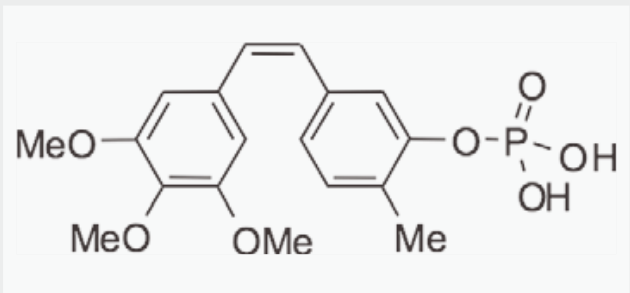
Abstract

In anti-cancer therapy, current investigations explore the possibility of two different strategies to target tumor vasculature; one aims at interfering with angiogenesis, the process involving the outgrowth of new blood vessels from pre-existing vessels, while the other directs at affecting the already established tumor vasculature. However, the majority of *in vitro* model systems currently available examine the process of angiogenesis, while the current focus in anti-vascular therapies moves towards exploring the benefit of targeting established vasculature as well. This urges the need for *in vitro* systems that are able to differentiate between the effects of compounds on angiogenesis as well as on established vasculature. To achieve this, we developed an *in vitro* model in which effects of compounds on different vascular targets can be studied specifically. Using this model, we examined the actions of the fumagillin derivate TNP- 470, the MMP-inhibitor marimastat and the recently developed tubulin-binding agent Ang-510. We show that TNP-470 and marimastat solely inhibited angiogenesis, whereas Ang-510 potently inhibited angiogenesis and caused massive disruption of newly established vasculature. We show that the use of this *in vitro* model allows for specific and efficient screening of the effects of compounds on different vascular targets, which may facilitate the identification of agents with potential clinical benefit. The indicated differences in the mode of action between marimastat, TNP-470 and Ang-510 to target vasculature are illustrative for this approach.

Introduction

A functioning and continuously expanding vascular network is essential for tumor development, growth, survival and metastasis. Given its pivotal role in these processes, tumor vasculature is a highly attractive target in anti-cancer therapy. Moreover, anti-vascular treatment may present with a low risk of developing drug resistance and promises to be effective against a broad spectrum of tumors[1,2]. Currently, two key approaches to target the tumor's blood vessel network have been developed[3,4]. One is directed at interfering with angiogenesis while the other aims to affect the already established tumor vasculature.

Angiogenesis is the process involving the outgrowth of new blood vessels from pre-existing vessels, and many compounds that affect tumor angiogenesis *in vitro* have been identified and are currently being investigated in clinical trials. Anti-angiogenic agents that have been tested interfere with different targets, such as angiogenic stimuli, receptor activity and endothelial cells[4-6]. The second approach aims at preferential targeting of the already established tumor vascular network and makes use of so-called vascular-disruptive agents (VDAs)[7-9]. All VDAs currently examined draw on the differences between tumor and healthy vasculature to allow for highly selective targeting of tumor blood vessels[10,11]. The VDAs can be divided into two categories: biologic and small-molecule agents (SMAs). Biologic agents include peptides and antibodies that deliver effectors to the tumor endothelium, where SMAs exploit the differences between healthy and tumor vasculature to induce selective vascular dysfunction[12-14]. Targeting angiogenesis and already established vasculature could both have their role in anti-cancer therapy. Where tumor angiogenesis is well suited for treating micrometastatic disease and early-stage cancer, disrupting established tumor vasculature



Figuur 1 Chemical structure of the tubulin-binding agent Ang-510.

leads to rapid vascular collapse, vessel congestion and tumor necrosis and is therefore more efficacious against large, already established tumors. Both approaches have shown promising results in ongoing preclinical studies, but treatments either targeting tumor angiogenesis or established tumor vasculature alone are not fully effective[10,15-19]. For this reason, current research explores the benefit of combining these anti-vascular treatment strategies[13,20-22].

When developing new anti-vascular compounds it would be of great benefit if one could determine if the overall anti-vascular action is mainly due to effects on inhibition of angiogenesis or to suppression of established vasculature or a combination of both. Therefore, in the present study, we developed an assay in which effects of substances on angiogenesis can easily be studied next to those on established vasculature in the same *in vitro* model. In order to validate this model system, we examined the actions of a number of different anti-vascular agents, among which a recently developed combretastatin like tubulin-binding agent Ang-510 (Fig. 1).

Materials and methods

CHEMICALS AND REAGENTS

Culture medium was α-MEM from Gibco-BRL, Breda, The Netherlands, supplemented with 10% FBS and penicillin/streptomycin. rhVEGF-A was from Oncogene, Sanbio, Uden, The Netherlands. ER-MP12 directed against murine PECAM-1 (CD31) was kindly provided by Dr. P. Leenen, Erasmus University, Rotterdam, The Netherlands. The MMP-inhibitor marimastat was kindly provided by Chiroscience Inc. (Cambridge, United Kingdom). TNP-470, a kind gift from W. Landuyt, University Hospital, K.U. Leuven, Belgium. The newly developed tubulin-binding agent Ang-510 was a kind gift from Graeme J. Dougherty and Peter D. Davis, Angiogene Pharmaceuticals Ltd. (United Kingdom).

IN VITRO VASCULARIZATION MODELS

In vitro angiogenesis was measured as outgrowth of endothelial capillary structures from cultures of 17-day-old fetal mouse metatarsal bone explants, as described previously[23]. In short, isolated metatarsals were cultured for 48 h in 24-well plates in 125 µl α-MEM medium to allow for attachment to bottom of the culture plate. Subsequently, medium was replaced by 500 µl fresh medium containing VEGF (50 ng/ ml) and the test substances and the medium was replaced every 3-4 days. After a total of 10 days of culture, the explants were fixed and stained for PECAM-1.

In the pre-culture experiments, the explants were treated for 24 h with the test substances, after attachment to the bottom of the plate, and were subsequently cultured for another 10 days in the presence of VEGF (50 ng/ml).

The area of PECAM-1-positive tubular structures was determined by image analysis using Image Pro Plus 3.0 for Windows 95/NT (Media Cybernetics, Carlsbad, CA). Images were obtained using a digital camera with a fixed window of 768 x 576 pixels. Data are depicted as number of pixels per area.

In vitro effects on newly established vasculature were examined in fetal mouse bone explant cultures that were first cultured for 10 days in the presence of VEGF (50 ng/ml) to stimulate capillary network formation. Subsequently, the medium was replaced with 500 µl fresh medium containing the test substances and were cultured for another 24 h after which they were fixed and stained for PECAM-1 and further analyzed as described above. After obtaining images for the quantification of PECAM-1 positive structures, the cultures were counterstained with Mayer's hematoxylin (H) for 30 s and eosin (1% in 96% ethanol) (E) for 90 s.

STATISTICS

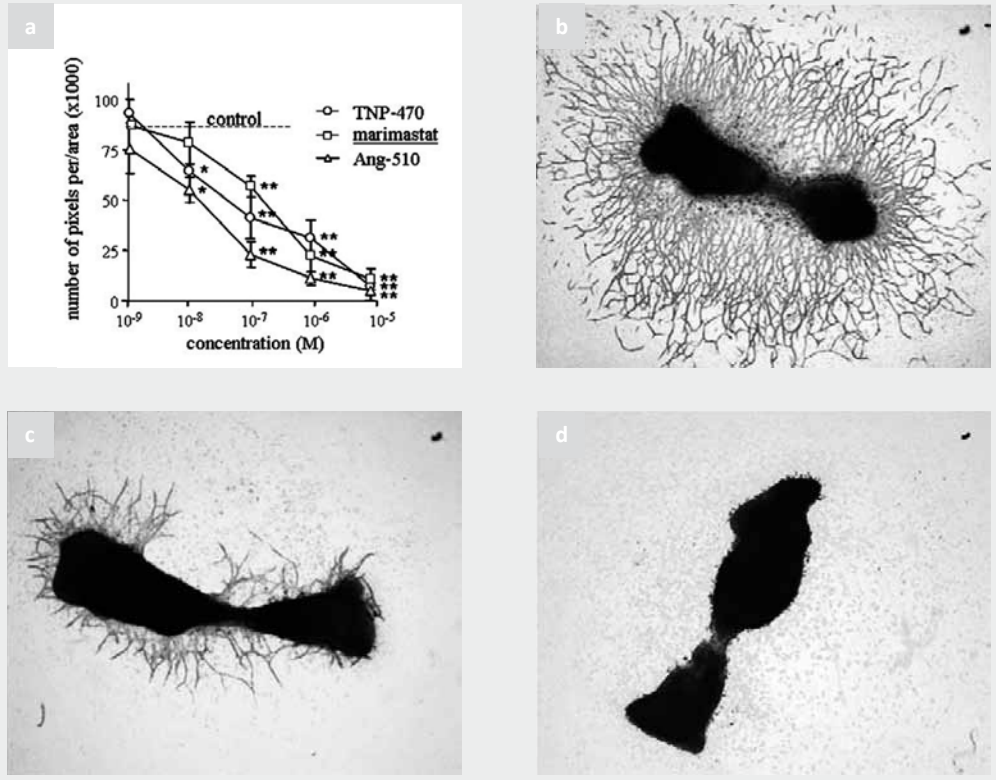
Results are depicted as mean value standard error of the mean (SEM). Differences between groups were determined by one-way analysis of variance for multiple comparisons followed by Fisher's LSD test.

Results

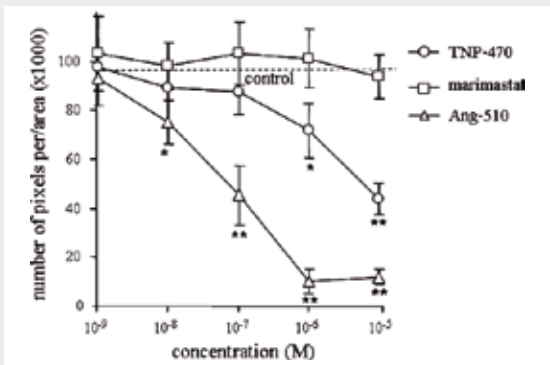
EFFECTS ON ANGIOGENESIS

Fig. 2A shows dose-inhibition curves of the effects of marimastat, TNP-470 and Ang-510 on VEGF (50 ng/ml) stimulated PECAM-1 positive capillary outgrowth from 17-day-old fetal mouse metatarsal bone organ cultures. VEGF-stimulated outgrowth was significantly and dose-dependently suppressed by marimastat, TNP-470 and Ang-510 with IC50s of approximately 0.6, 0.6 and 0.06 μM, respectively. The effects of these agents on endothelial outgrowth are further illustrated in Fig. 2B–D. Fig. 2B shows a large PECAM-1 positive endothelial network that has been formed after 10 days stimulation with VEGF. Fig. 2C and D show VEGF- stimulated cultures in the presence of 1 μM TNP-470 and 1 μM Ang-510, respectively. Both compounds inhibited the outgrowth of a capillary network, with Ang-510 being more potent than TNP-470. Explants cultured with 1 μM marimastat showed inhibition of vascular outgrowth, similar to those treated with TNP-470 (not shown).

In order to study the effect of the three agents in our anti-angiogenic model in more detail, we examined the effect of pre-treatment with these agents on subsequent VEGF-stimulated vascular outgrowth. At time of explantation, PECAM-1 positive endothelial precursor cells are located in the perichondrium of the explants, as previously shown[23]. From these precursor cells the capillary structures sprout and form the vascular network. To target these precursor cells, directly after adhesion to the culture plate, the fetal bone explants were pre-treated for 24 h with the different anti-vascular compounds and were than subsequently cultured for 10 days in the presence of VEGF. As shown in Fig. 3, pre-treatment with marimastat did not affect VEGF-stimulated capillary outgrowth, while both TNP-470 and Ang-510 significantly suppressed subsequent VEGF-stimulated outgrowth with IC50 values of approximately 0.7 and 0.08 μM, respectively.



Figuur 2 Effects on angiogenesis. **(a)** 17-day-old fetal mouse bone explants were stimulated for 10 days with VEGF (50 ng/ml) in the absence or presence of different concentrations marimastat, TNP-470 or Ang-510 (n = 6). Quantification of the number of PECAM-1 positive pixels per area is given as mean ± SEM. **p < 0.01 and *p < 0.05, compared to controls. **(b–d)** Endothelial outgrowth after stimulation with VEGF **(b)** and after simulation with VEGF together with TNP-470 (1 μM) **(c)** and together with Ang- 510 (1 μM) **(d)**.

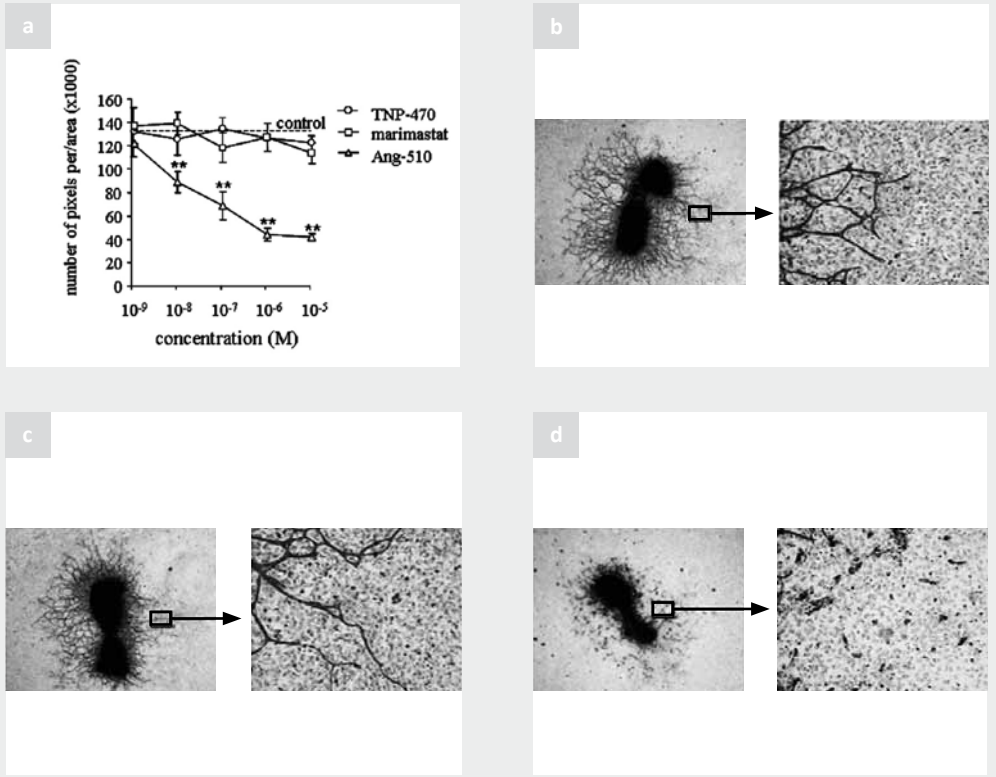


Figuur 3 Effects of pre-treatment on vascular outgrowth. 17-day-old fetal mouse bone explants were, directly after adhesion to the culture plate, cultured for 24 h with different concentrations of marimastat, TNP-470 or Ang-510 and subsequently stimulated for another 10 days with VEGF (50 ng/ml) (n = 6). Quantification of the number of PECAM-1 positive pixels per area is given as mean ± SEM. **p < 0.01 and *p < 0.05, compared to controls.

EFFECTS ON NEWLY ESTABLISHED VASCULATURE

To study the effects of the three compounds on newly established capillaries, first endothelial outgrowth was stimulated with VEGF (50 ng/ml) for 10 days and subsequently the cultures were treated for 24 h with the different anti-vascular agents. As shown in Fig. 4A, marimastat and TNP-470 did not affect the VEGF-stimulated newly formed vasculature while Ang-510 showed a significant suppression of newly established vasculature with an IC50 of around 0.01 μM.

Fig. 4B shows control capillary outgrowth after 10 days stimulation with VEGF, stained for PECAM-1 and counter stained with HE. Fig. 4C and D depicts newly formed vasculature after subsequent 24 h treatment with TNP-470 (10 μM) and Ang-510 (1 μM). As shown, TNP-470 did not affect the established capillary network (similar results were obtained with marimastat (10 μM) (not shown)). In contrast, 24 h treatment with Ang-510 caused a significant disintegration of the newly established capillary structures, with only fragments of the original network remaining. Histological HE staining revealed that this degenerative effect was specific for the capillary network, as the layer of fibroblastic cells, originating from the periosteum on which the capillary network grows and expands[23], remained morphologically fully intact.



Figuur 4 Effects on newly established vasculature. **(a)** 17-day-old fetal mouse bone explants were stimulated for 10 days with VEGF (50 ng/ml) followed by 24 h treatment with different concentrations marimastat, TNP-470 or Ang-510 (n = 6) Quantification of the number of PECAM-1 positive pixels per area is given as mean ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$, compared to controls. After culture, bone explant capillary outgrowth was visualized by staining for PECAM-1 in combination with HE. **(b–d)** The combined PECAM- 1 and HE staining are shown for explants stimulated for 10 days with VEGF without subsequent treatment (control) **(b)**, and for explants stimulated with VEGF with subsequent 24 h treatment with TNP-470 (10 μM) **(c)** or Ang-510 (1 μM) **(d)**.

Discussion

In this study, we developed an *in vitro* model that can distinguish between effects of compounds on angiogenesis and on newly established vasculature. We examined the effects of three anti-vascular agents, among which the recently developed tubulin-binding agent Ang-510. We showed that this compound effectively interfered with both angiogenesis as well as newly established vasculature, whereas the synthetic fumagillin derivate TNP-470 and the MMP-inhibitor marimastat selectively affected angiogenesis alone.

Angiogenesis is the process of generating new blood vessels from pre-existing vasculature, which is indispensable for solid tumor growth and metastasis. As such, targeting tumor angiogenesis, in anti-cancer therapy, is an intense field of interest. Current investigations towards the development of agents that inhibit tumor vascularization, however, not only focus on interference with the process of angiogenesis, but also on intervention with already established tumor vasculature[3,4]. Compounds that belong to this group are called vascular-disrupting agents (VDAs); agents that selectively target tumor vasculature on basis of structural and functional abnormalities of these vessels [7-9]. In the development of new and more effective anti-vascular agents, it is of importance to have model systems available that can give accurate information about their mode of action and vascular targets involved and thus can differentiate between effects on angiogenesis or newly established vasculature. For this, we adapted our previously developed angiogenesis assay consisting of the outgrowth of capillaries from cultured fetal mouse metatarsals and suited it to study effects on established vasculature as well[23]. To validate this *in vitro* model, we examined the effects of three different compounds in several experimental settings.

We examined the anti-angiogenic actions of two well-known inhibitors of angiogenesis, marimastat and TNP-470, respectively, and that of the newly developed tubulin-binding agent Ang-510. In previous studies, it has been shown that both the synthetic MMP-inhibitor marimastat, as well as the synthetic fumagillin derivate TNP-470 possess strong anti-angiogenic properties in various *in vitro* models by interfering with endothelial cell invasion and proliferation[24-28]. In concordance with these observations, in our model, marimastat and TNP-470 potently and dose-dependently inhibited angiogenesis, indicated by suppressed outgrowth of PECAM-1 positive capillaries. Moreover, the newly developed tubulin-binding agent Ang-510 also showed strong anti-angiogenic properties in our model system.

Previously, we have shown that PECAM-1 positive endothelial precursor cells are present in the perichondrium of the bone explants, before the outgrowth of vasculature[23]. In order to determine whether the observed anti-angiogenic effects might involve a direct action on these early-stage endothelial precursor cells from which the capillaries are formed, we pre-incubated the bone explants with the different agents for 24 h and subsequently cultured them for 10 days in the presence of VEGF. After pre-treatment of the bone explants with marimastat, at doses that actively suppressed angiogenesis, there was no effect on the subsequent outgrowth of vasculature. Recent studies have shown that MMP- inhibitors such as marimastat inhibit angiogenesis by blocking the invasion and migration of endothelial cells into the extracellular matrix[24,29,30], which might explain why in our model, marimastat does not have a direct effect on endothelial precursors and their subsequent vascular outgrowth after pre-treatment, but strongly inhibits angiogenesis when it is continuously present. In line with this, it was previously shown that in a three-dimensional rat aortic model, marimastat potently inhibited angiogenesis, without affecting the proliferation of rat aortic endothelial cells in monolayer cultures[24].

In contrast to marimastat, TNP-470, and even more potently Ang-510, inhibited vascular outgrowth after 24 h pre-treatment of the bone explants. TNP-470 is a known angiogenesis inhibitor, which has been shown to induce a cell cycle arrest in the G1-phase, resulting in inhibition of endothelial cell proliferation and network formation, indicating that this compound acts via a cytostatic rather than a cytotoxic mode of action[25-28]. However, in our model, at higher concentrations, inhibition of outgrowth of vasculature by TNP-470 was not reversible after stimulation with VEGF, suggesting that, at these doses, the mode of action is cytotoxic and not cytostatic. Interestingly, a similar dual mode of action of TNP- 470 has been described on the *in vitro* growth of human umbilical vein endothelial cells (HUVECs), showing cytostatic inhibition at lower doses and a cytotoxic suppression at higher doses[31]. Furthermore, we found that Ang-510 strongly inhibited the outgrowth of capillaries after pre-treatment of the bone explants, suggesting that this agent possess an irreversible cytotoxic mode of action on endothelial precursor cells. This observation is in line with findings of Ahmed et al. and Iyer et al. who showed that the combretastatin analog A4 phosphate (CA4P) was cytotoxic to proliferating HUVECs[6,32].

Finally, we studied the effects of the three compounds on newly established vasculature. In contrast to their actions on angiogenesis, marimastat and TNP-470 did not affect newly established capillaries. *In vitro* studies exploring the effects on established vasculature are very rare, however, in one study, using cultures of rat aorta, marimastat showed to stabilize rather than to inhibit existing microvessels and to prevent their regression, resulting in the prolonged survival of microvascular networks[33]. To date, no studies on the effects of TNP-470 on established vasculature have been published. However, our observations, that this agent has no effect on established vasculature may be perceivable, since TNP-470 has been shown to act on endothelial cells via a cytostatic action through suppression of the cell cycle[28]. Furthermore, as expected, next to its strong inhibitory effects on angiogenesis and capillary outgrowth after pre-treatment of bone explants, Ang-510 showed to have a marked disintegrative effect on newly established vasculature. This damaging effect of Ang-510 was most likely specific for endothelial cells, as concomitant HE staining revealed that the underlying layer of fibroblastic cells remained unaffected. In line with our findings, the VDA CA4P showed *in vitro* and *in vivo* rapid disruption of the tubulin cytoskeleton and changes in the three-dimensional shape of proliferating endothelial cells[34-36].

In conclusion, the current search for more specific and more active VDAs is hampered by a lack of *in vitro* models that can accurately distinguish between effects on angiogenesis and on established vasculature, urging the need for models which can specifically differentiate between the two. The overlap in action of VDAs on angiogenesis and on newly established vasculature illustrates the usefulness of this *in vitro* model, which is able to differentially recognize effects on both vascular targets. This *in vitro* model provides an efficient and rapid way to screen for biological activity of anti-vascular compounds, which could prove of great benefit in the field of vascular research. Moreover, the ability to make a clear distinction between different vascular targets may facilitate the identification of pharmacological compounds with potential clinical benefit. The indicated differences between marimastat, TNP-470 and Ang-510 in targeting vascular networks are illustrative for this approach.

ACKNOWLEDGMENTS

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Chapter 4

Celecoxib enhances doxorubicin-induced cytotoxicity in MDA-MB231 cells by NF-kappaB-mediated increase of intracellular doxorubicin accumulation

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) and cyclo-oxygenase (COX) inhibitors are anti-inflammatory agents that have also shown to be useful in anti-cancer therapy. In the present study, we show that the specific COX-2 inhibitor celecoxib enhances the inhibitory effect of doxorubicin (dox) on human MDA-MB231 breast tumor growth *in vivo* and *in vitro*. We also found that celecoxib increased the intracellular accumulation and retention of dox *in vitro*. Since the NSAID indomethacin and the specific COX-2 inhibitor NS398 did not affect the *in vitro* actions of dox, these effects are likely to be mediated via a COX-independent mechanism. It has been suggested that some COX-inhibitors can enhance the actions of cytostatics by overcoming multidrug resistance through the inhibition of ABC-transporter proteins. However, we found that the three main ATP-binding cassette (ABC)-transporter proteins, implicated in dox transport, were inactive in MDA-MB231 cells. Therefore, the finding that the P-glycoprotein (P-gp) blocker PSC833 also increased cellular accumulation of dox was unexpected. In order to unravel the molecular mechanisms involved in dox accumulation, we examined the involvement of NF-κB, as this transcription factor has been implicated in celecoxib action as well as in chemoresistance. We found that celecoxib and PSC833, but not indomethacin or NS398, almost completely inhibited basal- and dox induced NF-κB gene-reporter activity and p65 subunit nuclear translocation. Furthermore, the NF-κB inhibitor PDTC mimicked the actions of celecoxib and PSC833 on cell growth and on intracellular accumulation of dox, suggesting that NF-κB is functionally involved in the actions of these compounds. In conclusion, we show that structurally different compounds, among which are celecoxib and PSC833, increase the intracellular accumulation of dox and enhance dox induced cytotoxicity in MDA-MB231 breast cancer cells most likely via the modulation of NF-κB activity.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) and specific cyclo-oxygenase (COX)-2 inhibitors are widely used in the treatment of pain and rheumatoid arthritis and have shown promising results in the treatment of cancer in experimental and clinical studies.[1,2] COX-2 is overexpressed in many malignancies and is involved in tumor development and growth. The effects of NSAIDs and specific COX-2 inhibitors on tumor cells include inhibition of cell proliferation, induction of apoptosis and reduction of cell motility and adhesion.[2–10] Furthermore, both non-specific and specific COX-2 inhibitors have shown to significantly inhibit tumor angiogenesis.[11–14] These anti-cancer properties make it worthwhile examining the possible benefit of combining NSAIDs and COX-2 inhibitors with conventional anti-cancer therapies, such as chemotherapy.

Several preclinical and clinical studies have explored and are currently exploring the therapeutic benefit of combining NSAIDs and specific COX-2 inhibitors with chemotherapeutics and have been shown to improve treatment outcome. For example, in experimental and clinical studies, the COX-2 inhibitor celecoxib has shown to enhance the anti-tumor efficacy of several cytostatics, such as that of irinotecan, doxorubin (dox), bleomycin and 5-fluorouracil.[15–18] The mechanism by which COX-2 inhibitors enhance the action of cytostatics is, however, not clear and it is suggested that this may involve mechanisms other than suppression of the COX-2 enzyme. For example, it has been proposed that COX-inhibitors modulate the resistance of tumors to chemotherapeutic drugs by affecting the activity of plasma membrane transporter proteins of the ABC-transporter family, which behave as energy-dependent efflux pumps for cytostatics. The three key mammalian transporters involved in the transport of anti-cancer agents, such as the anthracyclines, are P-glycoprotein (P-gp/ABCB1), multidrug-resistance protein-1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/MXR/ABCG2).[19,20]

In recent years, much effort has been made to identify agents that are able to overcome MDR, in order to improve chemotherapeutic treatment. These agents, called chemosensitisers, belong to a variety of structural classes, such as calcium channel blockers, drug analogues, cyclic peptides and steroids.[21,22] It has been suggested that COX-inhibitors may also act as chemosensitisers and can overcome MDR by inhibiting P-gp[15] or MRPs.[23–26] However, conclusive evidence for the actions of NSAIDs and specific COX-2 inhibitors on these

transporters is lacking. Interestingly, apart from MDR, NF-κB has recently been described as another COX independent molecular target for actions of NSAIDs and COX-2 inhibitors, such as aspirin, indomethacin and celecoxib.[27-30] Moreover, NF-κB has also shown to be involved in chemoresistance in different cancer types[31-36], suggesting a possible role of this transcription factor in the chemosensitising effect of COX-inhibitors.

In the present study, we addressed these issues both in vivo and *in vitro* by studying the effects of NSAIDs, specific COX-2 inhibitors such as celecoxib and specific pump inhibitors in combination with dox in the breast cancer cell line MDA-MB231.

Materials and methods

CELL LINES, CHEMICALS AND REAGENTS

The human mammary carcinoma cell line MDA-MB231 was from the American Type Culture Collection (Rockville, MD) and was cultured in Dulbecco's modified Eagle's medium (Biochrom, Basel, Switzerland) and 10% FCS (p/s, Life Technologies, Breda, the Netherlands). Dox was from Pharmacia B.V., Woerden, The Netherlands. Indomethacin was from Bufa B.V., Uitgeest, The Netherlands; Sc-236, NS-398 and celecoxib were from Pharmacia, Skokie, USA. PSC833 was from Novartis, Basel, Switzerland. Probenecid was from Sigma, St. Louis, MO, USA. Ko143 (fumitremorgan C analogue) was a kind gift from A. van Loevezijn, Laboratory of Organic Chemistry, University of Amsterdam (Amsterdam, The Netherlands). The fluorescent pump substrates, Syto16 (for Pgp), calcein acetoxymethylester (for MRP1) and Bodipy-prazosin (for BCRP), were from Molecular Probes, Eugene, OR, USA, and actionomycin D (7-AAD) was from Pharmingen, San Diego, CA, USA. For transfections, Fugene 6 transfection reagent was from Roche, Basel, Switzerland, and the NF-κB reporter construct NF-κB-luc was from Stratagene, Amsterdam, The Netherlands, and Renilla luciferase (pRL-SV40) was from Promega, Madison, WI, USA. The NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC) was from Sigma, Zwijndrecht, The Netherlands.

ANIMAL STUDY

Female BALB/c nu/nu mice were from Iffa Credo (L'Arbresle, France) and were housed in individual ventilated cages under sterile conditions according to the Swiss guidelines for the care and use of laboratory animals. Sterile food and water were provided ad libitum. At start of the experiment, the mice were ≈10 weeks old. MDA-MB231 cells (2 x 10^6 cells/100 µl) were injected sc. in the left flank. After 2 weeks, animals containing a tumor with a volume (TV) of 100 ± 20 mm^3 were selected and divided into four groups of eight mice each. TV was assessed by using a calliper measuring the two major diameters by the formula $TV = \pi/6\ (d1 \times d2)^{1/2}$. Mice (8 per experimental group, n = 8) were treated with vehicle (DMSO), celecoxib (15 mg kg^{-1}), dox (0.5 mg kg^{-1}) or a combination of celecoxib and dox. All agents were injected i.p. in a total volume of 125 µl. The mice were treated every other day for 30 days. TV and body weight were measured twice a week.

MTS ASSAY

Cell proliferation was assessed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulphophenyl)-2H-tetrazolium) tetrazolium assay (Cell Titer96 Aqueous, Promega). For this, 2 x 10^3 cells per well were seeded in triplicate in 90 µl culture medium in 96-well flat-bottom microculture plates and one day later the additives were added. After 4 d of culture, viable cells were determined by adding 20 µl MTS to each well and measuring OD 490 (Thermomax, Molecular Devices) after 2 h incubation. The results were expressed as the mean optical density (OD) of each 3-well set.

DOX ACCUMULATION ASSAY

Cells (3 x 10^4) were seeded in 4-well Lab-Tek® glass Chamber Slides (Nalge Nunc International Corp. Naperville, IL) and incubated overnight after which additives were added for 24 h. Subsequently, the culture medium was removed and cells were washed three times with PBS. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS and mounted under glass coverslips with Vectashield (Brunschwig, Amsterdam, The Netherlands). The cells were examined for doxorubicin fluorescence using fluorescence

microscopy. Fluorescence was excited at 465–495 nm and detected at an emission maximum of 515–555 nm. Intracellular doxorubicin was quantified using computerised image analysis. Images of control and treated cultures were acquired using a colour CCD camera, digitised with a Matrix meteor frame grabber, filtered and analysed with Image Pro Plus 3.0. Cells (20) were measured and values were expressed as mean light intensity per cell ± SD, corrected for background.

FACS ANALYSIS

P-gp, MRP and BCRP activities were measured using a green fluorescent probe assay with specific substrate/modulator combinations as previously described.[20,37,38] In short, 3 x 10^5 cells/ml were incubated in DMEM at 37°C for 45–60 min with either: (i) Syto16 (0.6 nM) in the presence or absence of PSC833 (2 μM) for P-gp activity, (ii) calcein acetoxymethylester (1 nM) in the presence or absence of probenecid (3 mM) for MRP1 activity, or (iii) with Bodipy-prazosin (25 nM) in the presence of PSC-833 (2 μM) with or without Ko143 (200 nM) for BCRP activity. Subsequently, they were incubated with 7-amino actinomycin D and flow cytometric analysis was performed on a FACScalibur (Becton Dickinson, San José , CA). P-gp, MRP and BCRP activities were expressed as ratio of substrate fluorescence with modulator present and substrate fluorescence without modulator present after subtraction of the fluorescence of the control (cells in accumulation medium alone). Since these functional assays are highly sensitive, the ratios below 1.5–2.0 in cell lines are thought to have little practical meaning in terms of drug resistance.[37,38]

NF-κB REPORTER ASSAY

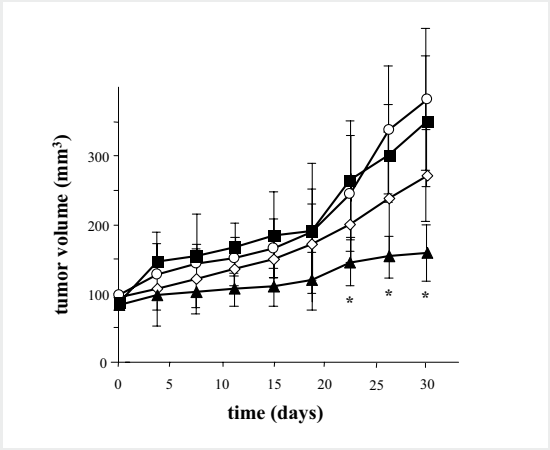
MDA-MB231 cells were seeded at a density of 1.25 x 10^4 cells/well in 24-well plates, and transiently transfected with 1 μg of the reporter construct using Fugene 6 transfection reagent. The NF-κB reporter construct NF-κB-luc was used. To correct for transfection efficiency, 100 ng of Renilla luciferase was co-transfected. 24 h after transfection, test substances were added to the cells for another 24 h. Hereafter, luciferase assays were performed with the Dual-Luciferase Reporter assay system (Promega) according to the protocol. Cell lysate (5 μl) was first assayed for firefly luciferase and then for Renilla luciferase activity, using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin–Elmer, Boston, MA, USA). Firefly luciferase activity was corrected for Renilla luciferase activity.

WESTERN BLOT ANALYSIS

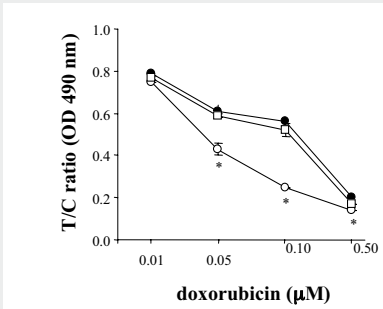
In all, 10 μg of cytoplasmatic or nuclear protein was prepared from cells treated under different conditions, separated on 8% SDS–PAGE and transferred to polyvinylidene difluoride membrane by wet blotting. The membrane was blocked for 1 h at ambient temperature with 2% milk powder in PBST (PBS containing 0.1% (v/v) Tween-20), followed by primary antibody incubations (anti-p65 and anti-p50, Santa Cruz Biotechnology, Heerhugowaard, The Netherlands) overnight at 4 °C in 0.2% milk powder in PBST. The membrane was washed and primary antibodies were detected with rabbit anti-goat IgG conjugated to horseradish peroxidase and the bands were visualised with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, England). Hereafter, blots were stripped using blot restore membrane rejuvenation kit (Chemicon, Hampshire, UK) according to the manufacturer's instructions. Hereafter, protein samples were checked for nuclear and cytoplasmatic specificity using anti-Lamin A (nuclear membrane structural component, Cell Signaling Technology, USA) and anti-protein disulphide isomerase (PDI, endoplasmatic reticulum protein, Stressgen Biotechnologies, Vic., Canada) antibodies.

STATISTICAL ANALYSIS

The data are expressed as means ± SD for comparing treated groups with control groups, a Student's t-test was used. A P-value of <0.05 was considered significant.



Figuur 1 Effects of celecoxib and doxorubicin, alone and in combination on the growth of MDA-MB231 xenografts in nude mice. Vehicle (DMSO) (filled squares); celecoxib (15 mg kg^{-1}) (open circles); dox (0.5 mg kg^{-1}) (open diamonds) and celecoxib + dox (filled triangles). Values represent mean tumor volume. *P < 0.05 (treatment versus control).

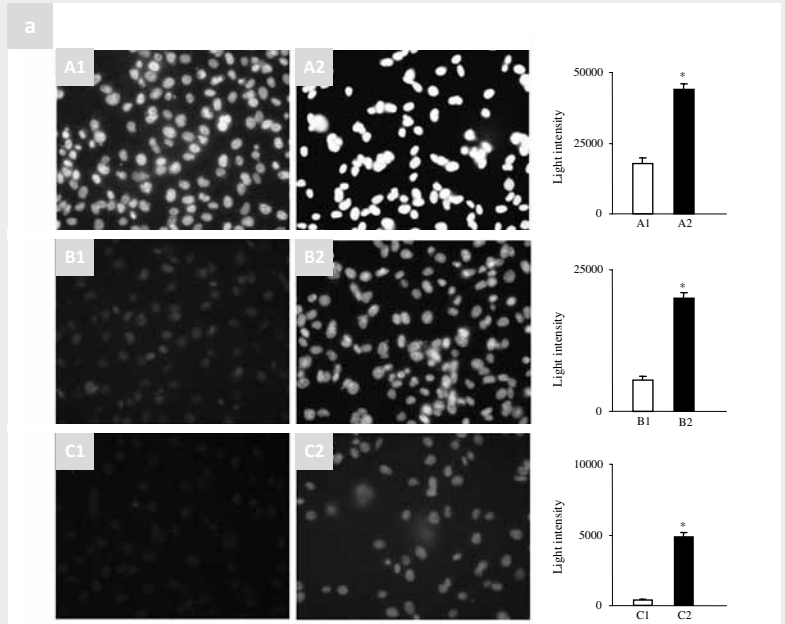


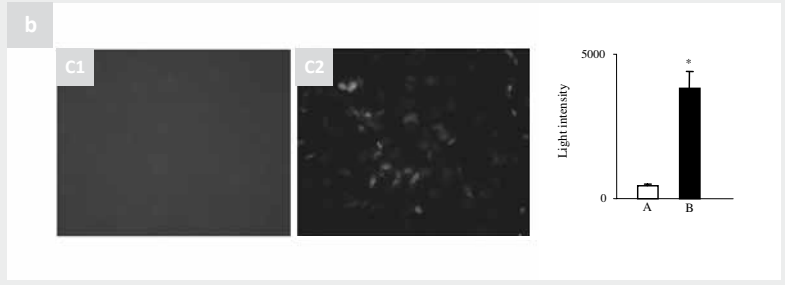
Figuur 2 Effect of dox alone, or in combination with celecoxib or indomethacin, on *in vitro* MDA-MB231 proliferation (OD 490). Dox (0.01–0.5 μM) (filled circles); Dox + celecoxib (10 μM) (open circles) and Dox + indomethacin (10 μM) (open squares). Results are expressed as treatment versus control ratio (T/C-ratio); mean ± SD *P < 0.05 (dox versus dox + celecoxib or versus dox + indomethacin).

Results

MDA-MB231 TUMOR GROWTH *IN VIVO*

Fig. 1 shows the effects of daily treatment with celecoxib (15 mg kg^{-1}) and dox (0.5 mg kg^{-1}) alone or in combination, on the subcutaneous growth of MDA-MB231 human mammary carcinoma xeno-transplants in nude mice. After 23 days of treatment, the average tumor volume of the mice treated with the combination of dox and celecoxib was, compared to controls, significantly suppressed (P < 0.05). Treatment with dox or celecoxib as single agents, with these doses, did not significantly affect tumor growth.





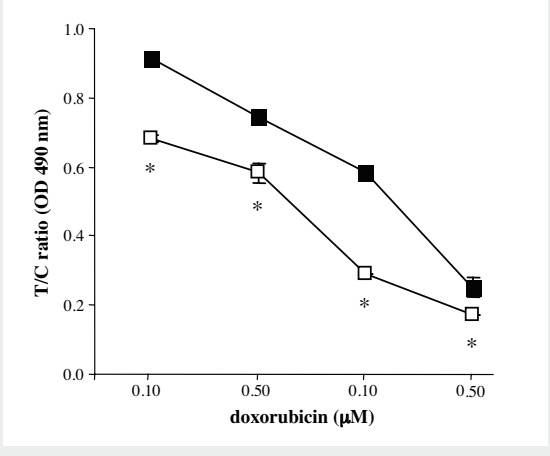
Figuur 3 Effect of celecoxib on the intracellular accumulation of doxorubicin. **(a)** Shows fluorescent microscopic pictures of MDA-MB231 cells that were incubated for 24 h in a medium containing dox (5 μM) **(A1)**; (1 μM) **(B1)** or (0.5 μM) **(C1)**, or these concentrations of dox in combination with celecoxib (50 μM) **(A2; B2; C2)**, respectively. Magnification: 200x. **(b)** Shows cells treated with dox (1 μM) for 24 h, followed by 24 h incubation in control medium **(A)** or medium containing celecoxib (50 μM) **(B)**. Magnification: 200x. The bar graphs show, for all experimental conditions, the mean light intensity per cell, measured by computerised image analysis, expressed as mean ± SD *P < 0.001.

MDA-MB231 CELL PROLIFERATION *IN VITRO*

Fig. 2 shows growth inhibition curves of MDA-MB231 cells by dox in the absence or presence of celecoxib (10 μM) or indomethacin (10 μM). Dox inhibited cell proliferation dose-dependently with an EC50 of around 0.2 μM. Co-treatment of the cells with celecoxib, which alone had no effect on cell growth, decreased the EC50 to 0.06 μM. In contrast, indomethacin did not affect the action of dox, and the same was true for the specific COX-2 inhibitor NS398 (10 μM) (not shown). Moreover, the specific COX-2 inhibitor Sc236 (10 μM), like celecoxib, enhanced the effect of dox, decreasing the EC50 from 0.2 to 0.08 μM (not shown).

INTRACELLULAR ACCUMULATION AND RETENTION OF DOX

Doxorubicin is an auto-fluorescent compound, which enables the visualisation of its intracellular presence by fluorescence microscopy. Fig. 3a shows the fluorescence of intracellular accumulated dox in MDA-MB231 cells after incubation for 24 h with different concentrations of dox (0.5, 1 and 5 μM) in the absence (A1, B1 and C1) or presence of celecoxib (50 μM) (A2, B2, and C2). As shown in the pictures and in the graphs, the intracellular fluorescence, which is mainly visible in the nuclei, is at all concentrations significantly ($P < 0.001$) higher in cells that were co-treated with celecoxib. Like celecoxib, the specific COX-2 inhibitor Sc236 (50 μM) also increased the cellular accumulation of dox (not shown). In addition, NS398 (50 μM) and indomethacin (50 μM) did not affect dox accumulation (not shown). Fig. 3b shows MDA-MB231 cells that were incubated for 24 h with dox (1 μM) followed by incubation for another 24 h in control medium (A) or in medium containing celecoxib (50 μM) (B), respectively. As shown in the pictures and the graphs, cells treated with celecoxib contained significantly ($P < 0.001$) more dox than those that were incubated in control medium during the second 24 h culture period.



Figuur 4 Effect of dox, alone or in combination with PSC833, on *in vitro* MDA-MB231 proliferation (OD 490). Dox (0.01-0.5 μM) (filled squares) or Dox + PSC833 (10 μM) (open squares). Results are expressed as treatment vs. control ratio (T/C-ratio); mean ± SD *p<0.05 (dox vs. dox + PSC833).

EFFECTS OF ABC-TRANSPORTER BLOCKERS ON DOX-SUPPRESSED CELL GROWTH AND ON INTRACELLULAR DOX ACCUMULATION

Dox is a substrate for proteins of the ABC-transporter protein family that enhance the efflux of cytostatics. Since our results would suggest that celecoxib and Sc236 modulate the cellular actions of dox via inhibition of these transporters, we examined their possible involvement on intracellular accumulation of dox and induction of cytotoxicity in MDA-MB231 cells using specific blockers.

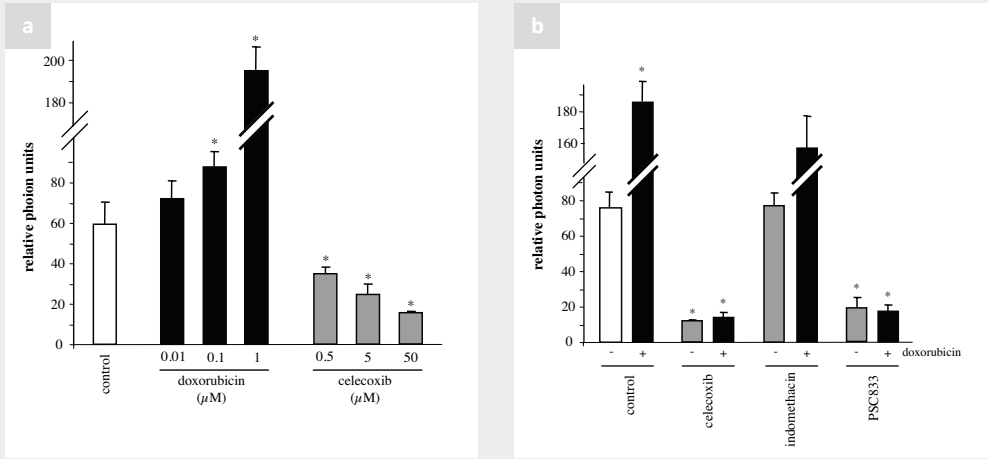
Fig. 4 shows a dose–inhibition curve of dox in the absence or presence of the P-gp-blocker PSC833 (10 μM) on MDA- MB231 growth. PSC833 significantly enhanced the effect of dox on cell growth, decreasing the EC50 from 0.2 μM to 0.08 μM. Moreover, PSC833 (50 μM) markedly increased the intracellular accumulation of dox (not shown). The MRP- blocker probenecid (0.1–5 mM) and the BCRP-blocker Ko143 (1–10 μM) did not affect the cell growth suppressive effect of dox, nor its intracellular accumulation (not shown).

Table 1 Activity of Pgp, MRP and BCRP in MDA-MB231 cells.

	Pgp-activity		MRP-activity		BCRP-activity	
	–cxb	+cxb	–cxb	+cxb	–cxb	+cxb
Control	1.16	1.00	1.34	1.39	1.13	1.08
Doxorubicin (0.1 I M)	1.00	1.00	1.29	1.22	1.25	1.25

FACSCAN ANALYSIS FOR P-GP-, MRP- AND BCRP- TRANSPORTER ACTIVITY

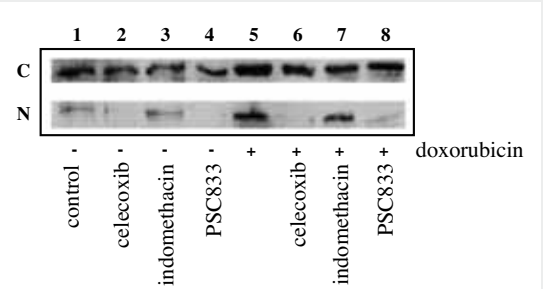
Table 1 shows the ratios for the activity of ABC-transporters P- gp, MRP1 and BCRP in MDA-MB231 cells under different treatment conditions. The measured ratio for each of the transporters, under all conditions, was hardly different from 1.0 and is considered to reflect a very low, if any, activity of these proteins since even very low resistant cell lines have a ratio of at least 2.0.[20,37,38]



Figuur 5 Effects of different additives on NF-kB-mediated luciferase expression (relative light units) in MDA-MB231 cells. **(a)** Dox (0.01-1 μM) (black bars) and celecoxib (0.5-50 μM) (grey bars). **(b)** Celecoxib, indomethacin and PSC833 (all at 50 μM) (grey bars) alone or in combination with dox (1 μM) (black bars). Results are expressed as mean ± SD * p<0.05 (control versus treatment).

EFFECTS ON NF-κB ACTIVITY

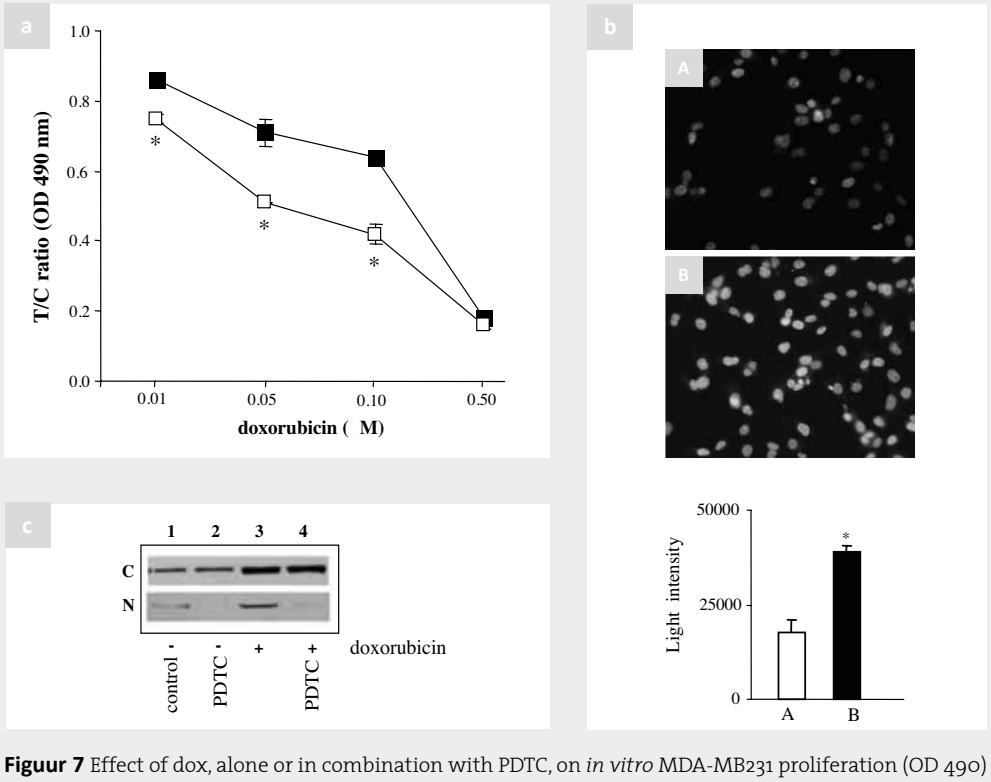
Fig. 5a shows the effects of dox (0.01–1 μM) and celecoxib (0.5– 50 μM) on NF-κB-luc activity in MDA-MB231 cells. Dox dose-dependently stimulated NF-κB-luc activity and celecoxib dose-dependently inhibited it. Fig. 5b shows the effects of celecoxib, indomethacin and the P-gp blocker PSC833 (all at 50 μM) alone or in combination with dox (1 μM) on NF-κB- luc activity. Both celecoxib and PSC833 almost completely blocked basal – as well as dox induced NF-κB activity, while indomethacin had no effect. In addition, Sc236 (50 μM) suppressed both basal and dox induced NF-κB activity and NS398 (50 μM) was totally ineffective (not shown).



Figuur 6 Western blot detection of NF-κB subunit p65 in nuclear (N) and cytoplasmatic (C) extracts from MDA-MB231 cells. MDA-MB231 cells were cultured for 24 h in the absence (lanes 1–4) or the presence of dox (1 μM) (lanes 5–8) and in combination with celecoxib (lanes 2 and 6), indomethacin (lanes 3 and 7) or PSC833 (lanes 4 and 8) (all at 50 μM).

NF-κB P65 AND P50 SUBUNIT TRANSLOCATION

Fig. 6 shows Western blot detection of NF-κB subunit p65 in nuclear (N) and cytoplasmatic (C) extracts from MDA-MB231 cells cultured for 24 h in the absence or the presence of different additives. As shown, the cytoplasmatic extracts of cells that were cultured in the presence of dox (1 μM) (lanes 5–8) stained significantly stronger for p65 than those cultured in the absence of dox (lanes 1–4). Moreover, compared to control (lane 1), celecoxib (50 μM) (lane 2) and PSC833 (50 μM) (lane 4) significantly inhibited nuclear p65 staining, whereas indomethacin (50 μM) (lane 3) had no effect. Furthermore, dox strongly enhanced nuclear p65 staining (lane 5) which was almost totally suppressed by celecoxib (lane 6) and PSC833 (lane 8) and was unaffected by indomethacin (lane 7). In addition, no effect of any of the additives on p50 nuclear translocation was observed, nor on IKKα, IKKβ and IκB-α (not shown).



Figuur 7 Effect of dox, alone or in combination with PDTC, on *in vitro* MDA-MB231 proliferation (OD 490) and effect of PDTC on intracellular dox accumulation. **(a)** Dox (0.010–0.5 μM) (filled squares); dox + PDTC (10 μM) (open squares). Results are expressed as treatment versus control ratio (T/C-ratio); mean ± SD * P < 0.05 (dox versus dox + PDTC). **(b)** Fluorescent microscopic pictures of MDA-MB231 cells that were incubated for 24 h in a medium containing dox (1 μM) **(A)** or dox in combination with PDTC (50 μM) **(B)**. Magnification 200 x. The bar graph shows, for both experimental conditions, the mean light intensity per cell, measured by computerised image analysis, expressed as mean ± SD *P < 0.001. **(c)** Western blot detection of NF-κB subunit p65 in nuclear (N) and cytoplasmatic (C) extracts from MDA-MB231 cells. MDA-MB231 cells were cultured for 24 h in the absence (lane 1) or the presence of PDTC (10 μM, lane 2), or in the presence of dox (1 μM, lane 3), and dox combined with PDTC (lane 4).

EFFECTS OF PDTC ON DOX SUPPRESSED CELL GROWTH AND ON INTRACELLULAR ACCUMULATION OF DOX

Fig. 7a shows the effects of dox in the absence or presence of PDTC (10 μM) on MDA-MB231 growth *in vitro*, and Fig. 7b shows its effect (10 μM) on intracellular dox accumulation. As shown, PDTC decreased the EC50 on cell growth from 0.2 μM to 0.09 μM and also significantly ($P < 0.001$) increased intracellular dox accumulation, see pictures and bar graph.

In addition, as expected, PDTC (10 μM) inhibited basal and dox (1 μM)-induced NF-κB-luc activity in MDA-MB231 cells from 82 ± 13 to 13 ± 4 and from 163 ± 18 to 39 ± 9 relative photon units, respectively (data not shown). Western blot detection of NF-κB subunit p65 in nuclear (N) and cytoplasmatic (C) extracts from MDA-MB231 cells cultured for 24 h in the absence (lane 1) or the presence of PDTC (10 μM, lane 2), doxorubicin (1 μM, lane 3), and treatment with dox and PDTC (lane 4) shows that PDTC inhibits both basal and dox-enhanced nuclear p65 staining (Fig. 7c).

Discussion

In this study we show that the specific COX-2 inhibitor celecoxib enhances the cytostatic effect of dox on MDA-MB231 tumor growth *in vivo* and *in vitro*. Celecoxib also significantly increased the intracellular accumulation and retention of dox *in vitro*. These effects of celecoxib were independent of COX-2 and of the activity of the ABC-transporters P-gp, MRP1 and BCRP and were most likely mediated by inhibition of NF-kB. Moreover, we show that the P-gp blocker PSC833, like celecoxib, increased the intracellular accumulation of dox and augmented the dox-induced cytotoxicity, independent of P-gp inhibition, mediated most likely via suppression of NF-kB activity.

Previous studies have shown that NSAIDs and specific COX-2 inhibitors are able to enhance the effects of certain cytostatic agents *in vitro* and *in vivo*. For example, celecoxib was shown to enhance the tumor growth inhibitory effect of dox in breast tumor bearing mice[15]. In line with this, we found that the combined treatment of human breast carcinoma MDA-MB231 xenografts in nude mice with dox and celecoxib synergistically inhibited tumor growth. *In vitro*, these compounds also synergistically inhibited the proliferation of MDA-MB231 cells. The mechanism by which celecoxib as well as Sc236 enhanced the action of dox are

most likely independent of suppression of COX, as we show that the specific COX-2 inhibitor NS398 and the NSAID indomethacin did not augment the cytotoxic actions of dox.

Some previous studies using less specific or sensitive assays, suggested that the enhancement of chemotherapeutic drug efficacy by COX-inhibitors may be due to inhibition of the ABC-transporters P-gp[15] or MRP[123-26]. In our study, we show that celecoxib and Sc236 augmented the *in vitro* intracellular accumulation and retention of dox, also suggesting the possible involvement of one of the dox transporters in this action. The activity of the key transporters of hydrophobic drugs, like the anthracycline dox, namely P-gp, MRP1 and BCRP can be inhibited by specific blockers, such as PSC833 for P-gp, probenecid for MRP1 and Ko143 for BCRP, respectively. Our results showed that, like celecoxib and Sc236, the P-gp blocker PSC833, but not the MRP-blocker probenecid, nor the BCRP blocker Ko143, stimulated the accumulation of dox in MDA-MB231 cells and also synergistically augmented the inhibitory effect of dox on MDA-MB231 proliferation. Collectively, this would suggest the involvement of the P-gp transporter in the actions of celecoxib and SC236 on dox inhibited cell growth and intracellular accumulation of dox. However, by using a very sensitive functional assay for P-gp, using the fluorescent dye Syto-16[20,38], we showed that the MDA-MB231 cells revealed no P-gp activity under all treatment conditions, ruling out its involvement in the actions of celecoxib, Sc236 and PSC833. A finding consistent with this result was reported recently by Wang and colleagues[39], who showed that ABC-transporter proteins were absent in MDA-MB231 cells. Therefore, another classical MDR phenotype-independent mechanism must be involved in the actions of celecoxib, Sc236 and PSC833 on dox induced cytotoxicity in these cells.

Previously, NF-κB has been shown to be involved in chemoresistance as well as to be a molecular target of celecoxib action. It has been shown that constitutive NF-κB activation in tumors protects the cells against apoptotic stimuli, such as those induced by chemotherapeutic treatment. Using a transfection luciferase assay, we found that celecoxib could completely inhibit NF-κB-luc activity, whereas dox increased it. This is in line with other studies, showing that celecoxib can inhibit and dox can specifically induce NF-κB nuclear translocation and activation of its target genes. In addition we showed that, in contrast to indomethacin and NS398, celecoxib, Sc236 and PSC833 can inhibit basal and dox-induced NF-κB activity. This is the first study that implicates NF-κB as a molecular target for the actions of the P-gp blocker PSC833. PSC833 is in general thought to modulate cellular responses to chemotherapy exclusively via the suppression of P-gp drug pumping, although some reports already

suggested the possibility that Pgp-independent effects might be involved in the chemomodulatory action of PSC833 on anthracycline cytotoxicity *in vitro* and in clinical studies.[40-42]

Overall, the NF-κB transfection studies were in complete concordance with findings in the MTS and dox intracellular accumulation assays. Moreover, Western blot analysis, showed that celecoxib, Sc236 and PSC833 all specifically inhibited constitutive and dox induced nuclear translocation of the p65 subunit of NF-κB, and not the nuclear translocation of the p50 subunit, nor did these compounds affect IKKα, IKKβ and IκB-α. Inhibition of p65 translocation has been implicated in enhancing chemosensitivity of chemotherapeutic drugs[43-45], however, the finding that only p65 is regulated is somewhat unexpected. Nonetheless, NF-κB inhibitors, among which are PDTC, have shown to be able to specifically inhibit nuclear translocation of p65, independently of p50 and in concordance with our findings, Sc236 has been reported to suppress p65 nuclear translocation independent of IKK activity and IκB-α gene transcription or degradation.[46,47] The molecular mechanisms involved in this specific regulation of p65 are not well understood, but it is suggested that Sc236 may directly target proteins that facilitate the nuclear translocation of NF-κB.

In order to further investigate the role of NF-κB in the observed enhancement of the anti-tumor efficacy of dox, we combined the NF-κB inhibitor PDTC with dox in the MTS and fluorescent dox accumulation assays. PDTC, which inhibited basal and dox-induced NF-κB activity in our transfection assay, also inhibited basal and dox-enhanced p65 nuclear translocation using Western blot analysis and increased the anti-proliferative effect of dox in the MTS assay as well as the intracellular accumulation and retention of dox. The ability of NF-κB activation to induce chemoresistance has previously been related to the escape of apoptosis, but the NF-κB pathway is linked to many aspects of cell growth and apoptosis.[34,45] Our results show that the inhibition of NF-κB activation may be involved in modulating intracellular chemotherapeutic drug accumulation and/or transport. However, to irrefutably implicate NF-κB as the sole and major target in the observed chemotherapeutic drug enhancement, further studies are warranted, as PDTC has been shown to not exclusively inhibit NF-κB and also to be able to affect the ubiquitin–proteasome pathway.[48]

Whether the enhanced intracellular dox accumulation is fully responsible for the observed, enhanced anti-tumor effect remains to be proven. The molecular mechanism linking the inhibition of NF-κB activity with the enhanced dox accumulation and retention may be related to membrane-related actions of dox[41,49] and is the subject of further investigations,

amongst which Suppression Subtractive Hybridisation-PCR50 and micro array studies are planned to identify genes and biochemical pathways involved.

Taken together, our results show a new mechanism by which COX-inhibitors can overcome anti-cancer drug resistance and enhance chemotherapeutic drug efficacy. The molecular mechanism by which the inhibition of NF-κB activity enhances the intracellular drug accumulation is the subject of further investigations and might have clinical implications.

CONFLICT OF INTEREST STATEMENT

None declared.

ACKNOWLEDGEMENTS

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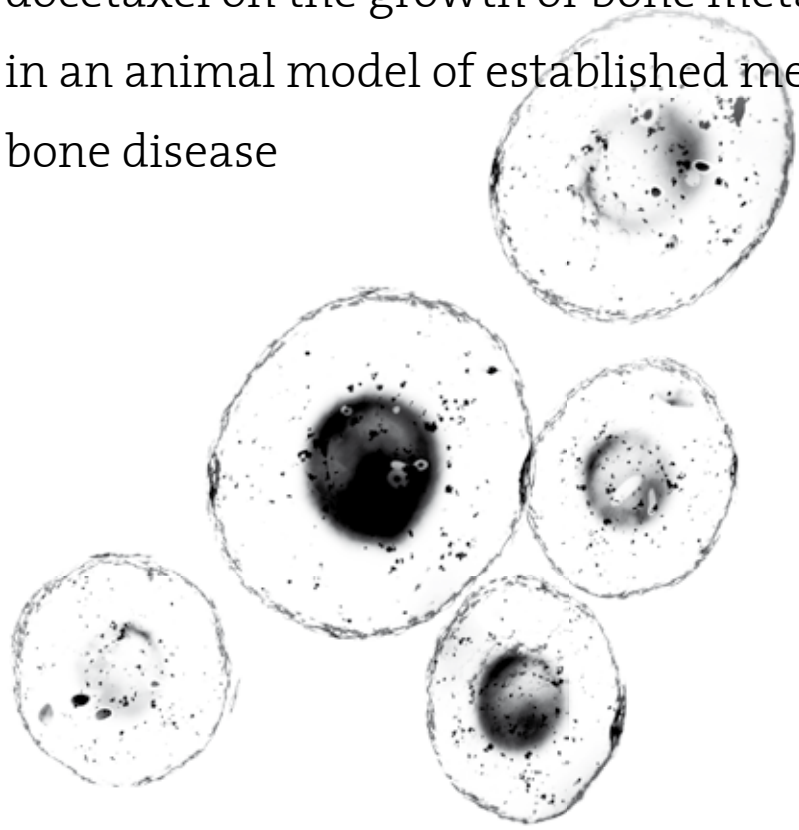
Chapter 5

Synergistic effect of bisphosphonate and docetaxel on the growth of bone metastasis in an animal model of established metastatic bone disease



Ermond van Beek, Clemens Löwik, Jens van Wijngaarden, Frank Ebetino, Socrates Papapoulos

Breast Cancer Res Treat. 2009 Nov;118(2):307-13. Epub 2008 Nov 7

Abstract

Bisphosphonates decrease bone resorption and reduce significantly the rate of skeletal complications in patients with metastatic bone disease. Bisphosphonates have also been shown to exhibit anti-tumor activity *in vitro* but *in vivo* results have been equivocal. In the present study, we investigated the effects of bisphosphonate treatment alone or in combination with the cytostatic docetaxel on the growth of breast cancer cells in bone. Tumor gowth was studied in an athymic nude mice model inoculated with MDA-231-B/luc+ breast cancer cells. Two days after the inoculation, mice were treated with risedronate, zolendronate or docetaxel alone or with a combination of risedronate and docetaxel. Bone destruction and tumor growth were evaluated radiographically, histologically and by whole-body bioilumines-cent reporter imaging (BLI). Five week treatment with high doses risedronate or zoledronate (37.5–150 μg/kg, 5 times/ week), fully protected the bones from osteolysis, but did not affect tumor growth. Docetaxel (2, 4, and 8 mg/kg, 2 times/week) inhibited tumor growth dose-dependently and after 5 weeks treatment with the highest dose, there was no detectable tumor in bone. The combination of a dose of docetaxel (4 mg/kg) that demonstrated only a minimal effect on tumor growth, with risedronate (150 μg/kg), protected bone integrity and nearly completely inhibited the growth of the cancer cells. Risedronate and docetaxel act synergistically to protect bone and decrease tumor burden in an animal model of established bone metastases from breast cancer cells.

Introduction

Metastatic bone disease is a major cause of morbidity in patients with different cancers including those of the breast and the prostate[1]. Despite differences in the pathogenesis of bone metastases from different cancer types, increased osteoclast-mediated bone resorption is the major mechanism for tumor-induced bone destruction[2, 3]. Bisphosphonates decrease bone resorption and reduce significantly the rate of skeletal complications in patients with metastatic bone disease[4]. In addition, several *in vitro* studies reported that bisphosphonates have direct anti-proliferative and proapoptotic effects on cancer cells and can inhibit the adhesion of cancer cells to mineralized matrices suggesting that these compounds may also have a favorable action on the growth and invasive behavior of cancer cells[5-8]. However, *in vivo* studies in animal models of bone metastasis have produced equivocal results[9-17].

These apparently discrepant results regarding an anti-tumor effect of bisphosphonates may be related to the timing of interference with bone turnover during the metastatic process. Decrease of bone turnover by bisphosphonates before colonization of bone by cancer cells, inhibits to a great extent the formation of bone metastases[15, 16]. However, when bisphosphonate treatment is given after the establishment of bone metastases, it has a minimal effect on the progression of cancer growth despite a substantial reduction of osteolysis. It was hypothesized that cancer cells metastatic to bone after an initial growth phase that depends on their interaction with the local stroma, they become independent of microenvironment's growth support and progress autonomously[15]. For the arrest of growth of established metastases, compounds with mechanisms of action different from that of bisphosphonates will be needed. Previous studies with concomitant administration of bisphosphonates and chemotherapeutics have shown a reduction in metastatic growth to bone[18-23]. However, toxicity of the latter precludes the application of fully effective doses. In the present study, we, therefore, tested the hypothesis that doses of a cytostatic that lack full anti-tumor efficacy when given alone, can act synergistically with bisphosphonates to reduce the growth of bone metastases from breast cancer cells.

Material and methods

CELL LINE AND CULTURE CONDITIONS

Luciferase positive human MDA-MB-231 breast cancer cells (MDA-231-B/luc+), were used for *in vivo* optical imaging as described previously[24]. MDA-231-B/luc+ cells were cultured in DMEM (Life Technologies, Breda, The Netherlands) containing 4.5 g/l glucose and supplemented with 10% FCS (Life Technologies) and 800 µg/ml geneticin/G418 (Life Technologies).

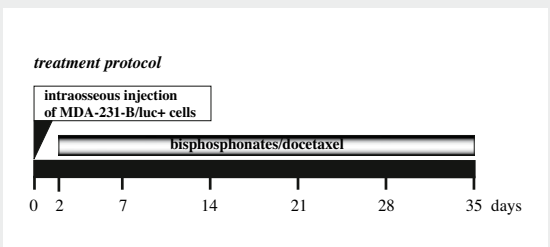
ANIMALS

Female nude mice (BALB/c nu/nu) were purchased from Charles River (Charles River, Maastricht, The Netherlands). Animals were housed in individual ventilated cages under sterile condition, and sterile food and water were provided ad libitum. Animal experiments were approved by the local committee for animal health, ethics and research of Leiden University and carried out in accordance with European Communities Council Directive 86/ 609/EEC.

EXPERIMENTAL ANIMAL MODEL

MDA-231-B/luc+ cells were harvested at about 80% confluence after changing to geneticin-free medium 24 h before inoculation.

The animals were anesthetized using the isofluorane anesthesia system (XGI-8, Xenogen) and a single-cell suspensions of 1.5 x 10^5 MDA-231-B/Luc+ cells/10 µl PBS were injected into the right tibiae of 6-week old mice as described previously[24].



Figuur 1 Schematic representation of the treatment protocol. The left tibial bones of nude mice were injected with MDA-231-B/luc+ cells ('day 0') and 2 days later treatment with i.p. injections of bisphosphonate (5 times/week), docetaxel (2 times/week) or the combination was started. The mice were treated for a total of 35 days.

Treatment of the animals started 2 days after intraosseous inoculation of MDA-231-B/Luc+ cells. From this time point (day 0) and during a subsequent period of 5 weeks, they received risedronate or zoledronate (5 times per week (100 µl by i.p. injection)) (dissolved in PBS), docetaxel (2 times per week (50 µl by i.p. injection) (dissolved in DMSO) or a combination of 5 times per week risedronate and 2 times per week docetaxel concurrently. The control animals received vehicle treatment. The different treatment schedules are illustrated in Fig. 1.

BIOLUMINESCENT REPORTER IMAGING (BLI)

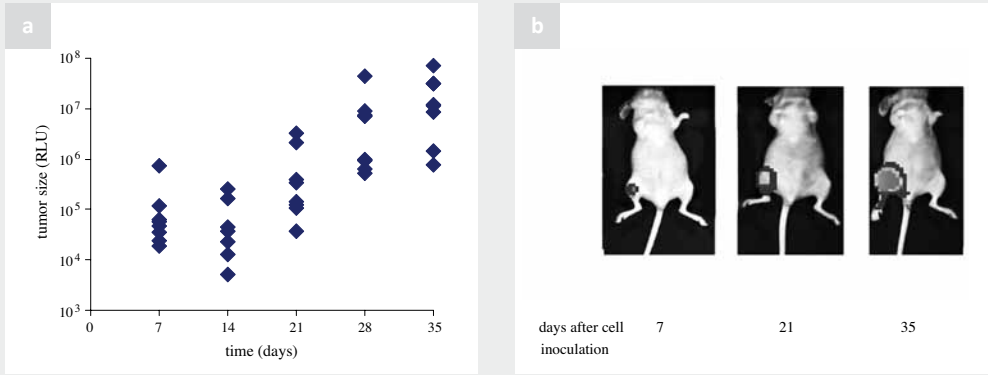
Tumor progression of intraosseous growth was monitored weekly by BLI. For this, the mice were anesthetized as described above and injected i.p. with 2 mg D-luciferin sodium salt (Synchem OHG) dissolved in PBS, and measurements were done 5 min after the injection of D-luciferin. Bioluminescence imaging was acquired with a 15-cm FOV, a medium binning factor, and exposure times of 10–60 s. Imaging data were analyzed by using the program living image (Xenogen). Values are expressed as relative light units (RLU) in photons per seconds.

RADIOGRAPHS

After the experimental periods, mice were sacrificed by cervical dislocation and the tumor bearing hind legs were removed and assessed for osteolytic lesions by radiography (Kodak X-OMAT TL film, Eastman Kodak Co.) using a Hewlett Packard X-ray system Faxitron 43805 and quantified using NIH Image 1.62b7 software as described earlier[25].

HISTOLOGY

The skin of the dissected hind legs was removed and the bones were fixed for 24 h in PBS with 4% formaldehyde; subsequently, the bones were decalcified in water containing 10% EDTA, pH 6.4 and embedded in paraffin and submitted to Masson-Goldner staining as previously described[26].



Figuur 2 (a) BLI measurements of tumor growth in control mice, monitored weekly during the 5-week experimental period. Results are expressed as individual mouse values. **(b)** Representative bioluminescent images of a control mouse at week 1, 3, and 5 after intraosseous inoculation of MDA-231-B/luc+ cells in the tibial bone.

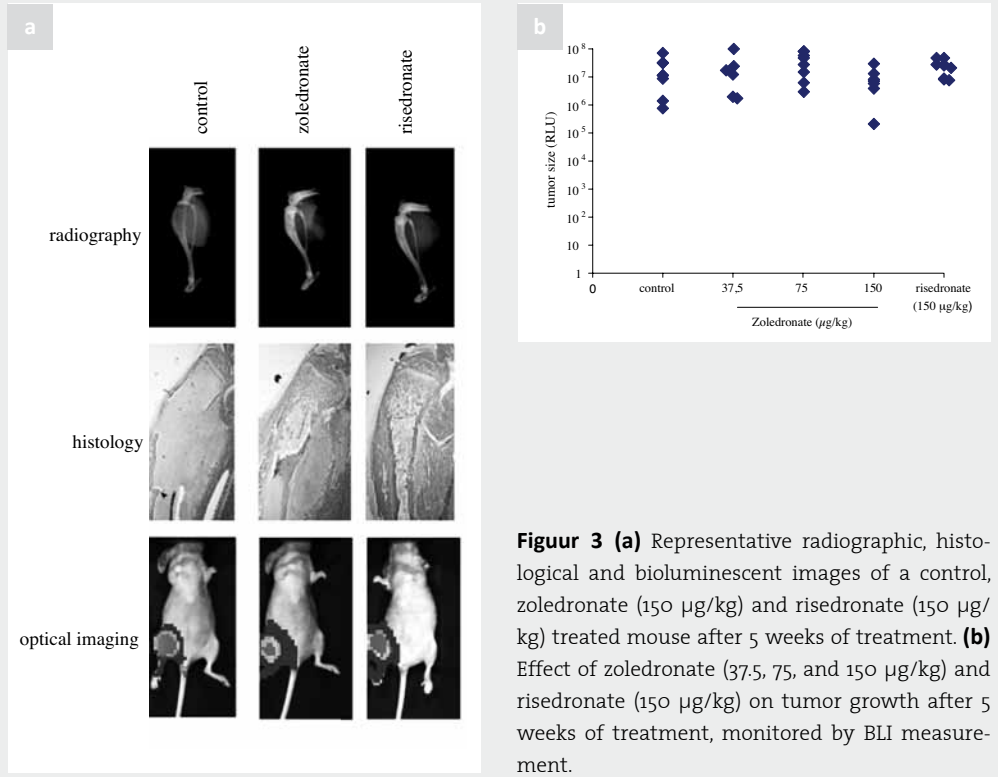
EFFECTS OF BISPHOSPHONATE TREATMENT ON METAPHYSAL DRY WEIGHT OF THE TIBIA

To determine the effectiveness of bisphosphonate treatment, at the end of the experiment, the dry weight of the metaphysis of the right tibia (not inoculated with cancer cells) was measured as previously described[27].

Results

TUMOR GROWTH KINETICS

Following inoculation of the left tibiae of athymic nude mice with MDA-231-B/Luc+ cells there was a progressive increase in tumor size with an increase of the BLI signal of more than 100-fold ($1.4 \cdot 10^5 \pm 2.5 \cdot 10^5$ to $2.1 \cdot 10^7 \pm 2.3 \cdot 10^7$ RLU) from day 7 to 35 (Fig. 2a). Figure 2b shows representative images of the BLI signal intensity in the tumor baring leg of a control mouse on day 7, 21 and 35, respectively.



Figuur 3 (a) Representative radiographic, histological and bioluminescent images of a control, zoledronate (150 µg/kg) and risedronate (150 µg/kg) treated mouse after 5 weeks of treatment. **(b)** Effect of zoledronate (37.5, 75, and 150 µg/kg) and risedronate (150 µg/kg) on tumor growth after 5 weeks of treatment, monitored by BLI measurement.

EFFECTS OF BISPHOSPHONATES

We first examined the efficacy of bisphosphonates on normal bone resorption. For this, we measured the dry weight of the metaphyses of contralateral tibiae, which were not inoculated with cancer cells, of the animals after 5 weeks of bisphosphonate treatment. Compared to controls, zoledronate (37.5, 75, and 150 µg/kg) and risedronate (150 µg/kg) (5 times/week) increased significantly the mean metaphysal weight of the tibae of the mice: 112.7 ± 19.2 mg (control); 177 ± 13.9 mg (zoledronate 37.5 µg/kg); 173.7 ± 19.4 mg (zoledronate 75 µg/kg); 186.1 ± 16.7 mg (zoledronate 150 µg/kg) and 155.3 ± 15.0 mg (risedronate 150 µg/kg), respectively ($P < 0.01$ for all bisphosphonate doses). The lack of a dose-dependent effect in the zoledronate treated animals is due probably to already maximal inhibition of osteoclastic

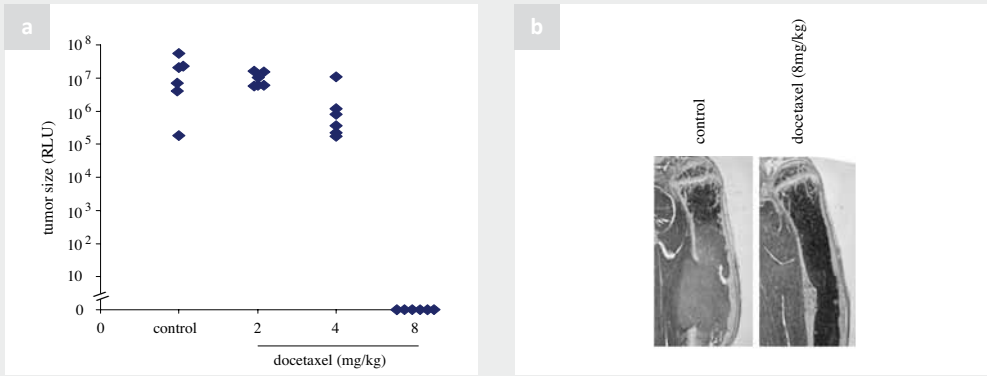
resorption by the lowest dose of this bisphosphonate used. Metaphysal weight in the risedronate-treated mice increased to the same extent as that in the zoledronate treated animals, indicating that resorption in these mice, was also maximally inhibited.

Both bisphosphonates prevented destruction of the tumor-bearing tibiae, assessed radiologically and histologically, but had no effect on tumor growth.

Figure 3 depicts representative radiographs, histological sections and BLI pictures of the tumor bearing legs, of these mice. Radiographically, the proximal tibia of the control animal was destroyed, whereas those of the bisphosphonate treated animals were intact, indicating protection of osteoclast-induced osteolysis by the bisphosphonates. Goldner stained histological sections demonstrated the presence of tumor in the legs of control mice. In bisphosphonate-treated mice there was a clear apparent reduction in the tumor within the bone whereas treatment had no effect on tumor load outside the bone collar. The bone marrow cavity of treated and untreated mice was invaded by the tumor which expanded outside the bone collar. Obviously, as result of their antiresorptive action, the tibial metaphysis of the bisphosphonate treated mice contained significantly more trabecular bone than that of controls. The results of BLI were consistent with the histological findings, as also shown previously[15, 24], and showed no difference in signal intensity between control and bisphosphonate-treated animals and neither bisphosphonate at any dose had any effect on tumor growth (Fig. 3b).

EFFECTS OF DOCETAXEL

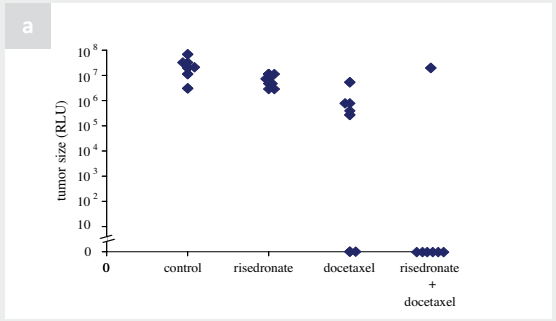
Figure 4a shows the effect of systemically administered docetaxel (2, 4 or 8 mg/kg, 2 times/week) on tumor growth after 5 weeks of treatment. Docetaxel inhibited tumor growth dose-dependently, with no BLI signal being measurable at the highest dose tested. Histological examination of the tibiae corroborated BLI findings. In contrast to controls, the tibiae of mice treated with 8 mg/kg docetaxel were intact and there was no detectable tumor tissue (Fig. 4b). Qualitative evaluation of the metaphyses revealed further that the amount of metaphyseal trabecular bone of docetaxel-treated mice appeared similar to that of controls and less than that of bisphosphonate-treated animals after 5 weeks.



Figuur 4 (a) Effect of docetaxel (2, 4, and 8 mg/kg, 2 times/week) on tumor growth after 5 weeks of treatment, monitored by BLI measurement. **(b)** Histology of the tibiae of a control and a docetaxel (8 mg/kg) treated animal.

EFFECTS OF COMBINED TREATMENT WITH RISEDRONATE AND DOCETAXEL

To examine the effect of the concomitant administration of bisphosphonate and docetaxel on tumor growth, docetaxel was given at minimally effective concentrations (4 mg/kg, twice/week) and risedronate at a dose of 150 µg/kg (5 times/week). As shown in Fig. 5a, risedronate alone did not affect tumor growth, as expected, while docetaxel alone failed to reduce tumor growth in five of the seven mice. Treatment with the combination of docetaxel and risedronate, however, resulted in a total absence of BLI signal in six out of seven mice. Histological examination confirmed the optical imaging findings, and only in one animal in the combined treatment group a tumor was present whereas in the other six mice no cancer tissue could be detected. In addition, like in the mice treated with risedronate alone, the tibiae of the animals treated with the combination of docetaxel and risedronate showed no osteolysis and contained a large quantity of trabecular bone (Fig. 5b).



Figuur 5 (a) Effect of risedronate (150 µg/kg, 5 times/week) and docetaxel (4 mg/kg, 2 times per/week) treatment, alone or in combination, on tumor growth after 5 weeks of treatment, monitored by BLI measurement. Difference among groups $P < 0.001$ (one-way ANOVA); combination therapy ($P < 0.001$) and docetaxel ($P < 0.05$) different from risedronate alone; combination therapy different from docetaxel alone ($P < 0.05$). **(b)** Representative bioluminescent, radiographic and histological images of a control, risedronate (150 µg/kg), docetaxel (4 mg/kg) and risedronate + docetaxel treated mouse after 5 weeks of treatment.

Discussion

We show here that combined treatment with a potent bisphosphonate and a cytostatic, at doses that have minimal effect on tumor growth when given alone, protects skeletal integrity and inhibits the growth of breast cancer cells in an animal model of metastatic bone disease. Animal and human studies have previously shown that increased bone resorption comprises the main mechanism responsible for bone destruction in metastatic disease and is related to the incidence and severity of skeletal complications in patients with malignancies[28, 29]. Breast cancer cells secrete factors, such as PTHrP, which stimulate the formation and activity of osteoclasts leading to bone destruction which causes bone pain, pathological fractures and hypercalcemia[1, 2, 30, 31]. This pathogenetic mechanism provided the rationale for the use of bisphosphonates in the management of patients with various tumors which metastasize to the skeleton, including those of the breast. However, during bone resorption induced by the osteoclasts, factors stored in the matrix of bone are also released in the bone marrow microenvironment and can act on cancer cells and stimulate further their growth as well as the production of bone resorbing factors[2, 31]. It was, therefore, thought that inhibitors of bone resorption, such as the bisphosphonates, may not only protect the integrity of bone at metastatic sites but may also have a favourable effect on the local growth of bone metastases. In addition, several *in vitro* studies have shown that bisphosphonates have direct effects on tumor cells, increase their rate of apoptosis, decrease angiogenesis and prevent their attachment on bone matrices[5, 6]. Thus, bisphosphonates, in addition, to their bone protective effect, may also reduce the growth potential of cancer cells in the bone-bone marrow microenvironment.

This attractive hypothesis has been, however, difficult to prove experimentally or clinically and appears to depend on the stage of the metastatic process as well as on the techniques used to assess cancer growth. For example, interference with the bone microenvironment with bisphosphonates before the establishment of bone metastases protects bone integrity and inhibits tumor growth. However, when bisphosphonates are given after the establishment of bone metastases, their effect on tumor growth is minimal as also shown in the present study. Furthermore, in studies reporting a beneficial effect on the tumor burden following bisphosphonate treatment, this is generally evaluated by histology of the area contained within the bones of animal models. However, it has been shown that tumor growth

outside the bone collar was not affected by treatment and that the apparent decrease in tumor growth within bone was rather due to the decreased space available due to the preservation of the bone structure[15]. Such histological findings were supported by studies which assessed directly tumor growth by molecular imaging techniques and showed no effect in the overall growth of cancer cells[15]. We confirmed this in the present study and we showed that treatment with the two very potent bisphosphonates risedronate and zoledronate given at high doses with similar anti-resorptive potencies to an animal model of established bone metastases were very effective in decreasing bone resorption and preventing bone destruction. However, bisphosphonate treatment given alone had only a minor effect on tumor growth assessed by histology and BLI once the tumor had been established in the bone marrow. In contrast, treatment with high doses of docetaxel did not only preserve the structure of bone but decreased also significantly the growth of the cancer cells within and outside the bone collar.

In recent years the significance of the interactions between tumor cells and cells of the bone marrow in the development of micrometastases to overt metastases has been increasingly recognized[2, 31]. In this process, increased bone resorption plays an important role and promotes the initial growth of cancer cells. However, once these cells develop into macrometastases mechanisms other than bone resorption contribute to their growth potential, such as for example angiogenesis. This sequence of events explains why a bisphosphonate given to animals for prevention of bone metastases is effective whereas when given to models with established metastatic disease has minimal effect on the further growth of the tumor. The lack of an anti-tumor effect of bisphosphonates on bone metastases *in vivo* despite the demonstration of such effects *in vitro* is probably attributed to the specific pharmacokinetics of these compounds. Bisphosphonates are cleared rapidly from the circulation and are taken up preferentially by the skeleton at active remodelling sites where they bind strongly to bone[32, 33]. This action allows only very limited, if any, exposure of the cancer cells in the marrow to bisphosphonates[34]. Therefore, for the adequate management of established metastatic disease in bone, bisphosphonates may have to be combined with other agents which specifically affect tumor growth and progression.

Previous studies with bisphosphonates in combination with anti-tumor drugs were effective in decreasing tumor growth in relevant animal models and *in vitro* evidence of a synergism has been reported[34–37]. The question, therefore, addressed in this study was

whether the combination of a bisphosphonate with a dose of a chemotherapeutic that has no effect on tumor growth when given alone, might act synergistically on tumor growth *in vivo*. Our results showed that a dose of docetaxel that affected tumor growth minimally, when dosed alone, had a profound effect on the growth of breast cancer cells in bone when dosed in combination with risedronate. In all but one of the treated animals with risedronate and the lower dose of docetaxel tumor cells were completely eliminated from bone. Thus, the combined treatment did not only preserve the structural integrity of bone but had a clear anti-tumor effect demonstrated both histologically and by BLI. Interestingly, trabecular bone of the animals treated with risedronate and docetaxel appeared to be better preserved than the bone of the animals which received the higher docetaxel dose. This should be attributed to the specific action of the bisphosphonate on bone.

In conclusion, bisphosphonates and chemotherapeutics act synergistically to protect bone and decrease tumor burden in an animal model of established bone metastases from breast cancer cells. This approach warrants further investigation in animal and human studies, as it may allow the use of less toxic dose of chemotherapeutics in the management of patients with bone metastases.

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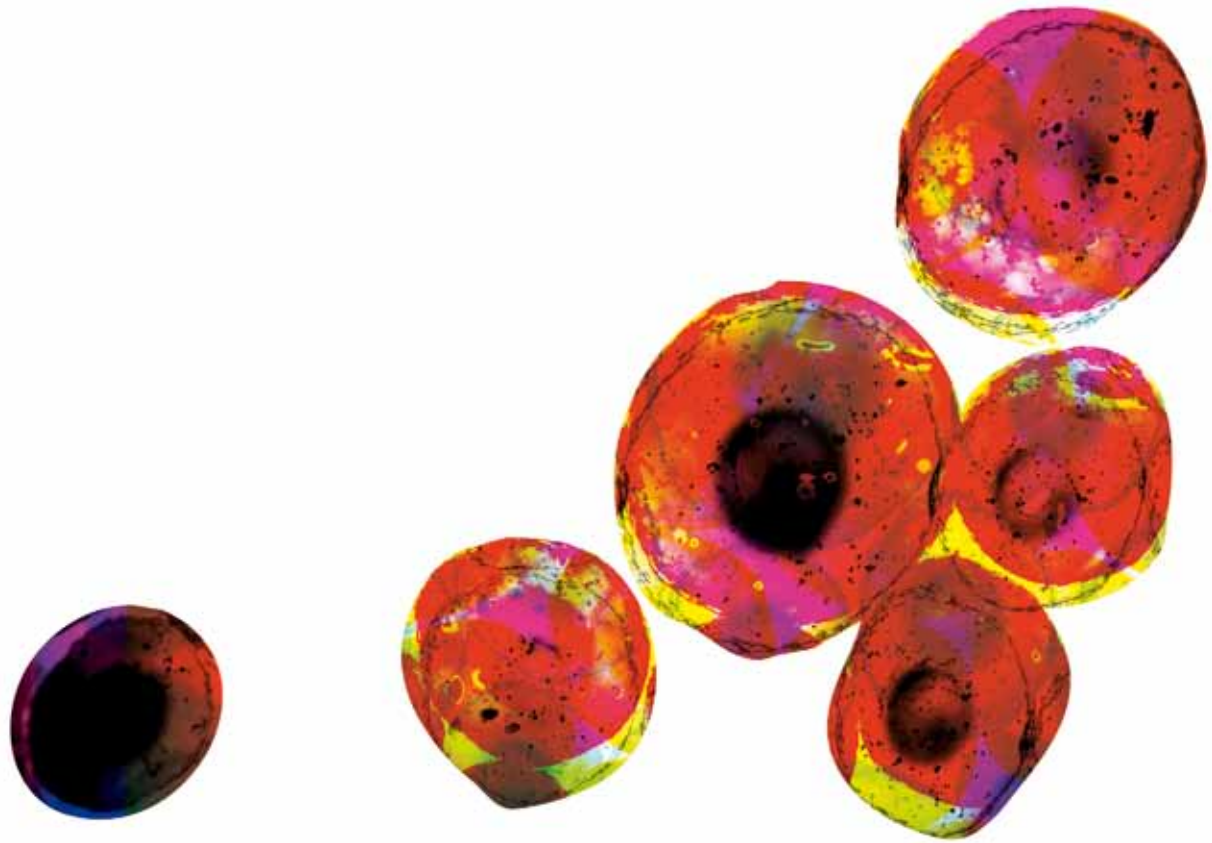
Chapter 6

General discussion



Cancer remains one of the leading causes of death worldwide and as such the development of new and improved anti-cancer therapies is of large importance. Despite numerous explorations and ongoing investigations, there still remain therapeutic gaps in the treatment of cancer. Focus of this thesis is to address and provide further suggestions for filling some of these remaining therapeutic gaps, by better understanding some of the molecular changes and processes behind the therapeutic treatment with chemopreventive agents **(chapter 2)**, using new models to investigate new candidate anti-angiogenic and vascular disruptive drugs **(chapter 3)** and exploring new combination treatment therapies of conventional chemotherapeutic strategies together with chemopreventive agents aimed at interfering with several important players in tumor development both at the level of the tumor itself as its microenvironment **(chapter 4 and 5)**.

1. Differential gene expression in a renal cell carcinoma model after treatment with endostatin

Chemopreventive agents are being widely explored to interfere with one or more rate limiting processes during tumor progression in order to prevent the promotion of tumor development at one stage or another. One of the most studied sorts of chemopreventive agents are anti-angiogenic agents. As discussed before, anti-angiogenic strategies may interfere both with the tumor directly, or with its microenvironment which plays a direct role in the further progression, invasion and metastasis of a tumor[1].

In **chapter 2**, we explored the effect of treatment of the human renal cell carcinoma RC-9 xenografts in nude mice with the chemopreventive anti-angiogenic agent endostatin. The treatment demonstrated to cause significant disintegration of blood vessels and subsequent tumor necrosis. In order to identify the effect of endostatin treatment on a molecular level, we applied the PCR based cDNA suppression subtractive hybridization (SSH) technique[2]. The SSH-technique compares two mRNA populations and identifies differentially expressed genes in one population. This technique is an all or nothing approach, or in other words, this technique allows for the identification of genes that are selectively 'turned on' in one population and 'turned off' in the other. Further to this, we implemented the Mirror Orientation Selection (MOS)-technique in the SSH technique, thereby significantly reducing the amount of false-positive genes[3]. Several genes were identified to be selectively induced

or suppressed by the treatment as described in **chapter 2**. Amongst the selectively induced genes were found calpain 2, insulin-like growth factor binding protein-3 (IGFBP-3), H2A histone family member Z; and amongst the selectively suppressed genes were found fibronectin (FN), tubulin alpha 1 and core binding factor alpha1 (cbfa1).

Selective suppression of FN and cbfa1, (also known as osf2, Runx-2, AML-3, PEBP2α-A, or NMP-2, known as a required transcriptional regulator of osteoblast differentiation and bone formation[4-6]) are further discussed in **chapter 2**. Since the time of publication an emerging picture of inflammatory processes in the tumor microenvironment has unfolded and knowledge in this field has expanded quite a bit. As such, the findings as described in **chapter 2** can now be viewed in a much broader perspective. At time, the downregulation of FN could be explained as a direct effect of endostatin treatment as endostatin binds to α5β1-integrin and FN being the ligand of α5β1-integrin[7, 8]. This still holds ground to date, however, it has further been shown that an indirect effect on the extracellular matrix (ECM) may have at least in part contributed to the suppression of FN[9-14].

An important regulator of normal tissue behavior is the ECM, which surrounds cells and is composed of many types of macromolecules. Most solid tumors exhibit a very different profile of ECM proteins in the stroma compared to their normal counterparts, and many of these proteins interact directly with tumor cells, via integrins and other cell surface receptors, to influence functions such as proliferation, apoptosis, migration and differentiation[9]. A number of these proteins are consistently up-regulated in solid tumors, including FN. Abnormal ECM remodeling in the tumor's microenvironment during tumor progression has been shown to contribute to, or is even required for, tumor formation and progression. Moreover, it has been shown that interaction of tumor cells with FN can enhance tumor survival[10, 11]. Likewise, as noted in the introduction of this thesis, it has been shown that 'normalization' of the stromal environment should be able to slow or even reverse tumor progression[12-14]. This has actually been shown after anti-angiogenic treatment with VEGF antibodies, where normalization of tumor border stroma took place. This normalization was found most likely due to a downregulation of matrix-degrading proteases such as MMP9 and MMP13 in the stroma, leading to a reduced turnover of crucial basement-membrane constituents like FN. As such, the specific downregulation of FN as found and described in **chapter 2** could well be, at least as well, explained by a normalization of the stromal environment as induced by the anti-angiogenic treatment[15].

As described in **chapter 2**, the finding of specific suppression of cbfa1 expressing granulocytes in untreated tumors was unexpected. It had, however, previously been shown that neutrophylic granulocytes can be a target for anti-angiogenesis therapy, because cbfa1 was reported to be a target for the anti-angiogenic effect of angiostatin, another naturally occuring inhibitor of tumor angiogenesis. As for the finding of specific suppression of FN, also the role of neutrophilic cells in tumor progression has become more apparent in recent years. Tumor cells are surrounded by an infiltrate of inflammatory cells, namely lymphocytes, neutrophils, macrophages and mast cells (MCs). In recent years, increased numbers of neutrophils in various human tumors compared with healthy tissues have found to be present. Moreover, their presence has been found to correlate with poor prognosis[16-20].

Fitting the emerging picture of tumorigenesis as a kind of inflammation process as described in the introduction, it is now widely believed that granulocytes play an important role in the tumor's microenvironment in the tumor's progression. These cells communicate via a complex network of intercellular signaling pathways, mediated by surface adhesion molecules, cytokines and their receptors[21]. Results point to the importance of a cross talk between several host cells for promoting angiogenic effects in tumor areas. Inflammatory cells cooperate and synergize with stromal cells as well as malignant cells in stimulating endothelial cell proliferation and blood vessel formation, tumor proliferation and invasion[22-24]. Granulocytes have been shown to be able to participate in tumor angiogenesis in many ways. Likewise, inhibition of angiogenesis, normalization of tumor stroma and tumor reduction is conceivable and has been shown to be able to block neutrophilic tumor infiltration, exactly coinciding with our observations[22].

The results of **chapter 2** further show the importance of the tumor's micro-environment as a potential therapeutic target as containing many different players contributing in the process of tumor progression, such as the ECM and the inflammatory infiltrate. As mentioned before, anti-angiogenesis therapy has, especially as a single agent, however, still live up to the clinical challenge. Moreover, cellular and molecular studies indicate that signals from the stromal compartment in the tumor's microenvironment play an important role in observed acquired resistance of tumors to anti-angiogenic therapy[25-27], which in earlier years of explorations of anti-angiogenesis therapy was thought to be impossible. One of the obstacles herein is, amongst others, that tumors acquire vasculature also via vessel co-option from existing vasculature in the microenvironment[28]. As anti-angiogenic

compounds do not affect incorporated pre-existent, or matured tumor vasculature, targeting of the existing tumor vessels is explored as an adjuvant approach to accomplish tumor regression via disruption of the tumor's blood supply, as is further discussed on in **chapter 3**.

2. A new model to identify and discriminate between new potential anti-angionic drugs and vascular disruptive agents

As described above, therapeutic vascular targeting has so far concentrated almost exclusively on anti-angiogenic approaches, which aim to prevent the neovascularization process in tumors. Current research also explores anti-vascular (vascular-disrupting) approaches using vascular-disrupting agents (VDAs), aiming to cause the rapid and selective shutdown of the established tumor vasculature, leading to secondary tumor-cell death[29-33].

VDAs are currently rapidly being developed and several clinical trials have been carried out or are ongoing[34-39]. Measuring both anti-angiogenic and anti-vascular efficacy, but moreover, discriminating between both targets, has proven to be difficult[40]. This is both due to an existing overlap in the effects of VDAs and anti-angiogenic agents as a lack of well-defined and validated models to study the efficacy of these agents. Indeed, some anti-angiogenic compounds have shown to cause vasculature remodeling and regression *in vivo*[41-45] and the VDA combretastatin-A4 phosphate has been described to be toxic to proliferating endothelial cells[46] and human umbilical vein endothelial cell (HUVEC) migration and tube formation *in vitro*[47]. The possibility of defining and discriminating between the specific effects of different anti-vascular compounds on different targets in vasculature is essential in optimizing therapeutic potential.

In **chapter 3**, we describe the development of an *in vitro* model that can distinguish between effects of compounds on angiogenesis and/or newly established vasculature. Further to this, this model allowed us for further specifying anti-angiogenic effects by being able to identify the effects of compounds on early stage endothelial precursor cells from which capillaries are formed. We examined the effects of three anti-vascular compounds, among which the new tubulin binding agent Ang-510. We showed this compound to effectively interfere with both angiogenesis as well as established capillaries, whereas the synthetic fumagillin derivate TNP-470 and the MMP inhibitor marimastat selectively affected angiogenesis alone. Further to this, where marimastat showed an effect on ongoing angiogenesis, no effect on endothelial precursor cells was seen and as such on the onset of angiogenesis. These results illustrate the ability of this *in vitro* model for the specific and efficient screening of the effect of compounds on different specific vascular targets, facilitating the identification of pharmacological compounds with potential clinical benefit.

VDAs specifically target established tumor vasculature, as it draws on the differences in architecture as opposed to its normal counterpart. In tumor vasculature walls are poorly developed, often with a discontinuous endothelial-cell lining, there exists a relatively poor investiture with vascular smooth muscle cells and there are poor connections between pericytes and endothelial cells where the endothelial cells themselves are often irregularly shaped, forming an uneven luminal layer, with loose interconnections and focal intercellular openings [33, 48-51]. It is possible that the cytoskeleton of tumor endothelial cells is particularly sensitive to disruption by VDAs due to expression of specific tubulin isotypes or posttranslational modifications to microtubule associated regulatory proteins[52]. In non-stabilized vasculature, as is often the case in tumor vasculature, tubulin interference causes endothelial cell detachment and subsequent vascular disruption, an effect that is not seen in normal stabilized vasculature. The vasculature in the described *in vitro* model in **chapter 3** shows similar architectural differences, (i.e. it also lacks SMCs and pericytes), which may well explain its predictive and discriminatory potential and suit it for studying the effects of agents on tumor vasculature.

Taken together, these observations also shed new light on the results as described in **chapter 2**. Interestingly, one of the selectively suppressed genes after endostatin treatment was shown to be tubulin alpha 1. This is further corroborated by recent findings that endostatin is promoting the disassembly of the actin cytoskeleton, disorders in cell-matrix interactions and decrease in endothelial mobility[53]. As such, it would be interesting to see whether the anti-vascular effect of endostatin is part of an anti-angiogenic effect, an effect on established vasculature as might be conceived based on the results as described in **chapter 2**, or both. One way or another, this further illustrates the existing overlap in actions and effects of anti-vascular agents, the difficulties in defining these effects and as such the need for the *in vitro* model as described in **chapter 3**.

The specific targeting of established vasculature provides VDAs with a unique and promising potential in treating cancer. It seems however inevitable that in order to fully exploit the therapeutic potential of these drugs, they need to be combined with other

therapies as on their own they leave a viable rim of surviving neoplastic cells at the periphery[54-56]. These residual areas of tumor tissue are believed to survive VDA treatment because their nutritional support is derived from vasculature in the adjacent normal tissue which can furthermore act as a source of tumor re-growth. As such, VDA treatment alone is highly unlikely to totally eradicate the tumor mass. This is corroborated by preclinical studies which have concluded that VDAs are ineffective at stopping tumor growth when used as single agents, and that combination with conventional therapies and/or anti-angiogenic therapies should be explored to gain in therapeutic potential and overcome treatment refractoriness and resistance as further discussed in **chapter 4** and **5**[57-62].

3. Combination therapies in overcoming treatment resistance: enhancing doxorubicin-cytotoxicity by NF-kB-mediated increase of doxorubicin accumulation

As mentioned above and in the introduction of this thesis, it is essential to design better and combined strategies to overcome treatment refractoriness and resistance. One of the therapeutic areas where resistance plays a major role in achieving full clinical potential is chemotherapy. As described in the introduction of this thesis, many different mechanisms exist by which tumor cells may become resistant to chemotherapeutic agents and the limitations of chemotherapy led to the exploration of chemopreventive approaches. These agents alone, however, have not yet provided full clinical benefit. Even more, resistance to these therapies is now also being observed[25]. As it points out combinational approaches, where more targets are simultaneously inhibited are proving to be an inevitable approach[57, 63, 64]. As such, research on combining conventional therapies such as chemotherapeutics with chemopreventive agents in order to overcome therapeutic resistance and escape is extensive, and includes combining anti-angiogenic agents with chemotherapy.

In **chapter 4**, we describe the combined effects of the combination of the specific COX-2 inhibitor and chemopreventive agent celecoxib with the chemotherapautic agent doxorubicin on the human breast cancer cell line MDA-MB231 both *in vivo* as *in vitro*. It has previously been shown that COX-2 inhibitors can significantly inhibit tumor angiogenesis[65-68], but also that these agents have an effect on tumor cells directly via inhibition of cell proliferation, induction of apoptosis and reduction of cell motility and adhesion[69-77]. These properties provide a rationale for examining the possible benefit of combining COX-2 inhibitors such as celecoxib with conventional anti-cancer therapies, such as chemotherapy, as is currently also being examined in clinical practice[78-81].

Combining these agents indeed showed a marked synergistic anti-tumor effect both *in vivo* as *in vitro* as opposed to applying these agents alone. This effect was found to be most likely independent of suppression of COX. Interestingly, we showed that celecoxib augmented the *in vitro* intracellular accumulation and retention of doxorubicin via a nuclear factor (NF)-kappa-B mediated mechanism. Whereas COX inhibitors have been described as chemosensitizers before[82-87], **chapter 4** describes a new mechanism by which COX-inhibitors can overcome anti-cancer drug resistance and enhance chemotherapeutic drug efficacy. Apart from the direct cytotoxic chemosensitizing effect on tumor cells as described, it is known that celecoxib as a COX-2 inhibitor in anti-cancer therapy also targets different therapeutic targets in the microenvironment such as on tumor angiogenesis as described above, or the inflammatory infiltrate[81, 88-91], which is of large importance as further elaborated on in **chapter 2**. As such, celecoxib may have different therapeutic roles in targeting both the tumor as its environment as well.

It has recently been shown that not just the tumor cells themselves contribute to chemotherapy resistance, but that the stromal microenvironment might also confer resistance to chemotherapy[92-95]. Amongst others, it has been shown that a stroma-related gene signature as found by gene expression profiling predicts resistance to neo-adjuvant chemotherapy in breast cancer. These findings show further the role the microenvironment can play in tumor progression and emphasizes the therapeutic potential of chemopreventive agents, as they may have a role not just in overcoming drug resistance to chemotherapy in tumor cells directly via an effect on drug transport but may perhaps also indirectly play a role via their effect on the stromal environment. Taken together, this further emphasizes the therapeutic potential of using chemopreventive agents in anti-cancer combination therapy. It however also makes clear, that it is possible to address multiple therapeutic targets at once with one drug, as is shown for celecoxib.

4. Combination therapies in overcoming treatment resistance: targeting tumor stroma of bone metastases with bone resorption inhibitors

Interference with the microenvironmental growth support is not only an attractive therapeutic target in primary tumor progression as described above but also in decreasing metastatic tumor growth[96]. Tumor cells must lodge, survive, extravasate, become established and grow at the secondary site before they become clinically relevant. Each stage is often rate limiting and the local microenvironment has a major role in every step in this path.

As touched upon in the introduction of this thesis, the secondary site must contain the microenvironment with specific local molecular mediators to support and allow for survival of the suitable type of cancer cells. This is both dependent on the properties of the tumor cells themselves as on the environment of the site of metastasis. For example, breast cancer frequently metastasizes to bone. Breast cancer cells themselves already express numerous bone-like properties, or they acquire bone cell-like properties along the way, a process which is referred to as osteomimicry[97]. In expressing these genes, the breast cancer cells are well equipped to home, adhere, survive and proliferate in the bone microenvironment. Next to this, bone contains numerous factors which make it a fertile soil for the survival and progression of breast metastases. These factors are continuously released in the environment due to osteoclastic bone resorption which is an integral part of the continuous remodeling that goes on in bone. The close interaction of the bone environment and breast cancer cells in breast cancer metastasis progression is illustrated by the phenomenon of the so-called 'vicious cycle'. In breast cancer 90% of metastasesin bone were found to express parathyroid hormone-related protein (PTHrP). PTHrP increases osteoclastic bone resorption, with consequent release and activation of matrix-integrated growth factors, such as TGF-β and IGFs. These factors in turn, stimulate the tumor growth and as such further secretion of PTHrP, starting the process all over again[98].

The essential properties of the miocroenvironment of a tumor's secondary site provide a rationale for not just targeting the microenvironment alongside conventional cytotoxic therapies in primary tumor development as discussed in **chapter 4**, but also during metastasis. One approach in doing so is by inhibition of chemokines and their receptors, which is currently in preclinical and clinical development[99, 100]. Another therapeutic strategy is to block the dissemination of tumor cells at the source, by inhibiting the development of

blood and lymphatic circulatory systems within the tumor. This hypothesis is supported by experiments with potent VEGF inhibition[101-103]. Another approach is by direct and specific interference with the secondary site, as has been explored amongst others by making use of bisphosphonates. Bisphosphonates are used clinically for the protection of bone destruction by metastases from different primary tumors[104-107]. The rationale in using bisphosphonates in targeting bone metastases lies in their inhibiton of osteoclastic bone resorption and as such in interference with the so-called 'vicious cycle' as discussed above.

In **chapter 5**, we investigated the effects of bisphosphonate treatment alone or in combination with the cytostatic agent docetaxel on the growth of breast cancer cells in bone. We showed that the bisphosphonate risedronate and docetaxel given at doses that have minimal effect on tumor growth when given alone, act synergistically to protect bone and decrease tumor burden in an animal model of established bone metastases from breast cancer cells. The role of bisphosphonates as an adjuvant treatment in decreasing breast cancer metastases has been subject to paradoxal results. In experimental settings, it has been shown that bisphosphonate treatment can prevent the establishment of bone metastases by using a preventive protocol[108], however, clinical studies have shown either a reduction of the establishment of new bone metastases, or no effect at all[109-111]. Most recent studies, however, point to a favorable effect of using bisphosphonates as an adjuvant treatment in primary breast cancer treatment in preventing the development of bone metastatses[112]. These different observations may, however, also be dependent on the potency of the bone resorption inhibiton and as such the specific bisphosphonate used.

In treating already established bone metastases from breast cancer, the results are emerging to be more apparent. For example, three clinical trials have evaluated the potential anti-metastatic efficacy of clodronate in patients with breast cancer. Two of these trials showed improvements in overall survival (OS) and bone metastases free survival in patients receiving clodronate[113, 114], where long-term follow-up showed improved OS at 8.5 years[115]. The third trial showed no effect, but this was most probably due to imbalances in patient characteristics between the two arms. As such, the use of bisphosphonates is now approved for use in metastatic breast cancer disease in the clinical setting[116]. Based on these promising results, clodronate is currently being evaluated for preventing bone metastases in two large randomized trials (NSABP-B34 and SWOG 0307), and the results are awaited. Our results as described in **chapter 5** add to large and growing body of evidence suggesting

benefits in an adjuvant setting of bisphosphonates in the treatment of established breast cancer metastases in bone.

Until now, data on the direct anti-tumor effect of bisphosphonates as single agents are limited and, thus far, provide conflicting evidence. Several large randomized clinical trials are ongoing with the next-generation bisphosphonate zoledronic acid to prospectively confirm an anti-tumor role for bisphosphonates in various tumor types[117]. Our experimental results in **chapter 5** show that when used in a non-adjuvant setting, when bisphosphonates are given alone after the establishment of bone metastases, the effect on tumor growth is minimal. These results are corroborated by studies that show that tumor growth outside the bone collar was not affected by treatment and that the apparent decrease in tumor growth within bone was rather due to the decreased space available due to the preservation of the bone structure[108]. Such histological findings were supported by studies which assessed directly tumor growth by molecular imaging techniques and showed no effect in the overall growth of cancer cells. We confirmed this in the present study and even more, showed that the combination treatment with high doses of docetaxel did also preserve the bone structure. These results show, again, as further elaborated on above, that the use of therapeutic agents targeting the microenvironment will most likely not be effective as monotherapy in most cases, but must be part of combinational therapies.

5. Conclusions and future perspectives

There is an unfolding picture of rate-limiting steps during tumor progression which can provide us with numerous potential therapeutic targets. Rate-limiting steps during tumor progression include for example limitless replicative potential, metastasis the establishment of a tumor's vasculature and further on the ability to invade and migrate in surrounding tissues. It is becoming increasingly clear both the tumor as its microenvironment contribute in these steps. Interfering with these rate limiting steps in both the tumor as its microenvironment via chemopreventive approaches provide us with therapeutic targets for preventing tumor progression. In the clinical setting, these approaches are extensively explored, amongst others in combination with chemotherapy.

Multiple examples of such agents interfering with different steps during tumor progression have been described and are being used in preclinical studies and phase I–III clinical trials and some in current clinical practice. For example, agents targeting angiogenesis include, amongst many others, integrin component antagonists Cilengitide, Abegrin, and Volociximab[118-121], the monoclonal antibody against VEGF-A Bevacizumab (Avastin®)[122-126], a large amount of VEGF tyrosine kinase inhibitors such as Semaxinib (SU5416) and Vandetanib (AZD6474) both used as first-line therapy in combination with chemotherapeutic regimens for metastatic colorectal cancer[127], thalidomide which is by the food and drug administration (FDA) and committee for human medicinal products (CHMP) approved for treatment in multiple myeloma, or the promising new class of agents of soluble VEGF receptors, of which Aflibercept (AVE0005, VEGF-trap) is an apparent example and being explored in several phase III trials in combination with different chemotherapy regimens (data from clinicaltrials.gov).

Another example of a class of agents being explored in anti-cancer therapy is VDAs, targeting the established tumor vasculature, of which combretastatin A4 phosphate is the most investigated, being currently explored in several clinical trials in combination with chemotherapeutic regimens and chemotherapy together with anti-angiogenesis therapy[128-131]. Targets currently which are being explored in the tumor microenvironment include interference with the inflammatory infiltrate, as being explored by using chemokine inhibitors[99], [100] and COX-inhibitors[81, 88-90], interference with matrix degradation by using for example MMP inhibitors marimastat and rebimastat[133-135] and TIMPs, interference with signaling pathways by using signaling and cytokine inhibitors[81] and achieving tissue and stromal normalization[136-138], which is in part the case in the use of bisphosphonates in prevention and reduction of breast cancer metastases.

The aforementioned examples are only a small exert of all therapeutic targets being explored, where there is an increasing focus in recent years on the role of the tumor microenvironment, following the understanding that the alterations that occur in the environment around a tumor might prove useful in generating new therapeutic targets. It has become clear, however, that using chemoprevention as monotherapy is in the vast majority of the cases likely not to be successful. An example of the observed lack of efficacy as monotherapy is the observed emerging resistance to anti-angiogenic therapies, which was previously thought to be impossible[25, 132]. As such, most of these targets are explored as part of combination strategies. One of the most investigated approaches in these combinational strategies is targeting the tumor cells themselves with chemotherapy together with che-

mopreventive approaches. In this thesis, we explore and address the therapeutic actions and potential of chemopreventive agents, either or not as part of a combinational approach with chemotherapeutic agents, where the role of the tumor itself and the tumors microenvironment is taken into account.

Future perspectives in exploring anti-cancer combinational strategies lay both in expanding the possibilities of conventional therapy by applying old agents in a new fashion, as in further exploring chemopreventive appoaches taking into account possible targets in the tumor microenvironment as well. For the first, one area that has not been addressed in this thesis is metronomic chemotherapy. Metronomic chemotherapy uses conventional chemotherapeutic agents as anti-vascular agents. Metronomic chemotherapy is defined as the chronic administration of chemotherapeutic agents at relatively low, minimally toxic doses and with no prolonged drug-free breaks. Previous research indicated that the therapeutic effect of metronomic chemotherapy is mainly due to a reduction of tumor angiogenesis rather than direct cytotoxicity[139, 140]. In addition, metronomic therapy with the chemotherapeutic drug cyclophosphamide selectively depleted T regulatory cells, which are key immune regulators in the tolerance and immune avoidance of tumors[141], thereby resulting in an enhanced tumor immune response[142, 143]. Further to this, the low dosage schedule significantly reduces undesirable toxic side-effects. Indeed, this promising and exciting new approach to the use of chemotherapy shows promising results in clinical settings[144].

Another example of new developments is the exploration of a new chemoprevention strategy in targeting the tumor microenvironment by making use of cancer vaccination against target antigens associated with tumor promotion and progression. In vaccinating against these antigens, the immune system is used as chemopreventive agent, instead of the promoting role it can normally have in tumor development and progression. In the case of a successful cancer-related response, antigen-specific T cells will be poised to destroy an aberrantly expressed protein even if the host is not exposed until years after the end of immunizations. After the abnormal cell is eradicated, T cells will lie in wait for the next exposure. The ability to develop effective cancer vaccines for prevention is fast becoming a reality as immunogenic aberrant proteins that drive malignant transformation are identified. Cancer vaccines have shown evidence of efficacy in controlled trials, and the type of immune response to a cancer vaccine that will be needed for cancer prevention is becoming increasingly known[145].

One way or another, applying rational approaches in which cytotoxic agents are administered with cytostatic anti-stromal agents hold considerable promise. For example, a three-step combinatorial approach could be investigated in which the tumor microenvironment is first normalized by anti-angiogenic or anti-stromal therapy, followed by treatment with cytotoxic therapies to shrink or even eradicate the tumor, then a maintenance regimen, such as low-dose chemotherapy or other anti-stromal drugs, could be administered to keep any remaining cancer cells in check[64]. In conclusion, an important step in this direction is the recognition that to effectively eliminate cancer, we should also consider targeting the normal cells that have been co-opted into supporting them.

The future will, according to the author of this thesis, lie in specific combinational therapies targeting both the tumor as its microenvironment, and being tailor made to both the tumor as the individual. This personalized regimen will be dependent on the type of tumor, the site of development or metastasis, the stage of the disease and gene expression patterns of the individual and the tumor itself. In doing so, it would be an attractive possibility to target more than one therapeutic area with one and the same agent. As we have shown, celecoxib proved to have a chemosensitizing effect on doxorubicin, apart from the previous described effects it has on tumor cells directly and the tumor microenvironment such as on the inflammatory infiltrate and malignant progression.

Taken together, this thesis explores the use of chemopreventive apporoaches as monotherapy and in combinational approaches and explores and discusses their effects on both the tumor as the tumor's microenvironment. It shows that these combinational approaches hold great promise, if both the role of the tumor as the tumor's microenvironment are taken into account as therapeutic targets. Where the term 'magic bullet' has not lived up to its promise, perhaps, in the future, the term 'personal strategic weapon's arsenal' could be found to fit the bill.

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Chapter 7

Summary
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Summary

Since the discovery of the first systemic anti-cancer treatments, enormous advances have been made in treatment efficacy. Despite these advances, however, cancer remains one of the leading causes of death worldwide. One of the most effective and commonly used conventional systemic therapies still is chemotherapy, however, both used as single agent and in combinational regimens, chemotherapy commonly faces the problem of therapeutic resistance. As such, the development of new and improved anti-cancer therapies is of large importance.

The remaining limitations of chemotherapy have led to the exploration of alternative anti-cancer approaches such as chemoprevention in order to improve efficacy. Chemopreventive agents interfere with rate-limiting steps in tumor progression, such as the establishment of a functional tumor vasculature. Currently, there is a large and unfolding picture of rate-limiting steps during tumor progression which can provide us with numerous potential therapeutic targets, where it is becoming increasingly clear that both the tumor itself and its microenvironment contribute to these steps. Interfering with these rate-limiting steps in both the tumor and its microenvironment via chemopreventive approaches provides us with therapeutic targets for preventing tumor progression.

In exploring chemopreventive therapies, it is becoming more and more apparent that using chemoprevention as monotherapy is in the vast majority of the cases likely not to be fully effective. An example of the observed lack of efficacy as monotherapy is the observed emerging resistance to anti-angiogenic therapies, which was previously thought to be impossible. As such, current research focuses on exploring chemopreventive approaches as part of combination strategies. One of the most investigated approaches in these combinational strategies is targeting the tumor cells themselves with chemotherapy together with chemopreventive agents.

In this thesis, we explore and address the therapeutic actions and potential of chemopreventive agents, either or not as part of a combinational approach with chemotherapeutic agents, where the role of the tumor itself and the tumors microenvironment is taken into account. In **chapter 2**, we used the anti-angiogenic agent endostatin as a treatment for human renal cell carcinoma RC-9 xenografts in nude mice and found this to cause significant disintegration of blood vessels and subsequent tumor necrosis. When examining the possible

molecular mechanism behind this effect, we found molecular evidence for the involvement of different targets within the tumor microenvironment, among others the inflammatory infiltrate by down regulation of a cbfa-1 expressing subset of granulocytes and the extracellular matrix by downregulation of FN. In **chapter 3** we addressed the difficulty in making a distinction between the effect of anti-angiogenic agents and vascular disruptive agents. For this we developed a specific new *in vitro* model which is able to distinguish between the onset of angiogenesis via measuring effects on endothelial precursors, ongoing angiogenesis and established vasculature. In doing so, this model could provide a great benefit in exploring the effect of newly developed drugs on tumor vasculature. In **chapter 4 and 5**, we explored the effect of two different treatment options combining a chemopreventive agent with a conventional chemotherapeutic agent. In **chapter 4** we explored the effect of the chemotherapeutic agent doxorubicin together with the chemopreventive agent and specific COX-2 inhibitor celecoxib. We found that celecoxib enhanced the anti-tumor potential of doxorubicin. The mechanism behind this was found to be that celecoxib can augment the intracellular accumulation of doxorubicin by a NF-kappa-B mediated mechanism. This showed a new mechanism by which celecoxib can interfere with tumor progression and provided new insights in the additional therapeutic potential of these combinational approaches. In **chapter 5**, we explored the effect of bisphosphonates together with the chemotherapeutic agent docetaxel on the establishment of breast cancer metastases in bone. In doing so, we found a synergistic effect of using this combinational approach and provided further evidence of the favorable role of using bisphosphonates as an adjuvant treatment in treatment of breast cancer metastases. This further showed the importance of not just interfering with tumor progression on the level of the tumor itself, but on its microenvironment as well.

Taken together, this thesis explores the use of chemopreventive approaches as monotherapy and in combinational approaches and explores and discusses their effects on both the tumor as the tumor's microenvironment. It shows that these combinational approaches hold great promise, if taking both the tumor as the tumor's microenvironment into account for its potential as therapeutic targets.

Samenvatting

Ondanks de grote spongen voorwaarts in de behandeling van kanker, blijft kanker wereldwijd een van de meest dodelijke ziekten. De belangrijkste systemische therapie in het bestrijden van kanker is chemotherapie. Sinds de ontdekking van chemotherapie wordt de behandeling echter gekenmerkt door resistentieproblematiek, waardoor de ontwikkeling van nieuwe en verbeterde therapieën van groot belang is.

De bestaande beperkingen van chemotherapie hebben geleid tot het ontwikkelen van nieuwe benaderingen in de behandeling van kanker om behandelingswinst verder uit te bouwen. Een van deze benaderingen is chemopreventie. Chemopreventie gaat uit van het principe dat in de ontwikkeling van een tumor verschillende fases zijn aan te wijzen die van essentieel belang zijn in de verdere progressie van de ziekte. Door in te grijpen in een van deze fases wordt de verdere progressie van de tumor voorkomen. Een voorbeeld van chemopreventie is anti-angiogenese therapie. De fases die van belang zijn voor een tumor om zich verder te ontwikkelen worden steeds duidelijker in kaart gebracht, waarbij het duidelijk wordt dat niet alleen de tumor zelf, maar ook de omgeving waarin de tumor zich ontwikkelt bijdraagt en ondersteunend is in zijn ontstaan en verdere groei. Ingrijpen in een of meerdere van deze essentiÎle fases op zowel het niveau van de tumor als zijn omgeving kan als aangrijpingspunt dienen voor nieuwe therapeutische methoden.

Chemopreventieve benaderingen hebben als monotherapie tot nu toe nog niet de beoogde klinische winst laten zien waarop gehoopt werd. Onderzoek richt zich daarom meer en meer op het toepassen van combinatie therapieën, waarvan chemotherapie met chemopreventieve benaderingen een van de meest onderzochte en inmiddels in de klinische praktijk toegepaste is.

In dit proefschrift bestuderen we het effect van verschillende chemopreventieve middelen al dan niet in combinatie met een chemotherapeuticum, waarbij niet alleen het effect op de tumor zelf maar ook op zijn omgeving bestudeerd en bediscussieerd wordt. In **hoofdstuk 2** tonen we aan dat behandeling met de angiogeneseremmer endostatine de groei van een experimenteel niercelcarcinoom in de muis remt door een significante desintegratie van de bloedvaten. Door het onderzoeken van het moleculaire mechanisme werd gevonden dat dit onder andere werd veroorzaakt door een effect op de tumoromgeving, waaronder een specifieke suppressie van het matrixeiwit fibronectine en een cbfa1 (core binding factor

alpha-1) expresserende subset van granulocyten, onderdeel van het inflammatoire infiltraat in de tumor.

Gezien het feit dat anti-angiogenese therapie nog niet de verwachte therapeutische resultaten laat zien wordt er steeds meer onderzoek gedaan naar zogenaamde 'vascular disruptive agents' (VDAs). VDAs pakken in tegenstelling tot angiogeneseremmers niet de ontwikkeling van nieuwe bloedvaten aan, maar zorgen voor de desintegratie van het reeds bestaande vaatnetwerk. De ontwikkeling van nieuwe VDAs kenmerkt zich door moeilijkheden in het maken van onderscheid tussen de effecten van angiogeneseremmers en VDAs. In **hoofdstuk 3** ontwikkelden we een nieuw screeningsmodel waarmee een specifiek onderscheid tussen het effect van VDAs en angiogeneseremmers kan worden gemaakt. Dit model is in staat onderscheid te maken tussen verschillende stadia van het ontstaan van een vaatnetwerk, zoals de start van het angiogenese proces via te meten effecten op endotheel precursors, het doorgaande angiogene proces en het effect op reeds bestaande vasculatuur. Dit model zou derhalve een grote rol kunnen hebben in de facalitering van de identificatie, nieuwe ontwikkeling en toepasssing van dit soort therapeutica.

In **hoofdstuk 4 en 5** wordt er verder ingegaan op de toepassing van combinatietherapieën van chemopreventie middelen met chemotherapeutica en de toepassing en het effect van deze combinatie op de tumor en zijn omgeving. In **hoofdstuk 4** wordt het effect van het chemopreventieve middel celecoxib onderzocht op de groei van mammacarcinoom in combinatie met het chemotherapeuticum doxorubicine. In dit hoofdstuk laten we zien dat celecoxib het effect van doxorubicine specifiek kan versterken, door een NF-kappa-B gemedieerde verhoging van de intracellulaire opname van doxorubicine. Deze nieuwe bevinding onderstreept de verscheidene mogelijkheden en therapeutische facetten in de toepassing van chemopreventieve middelen.

In **hoofdstuk 5** werd het effect van een combinatie van een bisfosfonaat, een botresorptieremmer, met het chemotherapeuticum docetaxel onderzocht op bestaande botmetastasen van mammacarcinoom. We toonden een synergistisch effect aan in de toepassing van de combinatietherapie, welke eens te meer aantoonde dat het niet alleen van belang is om de tumor zelf te remmen maar tevens in te grijpen op de omgeving waarin hij zich ontwikkelt.

Samenvattend onderzochten we in dit proefschrift het therapeutisch effect van chemopreventie benaderingen, zowel als monotherapie als in combinatietherpaie met

een chemotherapeuticum. Het laat zien dat dit soort combinatietherapie een grote belofte in de behandeling van kanker is, waarbij zowel de rol van tumor zelf maar ook die van zijn omgeving waarin hij zich ontwikkelt niet onderschat moet worden en van groot therapeutisch belang en potentieel is.

List of abbreviations

6-MP	6-mercaptopurine
ABC	ATP-binding cassette
AEC	amino-9-ethyl-carbazole
ALL	acute lymphoblastic leukaemia
BCRP	breast cancer resistance protein
BLAST	basic local alignment search tool
BLI	bioluminescent imaging
BMP	bone morphogenetic protein
BSP	bone sialoprotein
CA4P	combretastatin-analogue A4 phosphate
CAF	cancer-associated fibroblast
CBFA	core binding factor alpha
cDNA	complementary DNA
CHMP	committee for human medicinal products
COX	cyclo-oxygenase
CSC	cancer stem cell
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dox	doxorubicin
EC50	50% effective concentration
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial mesenchymal transition
FDA	food and drug administration
FGF	fibroblast growth factor
FN	fibronectin
HUVEC	human umbilical vein endothelial cell
IL	interleukin
IGF	insulin growth factor
IGFBP	insulin-like growth factor binding protein
IKK	I kappa B kinase
INF	interferon
LGTC	leiden genome technology center
MC	mast cell
MDR	multi-drug resistant
MMP	matrix metalloproteinase
MOS	mirror orientation selection
mRNA	messenger RNA
MRP	multi-drug resistant associated protein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NF	nuclear factor
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
OS	overall survival
OPN	osteopontin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDTC	pyrrolidinedithiocarbamate
P-gp	p-glycoprotein
PlGF	placental growth factor
PMN	polymorphonuclear cell
PTHrP	parathyroid hormone-related protein
RANKL	receptor activator of nuclear factor kappa B ligand
RLU	relative light units
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SMA	small molecule agent
SMC	smooth muscle cell

SSH	suppression subtractive hybridization
T/C	treatment vs control
TGF	transforming growth factor
TIMP	tissue metalloproteinase inhibitor
VDA	vascular-disruptive agents
VEGF	vascular endothelial growth factor
ZnMF	zinc-macrodex formalin

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Curriculum Vitae

De auteur van dit proefschrift werd 14 april 1977 geboren te Purmerend. Na het behalen van het gymnasium diploma aan het Newman College te Breda in 1995, begon hij in datzelfde jaar aan de opleiding Biomedische Wetenschappen aan de Universiteit Leiden alwaar in 2000 het diploma werd behaald. Datzelfde jaar begon hij aan het onderzoek op de afdeling Endocrinologie en stofwisselingsziekten van het Leids Universitair Medisch Centrum onder leiding van Prof. dr. C.W.G.M. Löwik wat uiteindelijk heeft geleid tot de behaalde resultaten als beschreven in dit proefschrift. Dit onderzoek werd onder andere gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, beurs: PGN 902-17-090) en het Koningin Wilhelmina Fonds (KWF, beurs: RUL2000-2196). Vanaf 2007 is hij werkzaam als Regulatory Project Leader (RPL) bij het College ter Beoordeling van Geneesmiddelen, waar hij sinds 2010 als senior RPL zijn werkzaamheden voortzet.

List of publications

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