universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

"Mechanism of breast cancer in vitro metastasis"

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer. nat.)

Verfasserin:	Caroline Vonach
Matrikel-Nummer:	0305373
Studienrichtung /Studienzweig:	A 437 Biologie A 442 Anthropologie
Betreuerin:	Ao. Univ. Prof. Dr. Sylvia Kirchengast

Wien, am 09.02.2010

Für meinen Vater
(† Juni 2009)

Danksagung

Ein besonderer Dank gilt meinem Betreuer Ao. Univ. Prof. Dr. Georg Krupitza für die interessante Themenstellung der Diplomarbeit, seine ausgezeichnete Betreuung während des gesamten Jahres und seine engagierte Unterstützung in wissenschaftlichen sowie in privaten Angelegenheiten.

Weiters möchte ich mich bei Ao. Univ. Prof. Dr. Sylvia Kirchengast und Ao. Univ. Prof. Dr. Wolfgang Mikulits für die Betreuung und Unterstützung während meiner Diplomarbeit bedanken.

Ganz besonders möchte ich mich bei Dr. Sibylle Madlener, Dr. Thanh-Phuong Vo Nha, Mag. Benedikt Giessrigl, Sabine Bauer und Mag. Katharina Viola für die ausgezeichnete Zusammenarbeit in unserer Arbeitsgruppe und die stets gute Atmosphäre im Labor bedanken. Ebenfalls bedanken möchte ich mich beim Team der Ebene 00 für die gute Zusammenarbeit.

Mein ganz persönlicher Dank gilt meinen Eltern, Edgar und Siegrid Vonach, die mir das Studium in Wien ermöglicht haben und mich während meiner gesamten Studienzeit immer unterstützt haben. Ein weiterer Dank gilt meinen Schwestern, Angelika und Susanne und meinem Schwager Andreas, die mir stets mit Rat und Tat zur Seite standen.

Abschließend möchte ich mich noch bei meinem Freund Martin Kiene für die stetige Unterstützung in den letzten Jahren bedanken.

Table of Contents

ABBREVIATIONS

AA	Arachidonic acid
BRCA	Breast cancer gene
CAM	Cell adhesion molecule
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
COX	Cyclooxygenases
CYP	Cytochrome P450
Di-GA	Di-galloyl-resveratrol
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth factor
GA	Gallic acid
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HER2	Human epidermal growth factor type 2
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HTLV-1	Human T-cell leukaemia virus 1
HUVECs	Human umbilical vein endothelial cell
IC_{50}	Concentration which inhibits by 50 %
ICAM	Intracellular adhesion molecule 1
IL-1	Interleukin 1
INK4	Inhibitors of Cdk4
LEC	Lymphendothelial cell
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MMP	Matrixmetalloproteinase
MT-MMP	Membrane-type matrixmetalloproteinase
NDGA	Nordihydroguaiaretic acid
NEAA	Non essential amino acid
NF-κB	Nuclear factor kappa B

PDGF	Platelet-derived growth factor
PECAM	Platelet-endothelial cell adhesion molecule 1
PG	Prostaglandins
PIC	Piceatannol
RB	Retinoblastoma tumor suppressor gene
RNA	Ribonucleic acid
RR	Ribonucleotide reductase
RHD	Rel homology domain
RV	Resveratrol
TNF	Tumor necrosis factor
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

Zusammenfassung

Wie in vorausgehenden Studien beschrieben wurde, ist Brustkrebsmetastasierung abhängig von der Aktivität von Lipoxigenasen, welche 12(S)-HETE generieren. 12(S)-HETE wird von MCF-7 Tumorzellspheroiden produziert und freigesetzt und induziert dabei eine Spaltformation in Lymphendothelzell Monolayers. Ein 3D Ko-Kultur System imitiert das Eindringen von Brustkrebszellen in das lymphatische Gefäßsystem und fasst den Mechanismus der Metastasierung zusammen.

In dieser Arbeit untersuchten wir einen spezifischen 12/15 Lipoxigenase Hemmer, Baicalein, und das strukturell gleich gebaute Wogonin um Spaltformationen zu hemmen.

Neben 12(S)-HETE haben wir auch NF-κB als wichtigen Mitwirkenden in der Spalformation identifiziert. Demzufolge testeten wir Verbindungen wie Di-galloyl-resveratrol, Resveratrol und Gallussäure sowie deren Derivate mit vermeintlicher Hemmwirkung von NF-κB in diesem 3D Metastasierungs Ko-Kultur Model.

Unter den synthetischen Drogen untersuchten wir auch natürlich vorkommende Substanzen, wie Hyperforin, welches ein Hauptbestandteil von *Hypericum perforatum* ist.

Die Aktivierung von NF-κB ist verbunden mit Zellproliferation, Zellüberleben, Zelleindringen und Angiogenese. Dies macht NF-κB zu einem interessanten Ziel in der Krebsbekämpfung.

Wir untersuchten einen irreversiblen Hemmer von NF-κB – Bay11-7082.

Aufgrund der Hemmwirkung von Bay11-7082 in der Spaltformation untersuchten wir die NF-κB vermittelte Lymphendothelzellbewegung, welche die ZEB-1 abhängige Expression von VE-cadherin und die Anhaftung von Tumorzellen an Endothelzellen miteinbezogen hat. Die Anhaftung von Tumorzellen an das Endothelium stellt den ersten Schritt der Metastasierung dar. Wir beschrieben die mögliche Rolle von drei NF-κB regulierten Ligand – Rezeptor Pärchen in Tumorzelladhesion an das Endothelium, ICAM-1/$\alpha_L\beta_2$, CD31/ $\alpha_V\beta_3$ und E-selectin/ CD44.

Summary

Ductual breast cancer metastasis depends on the expression and activity of lipoxygenases, which generate 12(S)-HETE. 12(S)-HETE is produced and released by MCF-7 tumor cell spheroids and induces gaps in a LEC monolayer. A 3D co-culture system mimics the invading of breast cancer cells to the lymphatic vasculature and recapitulates the mechanisms of metastasis. In this work I investigated a specific inhibitor of 12/15-Lipoxygenase, Baicalein, and the structurally highly related Wogonin on inhibition of gap formation.

Besides 12(S)-HETE, we could identify also NF-κB as a major player in MCF-7/LEC gap formation. Therefore, we tested compounds, such as Di-galloyl-resveratrol, Resveratrol and Gallic acid and their derivatives, with putative NF-κB inhibitory properties in this 3D metastasis co-culture model.

Among the synthetic drugs we also studied natural compounds, such as Hyperforin, a main constituent of *Hypericum perforatum*.

NF-κB activation is associated with tumor cell proliferation, survival, invasion and angiogenesis. This makes NF-κB an interesting target for the treatment of cancer.

We investigated an irreversibly inhibitor of NF-κB – Bay11-7082.

Due to the Bay11-7082 inhibitory effects on gap formation we studied the NF-κB mediated LEC motility, including ZEB1 dependent expression of VE-cadherin and the adhesion of tumor cells to the endothelial cells which represents the first important event in the process of metastasis. We described the possible role of three bonafide NF-κB-regulated ligand – receptor pairs in tumor cell adhesion to the endothelium, ICAM-1/$\alpha_L\beta_2$, CD31/ $\alpha_V\beta_3$ and E-selectin/ CD44.

1. Introduction

1.1. Hallmarks of Cancer

Hanahan and Weinberg described in the year 2000 six essential alterations in cell physiology that identify malignant growth.

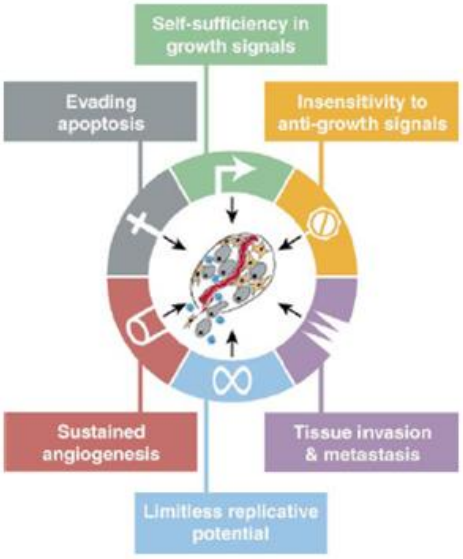


Figure I: The six hallmarks of cancer (Hanahan and Weinberg 2000)

1.1.1 Self-sufficiency in growth signals

Each normal cell needs a mitogenic growth signal (GS) before they can move from the G0/G1 state of the cell cycle into a proliferative state.

These signals are for example diffusible growth factors, extracellular matrix components and cell-to-cell adhesion molecules. They are transmitted into the cell by transmembrane receptors that bind these signals. Many of the oncogenes imitate the activation of such growth signals and the conclusion is that tumor cells can start the proliferation machine on their own (Hanahan and Weinberg 2000).

1.1.2 Insensitivity to growth-inhibitory (antigrowth) signals

In a normal tissue it is necessary to maintain cellular quiescence and tissue homeostasis. Growth-inhibitory signals operate to remain this and are received by transmembrane cell surface receptors coupled to intracellular signaling circuits. They can stop proliferation in two different mechanisms.

Some signals can force the cell to leave the active proliferate cycle and join the G0 raste state. They may reemerge in future when extracellular signals permit.

Other signals may induce the cells to cancel their proliferative potential by entering postmitotic states, which usually associated with acquisition of specific differentiation-associated traits (Hanahan and Weinberg 2000).

Loss of an antigrowth signaling circuitry leads to the development of cancer.

1.1.3 Evasion of programmed cell death (apoptosis)

Apoptosis represents a major source of cell attrition. To achieve homeostasis the rate of cell proliferation and cell attrition should be in balance. An insufficient amount of apoptosis results in uncontrolled cell proliferation, and further cancer.

Two major components are important for the apoptotic machinery. The sensors are necessary to control the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die (Hanahan and Weinberg 2000).

The effectors of apoptotic death are regulated from intracellular sensors. They activate the death pathway in response to detection abnormalities, including DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency or hypoxia (Evan and Littlewood 1998). Most and perhaps all types of cancer cells are able to evading apoptosis.

1.1.4 Limitless replicative potential

Self-sufficiency in growth signals, insensitivity to antigrowth signals and evasion of programmed cell death lead to an uncoupling of a cells growth program from signals in its environment. Many types of cells carry an intrinsic, cell-autonomous program that limits their multiplication. They stop growing after a certain number of

doublings. Tumor cells appear to be immortalized because they have a limitless replicative potential, which is essential for the development of their malignant growth (Hanahan and Weinberg 2000).

1.1.5 Sustained angiogenesis

Oxygen and other nutrients are supplied by the vasculature and they are important for cell function and survival. Thereby cells are obligating to reside within 100 µm of a capillary blood vessel. Because of this dependence on nearby capillaries, proliferating cells within a tissue would have an intrinsic ability to encourage blood vessel growth (Hanahan and Weinberg 2000).

The initiating signals for angiogenesis are the vascular endothelial growth factor (VEGFC) and acidic or basic fibroblast growth factors (FGF1/2). These factors bind to transmembrane tyrosine kinase receptors displayed by endothelial cells (Fedi et al. 1997, Veikkola and Alitalo 1999). In many tumors the expression of VEGF and/or FGF is increased.

1.1.6 Tissue invasion and metastasis

The final capability during tumorgenesis is tissue invasion and metastasis. These are complex processes, and their genetic and biochemical determinants are not completely understood.

In the development of most types of human cancer, cells remove from the primary tumor masses and move out, invade adjacent tissues and thence travel to distant sites to colonize new terrain in the body. This process is called metastases (Hanahan and Weinberg 2000).

Metastases, the distant settlements of tumor cells are the cause of 90% of human cancer deaths (Sporn 1996).

The activation of extracellular proteases and the binding specificities of cadherins, cell adhesion molecules (CAMs) and integrins are the most important steps for the acquisition of invasiveness and metastatic ability (Hanahan and Weinberg 2000).

1.2. Oncogenes and tumor suppressor genes

Most types of cancer occurs form mutations in genes that are involved in cell growth, differentiation and death. Oncogenes and tumor suppressor genes are two major classes of mutated genes that play a pivotal role in carcinogenesis.
An oncogene is a mutated gene and the produced protein of this oncogene has increased activity. Mutations in only one allele are sufficient for an effect.
In tumor suppressor genes the mutation caused a loss of function. Both alleles must be mutated (Pecorino 2008). In this chapter I want to give a short overview of the oncogenes and the most important tumor suppressor genes p53 and RB.

1.2.1 Oncogenes

The produced proteins of an oncogene control cell proliferation, apoptosis or both. Oncogenes can be activated by chromosomal rearrangements, mutations and gene amplification and can thereby cause a rearrangement in the oncogene structure, an increase in its expression or a deregulation of its expression (Bishop 1991).
The products of oncogenes include:

- growth factors: the product of the proto-oncogene c-sis is platelet-derived growth factor (PDGF). It is normally secreted by platelets and stimulates epithelial cells around the wound to proliferate and repair the damage. The oncogenic form is located in the cytoplasmic and leading to unregulated growth.
- growth factor receptors: the product of the proto-oncogene c-erbB is epidermal growth factor receptor (EGFR). This receptor is mutated in many tumors and triggers cell division in the absence of EGF.
- signal transducers: e.g. mutation in the ras gene leading to constitutive activation of RAS protein because of a loss of GTPase activity. Signals were continuously transducer and this induces limitless cell growth.
- transcription factors: e.g. AP-1 is an important regulator of cell growth, cell differentiation and cell death (Pecorino 2008).

- apoptosis regulators: BCL2 is up-regulated in many cancers and inhibit apoptosis (Adams and Cory 2007)

1.2.2 Tumor suppressor genes

RB (retinoblastoma tumor suppressor gene)

The retinoblastoma tumor suppressor gene was first detected in 1971 by Knudson. The major role of RB is to regulate the cell cycle by inhibiting the G1 to S-phase transition. Thereby it interacts with transcription factors such as E2Fs. These are important for regulating several genes which are essential for DNA metabolism and replication (Nevins 2001, Trimarchi and Lees 2002). The bound of RB to E2F resulted in inhibition of transcription and is regulated by cyclin-dependent kinases. Phosphorylation of RB by CDKs leads to the inactivation of RB and as a consequence to limitless cell proliferation. In the majority of cancers the RB protein is mutated or inactivated (Van den Heuvel and Dyson 2008).

p53 (Protein 53)

The tumor suppressor gene p53 was first detected in 1979 (Sherr 2004). It is a transcription factor and has a pivotal role in cellular response to stress signals, including DNA damage, oncogenic activation, hypoxia or irradiation (Vogelstein et al. 2000). Therefore the activation of p53 leads to cell cycle arrest or DNA repair, apoptosis or senescence. In normal tissues the p53 levels are very low. In tumor cells the p53 gene was either deleted or mutated (Baker et al. 1990).

1.3. Cell cycle

Disorders in the regulation of the cell cycle are important events in the development of cancer. Complex networks of regulatory factors decide whether cells proliferate or die. The DNA in every mammalian cell is under constant attack by agents, including solar radiation, polycyclic aromatic hydrocarbons and cigarette smoke etc. Cellular DNA-damage can result in the development of cancer (Meeran and Katiyar 2008).

1.3.1 Cell cycle progression

The cell cycle includes the duplication of cell contents and subsequent cell division. It consists of four distinct phases:

Gap1 phase (G1): cells preparing for DNA synthesis

S-phase (S): DNA synthesis and replication

Gap2 phase (G2): responsible for protein synthesis

M-phase (M): chromosome separation and cell division

The Gap0 phase is a resting state, in which cells do not grow and proliferate. Among these phases there are several checkpoints, which protect cells from cellular damage and stress signals and maintain the fidelity of DNA replication, repair and division (Pietenpol and Stewart 2002). One checkpoint is between G1 and S-phase, the next checkpoint is between G2 and M-phase and the last one is the spindle checkpoint in mitosis phase.

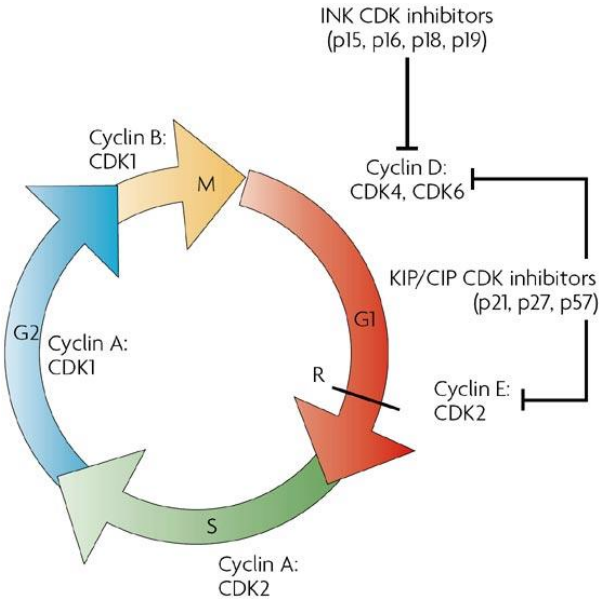


Figure II: Cell cycle control of eukaryotic cells. Key regulators are cyclin-dependent kinase complexes (CDKs) which ensure that all phases of the cell cycle are executed in the correct order. CDKs are activated when they become bound to regulatory proteins (cyclins). CDK activity is suppressed through interactions with inhibitory proteins (INK CDK inhibitors) (Dehay and Kennedy 2007).

1.3.2 Control of the cell cycle

Cellular responses to DNA damage result in the arrest of the cell cycle in order to provide time for DNA repair. Therefore the transition from one cell cycle phase to another is regulated by the activities of protein kinase complexes. Key regulators are cyclin-dependent kinase complexes (CDKs), which are activated when they become bound to regulatory proteins (cyclins). During cell cycle CDK4, CDK6, CDK2 (in G1 phase) CDK2 (in the S phase) and CDK1 (in G2 and M phase) are active. Their regulatory proteins, cyclins, can be divided in cyclin A, B, D1-D3 and E.

There are also cell cycle inhibitory proteins, called CDK inhibitors (CKI). They can bind to CDK alone or to the CDK-cyclin complex and regulate their activity. The CKI can be differentiating in two different families, the CIP and the INK4 family. The most important member of the CIP family is p21, which is under transcriptional control of the p53 tumor suppressor gene (Vermeulen et al. 2003).

1.4. Cell death

Cell death is a fundamental process in development, homeostasis and immune regulation. Dysregulation of cell death is often associated with numerous pathologies (Duprez et al. 2009). There are three types of cell death:

- The programmed cell death – apoptosis
- Necrosis
- Autophagic cell death

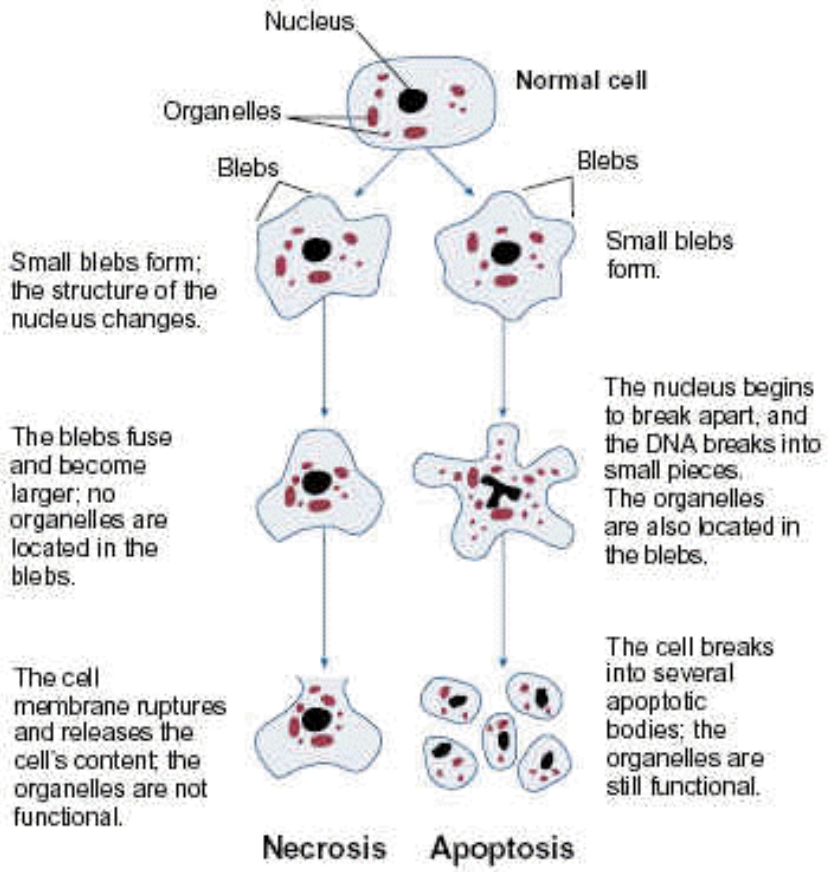


Figure III: The apoptotic and necrotic cell death process (Goodlett and Horn 2001).

1.4.1 Apoptosis

In 1972, the term apoptosis was described for the first time. It is a programmed suicide mechanism that results in the controlled breakdown of the cell into apoptotic bodies. These apoptotic bodies are recognized and engulfed by surrounding cells and phagocytes. Apoptosis is characterized by cell shrinkage, membrane blebbing and chromatin condensation (Duprez et al. 2009).

1.4.2 Necrosis

Necrosis is associated with cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space (Duprez et al. 2009).

1.4.3 Autophagic cell death

Autophagy allows eukaryotes to degrade and recycle cellular components. Proteins and organelles are excreted in specialized double-membrane vesicles, which are typical of autophagic cells. Many studies have revealed diverse functions of autophagy in maintenance of intracellular homeostasis, differentiation, development, longevity and immune defense. Massive autophagic activity could result in cellular demise (Maiuri et al. 2007, Duprez et al. 2009).

1.5. Breast cancer

Breast cancer is the most common cause of cancer death among women worldwide. Approximately 519.000 women died in 2004 due to breast cancer. Breast cancer is a disease of the developed world. 69 % of all breast cancer deaths occur in developing countries where the majority of cases are diagnose in late stages (WHO Global Burden of Disease 2004).

1.5.1 Risk factors of breast cancer

- The incidence of breast cancer increases rapidly with age.
- Breast cancer in one breast increases the risk of cancer in the other breast.
- The risk of breast cancer increases two fold if the mother, father, sister or daughter had breast cancer.
- Mutations in the gene BRCA1 or BRCA2 increase the risk of breast cancer
- The risk of breast cancer increased in women who had radiation therapy to the chest before age 30.
- The younger a woman is when she begins menstruating, the higher her risk of breast cancer.
- With increasing age at first full term pregnancy the risk of breast cancer is increased. Nulliparous women have an increased risk of breast cancer.
- Women who went through menopause at a late age and women who use hormonal therapy for the menopause are at higher risk of breast cancer.
- The risk of breast cancer is higher in women who are overweight or obese after menopause.
- Women who are physically inactive throughout life have a higher risk of breast cancer.
- The more alcohol a woman drinks, the greater her risk of breast cancer (http://www.cancer.gov/cancertopics/wyntk/breast/page4 last access 10 January 2010).

1.5.2 Treatment of cancer

There are different types of treatment for patients with breast cancer. Surgical resection is mostly the first step in treatment of breast cancer. After surgery patients get radiation therapy, chemotherapy or target-tailored therapy (e.g. Herceptin) to kill any cancer cells that are left. Thereby the prognosis and treatment options depend on the stage of cancer, the type of breast cancer and estrogen-receptor and progesterone-receptor levels in the tumor tissue. Other treatment options depend on how fast the tumor is growing, the age of the women, general health and menopausal status and whether the cells have high levels of human epidermal growth factor type 2 receptors (HER2/neu) (http://preview.cancer.gov/cancertopics/wyntk/breast/page8#f last access 10 January 2010).

60% of all drugs used in western medicine are derived from natural compounds (Cragg et al. 2006). Pharmaceutical companies collect marine organisms, bacteria, fungi and plants at random to test them in robot-screenings regarding their anti-cancer-, anti-inflammatory-, anti-bacterial- etc. activities. In Turkey a rich botanical biodiversity is found.

The genus *Scutellaria* has acquired considerable interest concerning anticancer activities (Yin et al. 2004). *Scutellaria baicalensis* is known as Huang-qin and is a most commonly used plant in Traditional Chinese Medicine (TCM). The root of *Scutellaria baicalensis* is use to treat various inflammatory conditions (Ma et al. 2005). Lee et al. 2008 have studied baicalein in detail and showed anticancer effects in human hepatoma cell lines (Himeji et al. 2007). Baicalein is reported to be a specific inhibitor of 12/15 lipoxygenases and is a major constituent of the plant *Scutellaria baicalensis* (Chen et al. 1999, Hsieh et al. 2007) and *Scutellaria orientalis* (Özmen et al 2009). The genus *Hypericum* is also used in different folk medicines worldwide and is tested against cancer cell growth. Hyperforin, a main constituent of *Hypericum perforatum* and *Hypericum adenotrichum* was shown to inhibit tumor growth, induce apoptosis of tumor cells and reduce tumor vascularisation (Schempp et al. 2005).

In my work I examine both these constituents of natural compounds and also synthetic compounds to study the treatment of cancer.

1.6. Metastasis mechanism

Metastasis is the cause of more than 90 % of all cancer deaths (Sporn 1996). It is the spread of tumor cells to distant sites, usually via the bloodstream, lymphatic system or through body spaces (Willis 1973).

Geiger and Peeper (2009) described the classical view on the metastatic cascade:

- Creation of a „premetastatic niche“
- EMT (epithelial-mesenchymal transition) and degradation of the basement membrane
- Dissociation of tumor cells from the bulk tumor
- Invasion of tumor cells into surrounding tissue
- Intravasation into blood and lymph vessels
- Transport through vessels
- Extravasation
- Establishment at a secondary anatomical site
- Outgrowth of secondary tumors

1.6.1 Epithelial-mesenchymal transition (EMT)

EMT is a biologic process that allows a polarized epithelial cell to undergo multiple biochemical changes to assume a mesenchymal cell phenotype. Carcinoma cells which are seen at the invasive front of primary tumors can get a mesenchymal phenotype. Thereby, the expression of proteins such as E-cadherin and γ-catenin can be lost and mesenchymal markers such as vimentin, S100A4, fibronectin, MMP-2 and MMP-9 can be acquired (Min et al. 2008, Zeisberg and Neilson 2009). This result in enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components (Kalluri and Neilson 2003).

Distinct molecular processes are necessary to initiate an EMT and enable it to reach completion.

EMT is classified in three different biological settings that carry various functional consequences. Type 1 EMTs are associated with implantation, embryogenesis and organ development. Type 2 EMTs are associated with wound healing, tissue regeneration and organ fibrosis.

The third type of EMT is the most important one for my diploma thesis. It is associated with cancer progression and metastasis (Kalluri and Weinberg 2009).

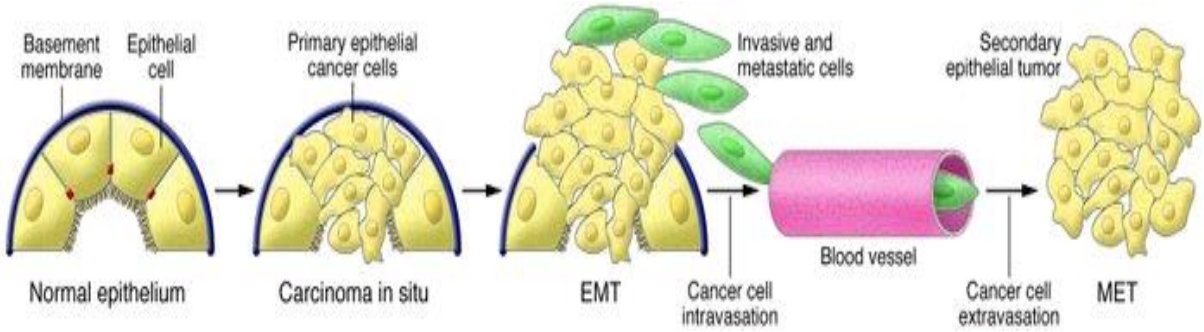


Figure IV: Type 3 of EMT. Epithelial cells losing their polarity and detaching from the basement membrane. The composition of the basement membrane is changing. Then, epithelial to mesenchymal transition permit cancer cells to enter the circulation and exit the blood stream. There the cancer cells may form micro- and macro-metastases (Kalluri and Weinberg 2009).

1.7. Arachidonic acid metabolism

The arachidonic acid metabolism leads to the generation of various biologically active eicosanoids. They have been shown to regulate numerous aspects of tumor development.

Three types of enzymes can metabolize arachidonic acid (AA). These enzymes are cyclooxygenases (COX), epoxygenases (cytochrome P450) and lipoxygenases (LOX). They insert oxygen at different positions in AA to generate eicosanoids (Moreno 2009, Pidgeon et al. 2007).

1.7.1 Lipoxygenases

5-LOX, leukocyte and platelet-type 12-LOX and reticulocyte-type 15-LOX-1 are the most studied LOX enzymes. These four LOXs metabolize arachidonic acid (AA) to the hydroperoxyeicosatetraenoic acids (HPETEs) which are subsequently converted to hydroxyeicosatetraenoic acid (HETE) (Moreno 2009, Pidgeon et al. 2007).

Type	Synthesize	
5-LOX	5(S)-HETE	Epithelial cancers (breast cancer, prostate, lung etc)
Platelet-type 12-LOX	12(S)-HPETE 12(S)-HETE	Smooth muscle cells, keratinocytes, endothelial cells and tumor cells
Leukocyte type 12-LOX	15(S)-HETE 12(S)-HETE	
15-LOX-1	13-(S)-HODE 15-(S)-HETE	Reticulocytes, eosinophils and airway epithelial cells, macrophages, atheriosclerotic lesions
15-LOX-2	15-HETE	

(Moreno 2009, Pidgeon et al. 2007)

12(S)-HETE

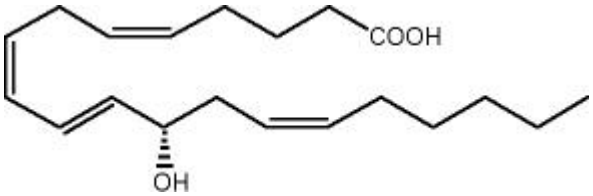


Figure V: Chemical structure of 12(S)-hydroxyeicosatetraenoic acid

12(S)-HETE is the 12-lipoxygenase metabolite of arachidonic acid and induces a reversible retraction of cultured endothelial cells. 12(S)-HETE have an important role in facilitating cancer metastasis (Chen et al. 1994, Honn et al. 1994).

12(S)-HETE is produced and released by MCF7 tumor cell spheroids and induced gaps in LEC monolayer (Kerjaschki et al. 2010). In our study we used 1 µM 12(S)-HETE to stimulates LECs. This mimics the 3D co-culture system of MCF-7 spheroids and LECs and further the invading of breast cancer cells to the lymphatic vasculature (Uchide et al. 2007, Kerjaschki et al. 2010, Kramer and Nicolson 1979).

Numerous of plant phenols, such as Baicalein, Resveratrol and Nordihydroguaiaretic acid (NDGA) are known for their chemo-preventive and LOX-inhibitory activity in many tumor models (Bednar et al. 2006). Wang et al. (2010) showed the strong anti-proliferative effect of Baicalein on MCF-7 human breast cancer cells.

Baicalein (figure VI, left) is a major constituent of the plant *Scutellaria baicalensis* (Chen et al. 1999, Hsieh et al. 2007) and *Scutellaria orientalis* (Özmen et al. 2009). It is reported to be a specific inhibitor of 12/15-LOX (Chen et al. 1999, Hsieh et al. 2007) and the root of *Scutellaria baicalensis* is use to treat various inflammatory conditions (Ma et al. 2005). Ductual breast cancer metastasis was shown to depend on the expression and activity of lipoxygenases in particular 12-LOX and to a minor extent 15-LOX. Hence, we assumed that Baicalein, the specific inhibitor of 12/15-LOX, have a strong inhibitory effect on gap formation.

In fact, figure VI (right) shows an inhibition of gap formation with a maximum inhibition of about 60 %.

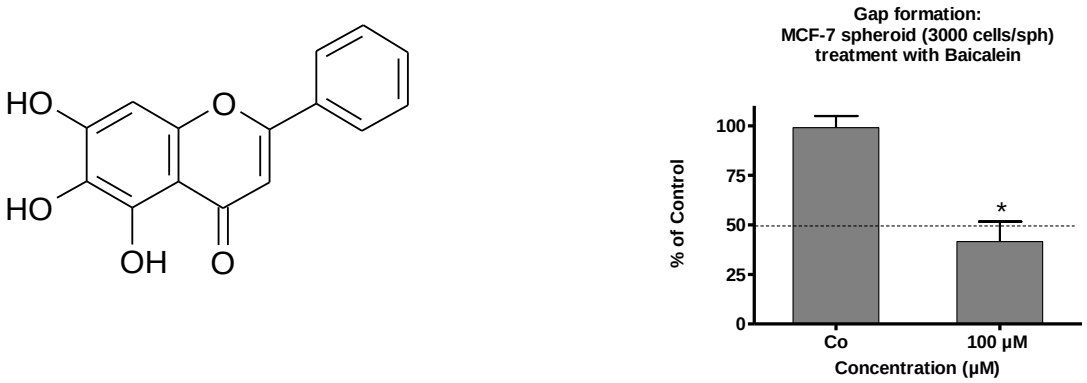


Figure VI: Chemical structure of Baicalein (left) and gap formation assay (right). LECs were seeded in 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within the following two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 100 µM) of Baicalein for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LECs monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$)

1.7.2 Cyclooxygenases

There are two isoforms of cyclooxygenases (COX). COX-1 is expressed in most tissues and appears to generate prostaglandins (PG). Thus control platelet aggregation, regulation of renal blood flow and maintenance of the gastric mucosa. COX-2 increased rates of PG formation during tissue injury and repair (Fürstenberger et al. 2006). It is shown as a promising target for chemoprevention and treatment of several cancers (Fürstenberger et al. 2006, Krysan et al. 2006).

The cyclooxygenases pathway metabolizes arachidonic acid to the unstable cyclic endoperoxides PGG_2 and PGH_2. In the next step they are converted to prostaglandins and thromboxanes by tissue specific synthases (Pidgeon et al. 2007).

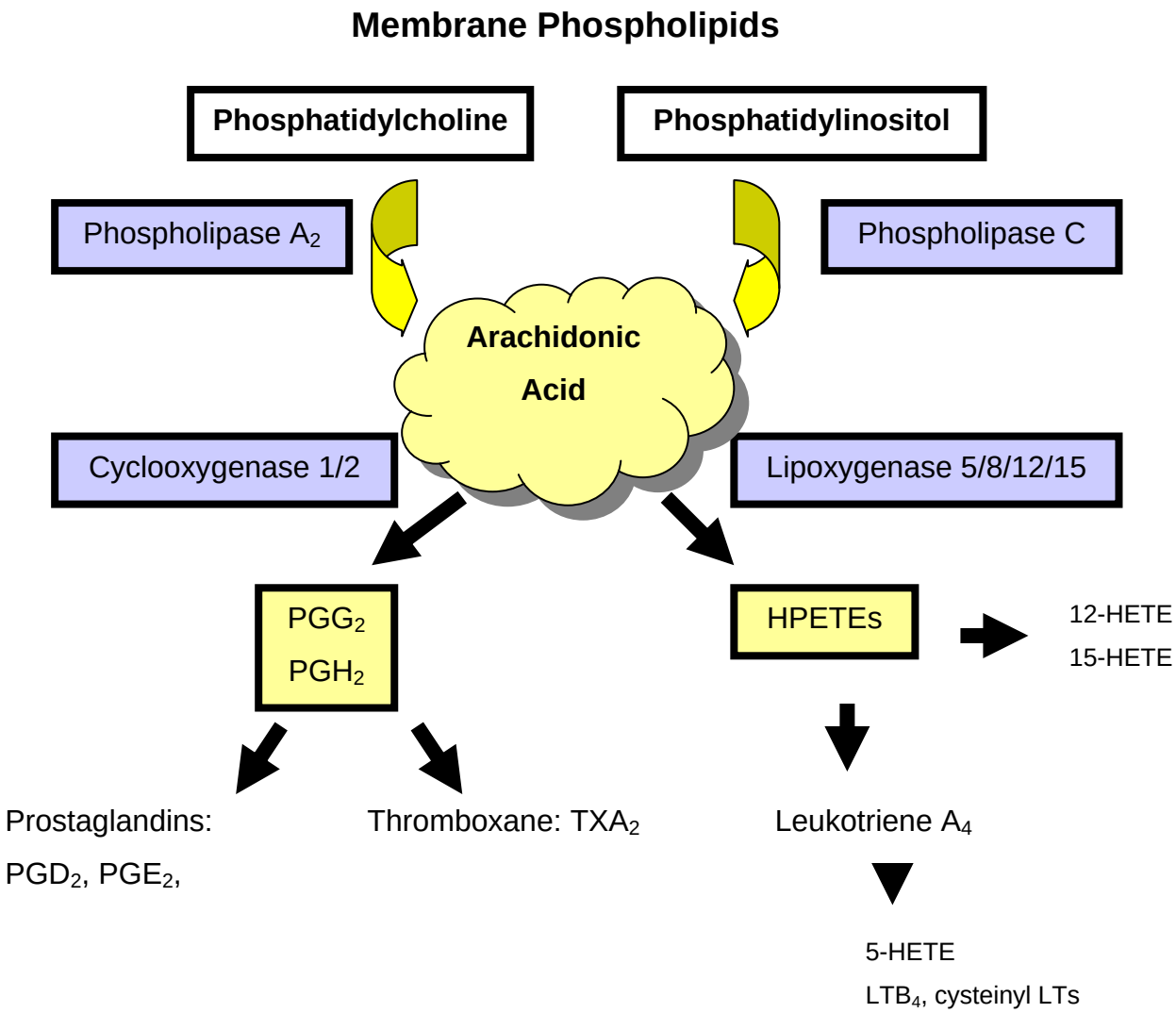


Figure VII: Lipoxygenase- and cyclooxygenase-catalyzed arachidonic acid metabolism.

1.7.3 Epoxygenases (Cytochrome P450)

Cytochrom P450 (CYP) proteins also metabolize arachidonic acid to produce 9-, 11-, 16-, 17-, 18-, 19- and 20-HETEs. CYP are primarily expressed in the liver but they are also detected in the heart, vasculature, gastrointestinal tract, kidney, lung, in vascular smooth muscle cells and endothelium (Scarborough et al. 1999, Lin et al. 1996).

1.8. Matrixmetalloproteases

Matrixmetalloproteinases (MMPs) have a crucial role in late stages of tumor progression. Tumor cell migration, invasion and metastasis are controlled by MMPs through ECM (extracellular matrix) degradation (Chabottaux and Noel 2007). MMPs are multidomain proteins which can degrade virtually all ECM components and activate (or inactivate) and release a number of modulators of cell functions (Cauwe et al. 2007, Overall and Dean 2006, Egeblad and Werb 2002, Greenlee et al. 2007). Most of the MMPs are secreted as soluble enzymes but there are also membrane-type MMPs (MT-MMPs). They are associated with the cell membrane (Zucker et al. 2003). In vitro studies showed the effect of MMPs in tumor growth, angiogenesis and metastasis (Overall and Dean 2006, Noel et al. 2007). MMPs are overexpressed in human cancer tissues (Egeblad and Werb 2002). MMP-1, -2, -9, -11 and MT1-MMP are the most common in breast cancer tissues (Chabottaux and Noel 2007).

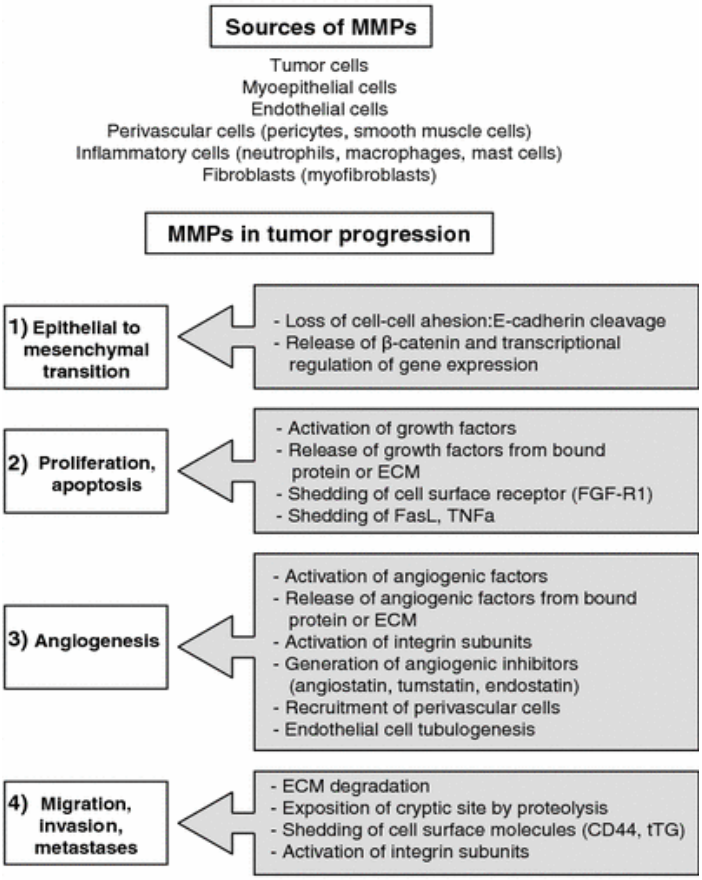


Figure VIII: Sources of MMPs and implication in tumor progression (Chabottaux and Noel 2007).

1.9. NF-κB

NF-κB is a transcription factor and plays a major role in inflammation, cell survival, transformation and cancer. NF-κB seems important in regulating the transcription of cytokines, adhesion molecules and other mediators (Sun and Andersson 2002). Five NF-κB members in mammals are identified: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and p105), and NF-κB2 (p52 and p100) (Ghosh and Karin 2002). These NF-κB/Rel proteins can exist as homo- or heterodimers and share a highly conserved 300 amino acid long N-terminal Rel homology domain (RHD). It is responsible for DNA binding, dimerization and association with the I-κB inhibitory proteins (Gosh et al. 1998). In the cytoplasm NF-κB exist in an inactive form that is bound to I-κB. The most important ones of these inhibitory proteins are I-κBα, I-κBβ and I-κBε. These I-κB family members share ankyrin-like repeats, which are protein-protein interaction domains that interact with NF-κB via the RHD (Ghosh et al 1998).

1.9.1 NF-κB activation and inhibition

In cells NF-κB can be activated by a wide variety of stimuli associated with stress or injury. Inducers of NF-κB are cytokines such as IL-1β and TNF-α, bacterial and viral products such as LPS, sphingomyelinase and the double-stranded RNA. Other inducers of NF-κB are the Tax protein from human T-cell leukaemia virus 1 (HTLV-1), proapoptotic and necrotic stimuli such as oxygen free radicals, and ultraviolet light and γ-irradiation (Tran et al. 1997). NF-κB is associated with the signal-induced proteolytic degradation of I-κB in the cytoplasm. Proinflammatory cytokines such as IL-1β and TNF-α initiate a signalling cascade leading to activation of two I-κB kinases (IKK), IKKα and IKKβ, which phorsphorylate I-κB at specific amino-terminal serine residues (Zandi et al. 1997). Phosphorylated I-κB is then ubiquitinated and this phosphorylated and ubiquitinated I-κB, which is still associated with NF-κB in the cytoplasm, is selectively degraded by the 26S proteasome. This process was associated with the translocation of NF-κB to the nucleus. There it binds its target genes to initiate transcription (Sun and Andersson 2002).

NF-κB protein activity is regulated by two major pathways. NF-κB proteins can form homo-and heterodimers. RelA (p65), RelB, c-Rel, NF-κB1 (p50 and p105), and NF-κB2 (p52 and p100) are the five NF-κB members which were identified (Ghosh and Karin 2002). The classical pathway leading to activation of p50/RelA or p50/c-Rel and the alternative pathway leading to activation of p52/RelB or p50/RelB (Min et al. 2008, Cao and Karin 2003).

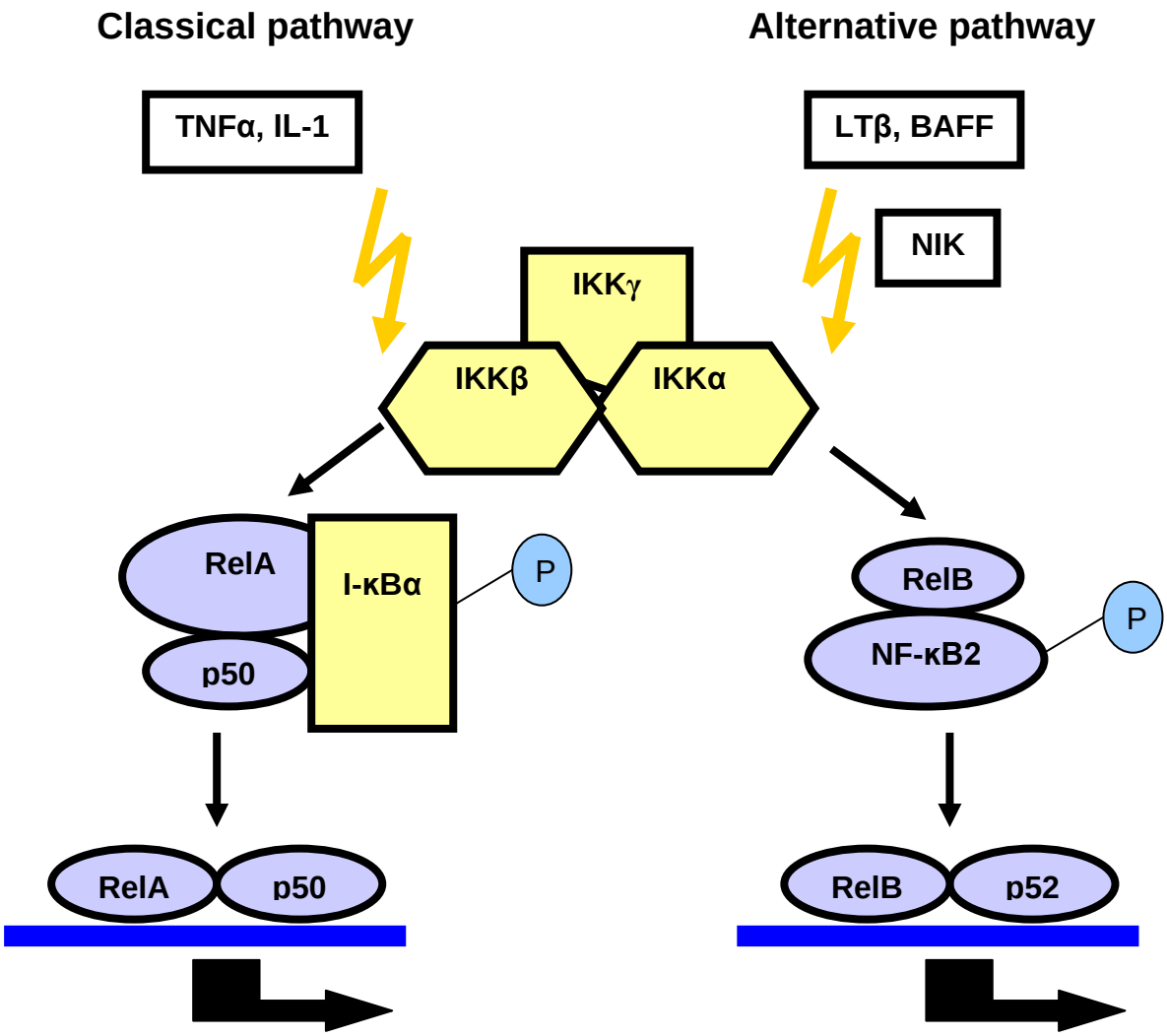


Figure IX: Two major pathways of NF-κB activation. Classical pathway: Stimuli such as TNFα and IL-1 activate the IKK complex, which phosphorylates the I-κB protein. This process triggers the upiquitin-dependent degradation of I-κB by the 26S proteasome and results in nuclear translocation and of NF-κB1 (p105) which is processed to p50 and bound to RelA (p65) and I-κB in the cytoplasm. Alternative pathway: Stimuli such as LTβ (lymphotoxin β receptor) and BAFF lead to activation of NF-κB inducing kinase (NIK). NIK induces IKKα which activates the processing of p100/RelB into NF-κB2 p52/RelB via the proteasome (Cao and Karin 2003, Brown et al. 2008).

1.9.2 NF-κB and inflammatory disease and cancer

Nuclear factor kappa B plays a crucial role in inflammatory diseases such as rheumatoid arthritis and asthma. The production of IL β, TNF-α and Interleukin 6 is increased in these patients.

The activation of NF-κB increases the expression of many genes involved in immune and inflammatory responses (Epstein et al. 1997).

In all chronic inflammatory diseases adhesion molecules hire inflammatory cells such as neutrophils, eosinophils and T-lymphocytes from the circulation to the site of inflammation (Albelda et al. 1994). NF-κB regulates the expression of such genes that encode adhesion molecules. An important aspect of NF-κB in these diseases is the fact that it is activated by inflammatory cytokines and it induces the expression of inflammatory cytokines (Epstein et al. 1997).

Numerous studies report that NF-κB could also be important in breast carcinogenesis (Cogswell et al. 2000, Nakshatri et al. 1996, Sovak et al. 1997). Thereby NF-κB activation has been linked to tumor progression via stimulation of cell proliferation, prosurvival, angiogenesis pathways and metastasis (Haffner et al. 2006).

2. Material and Methods

2.1. Material

2.1.1 Chemicals

Digalloylresveratrol, Gallic acid, Amidox, Didox, Trimidox, Resveratrol, M5 and M8 were from Thomas Szekeres. Piceatanol was purchased from Sigma (MO, USA).

Wogonin Cat# 681670 was purchased from Calbiochem (Darmstadt, Germany). Baicalein (El-106) and Bay11-7082 Cat# EI-278 were from Biomol (Hamburg, Germany). Hyperforin was purchased from Sigma (MO, USA). 12(S)-HETE Cat# 34570 was purchased from Cayman Chemical (MI, USA).

Monoclonal mouse anti-CD31 (JC70A) Cat# M0823 was purchased from DAKO (Glostrup, Denmark). Rabbit polyclonal anti-ZEB1 (H-102) sc-25388 and mouse monoclonal anti-α_V Integrin (P2W7) sc-9969, mouse monoclonal anti-β_2 Integrin (CTB104) sc-8420 and mouse monoclonal anti-α_L Integrin (CRIS-3) sc-59741 were from Santa Cruz (CA, USA). Monoclonal mouse anti-β-Actin Cat# A5441 (AC-15) was purchased from Sigma-Aldrich (Munich, Germany).

Rabbit anti-S100A4 was purchased from Sigma (MO, USA). Mouse monoclonal E-selectin (CD62E) (BBIG-E4(5D11)) Cat# BBA16, was from R&D Systems (MN, USA). Mouse monoclonal antibody against Integrin β_3 (CD61) (NCL-CD61-308) was purchased from Novocastra Laboratories Ltd. (Newcastle, UK). Mouse monoclonal anti-CD54 (ICAM-1) was purchased from Immunotech (Marseille, France). VE-cadherin (PN-IM1597) was purchased from Beckman Coulter (CA, USA). Mouse monoclonal CD44 (HCAM Ab-2 Clone 5F12) Cat# MS178-P0 was purchased from Thermo Scientific (MA, USA).

PVDF-membrane was from GE-Healthcare (Amersham, Buckinghamshire, UK). Pierce-ECL Western Blotting Substrate Prod# 32106 was from Thermo Scientific (Portsmouth, USA). Hyperfilms ECL - High performance chemiluminescence film was from GE-Healthcare (Amersham, Buckinghamshire, UK). Polycolonal rabbit anti-mouse IgG and anti-rabbit were from Dako (Glostrup, Denmark). Lab-Tek II chambered coverglasses (*155382, size 4-well) were purchased from Nalge Nunc International (Wiesbaden, Germany).

Alexa-Fluor 488 (green) goat-anti-rabbit and Alexa-Fluor 594 (red) goat-anti-mouse labelled antibodies were purchased from Molecular Probes, Invitrogen (Karlsruhe, Germany).

2.1.2 Cell culture

Two different cell types were used. MCF-7 mock cells were grown in MEM medium (Gibco #61100-087, Invitrogen, Karlsruhe, Germany) supplemented with 10 % FCS, 1% Penicillin/Streptomycin, 1% NEAA (Gibco #11140-035 Invitrogen, Karlsruhe, Germany), 150 µg Hygromycin B (Roche #10843555 Vienna, Austria) at 37°C in a humidified atmosphere containing 5% CO_2.

LECs (telomerase immortalized human lymphendothelial cells; T1S1) were grown in EGM 2 MV (Clonetics #CC-4147) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Methods

2.2.1 Proliferation-Assay

To determine the anti-proliferative effect of different chemicals MCF-7 cells were seeded at a concentration of 1 x 10^4 cells per ml into 24 well plates and grown for 24 h to enter a logarithmic growth phase. Then they were incubated with increasing concentrations of the different chemicals (5, 25, 50, 75 µM) for 72 h. After 48 h and 72 h the cells had to be washed with PBS, trypsinised and then they were counted with a Sysmex microcellcounter (Kobe, Japan). IC_{50} values for the chemicals were determined. All experiments were done in triplicate.

Calculation to determine the percent of cell divisions compared to the untreated control:

$[(C_{72\ h\ +\ drug} - C_{48\ h\ +\ drug}) / (C_{72\ h\ -\ drug} - C_{48\ h\ -\ drug})] \times 100 = \%$ cell division

$C_{72\ h\ +\ drug}$ ------ cell number after 72 h of drug treatment (5, 25, 50, 75 µM)

$C_{48\ h\ +\ drug}$ ------ cell number after 48 h of drug treatment (5, 25, 50, 75 µM)

$C_{72\ h\ -\ drug}$ ------ cell number after 72 h without drug treatment

$C_{48\ h\ -\ drug}$ ------ cell number after 48 h without drug treatment

2.2.2 MCF-7 spheroid generation

MCF-7 mock cells were transferred to 30 ml MEM medium with hygromycin B containing 20 % methylcellulose (6 ml). 150 µl of this cell suspension were transferred to each well of a 96 well plate (greiner bio-one, Cellstar 650185) to allow spheroid formation within the following two days.

2.2.3 MCF-7 spheroid/LEC monolayer co-cultivation

Method 1:

LECs were seeded in 2 ml EGM 2 MV medium into 24 well plates (costar 3524) and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution, Invitrogen, Molecular Probes #C2925) for 1 hour at 37°C. MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of chemicals for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LECs monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured.

Method 2:

MCF-7 tumor spheroids were grown as described above (MCF-7 spheroid generation). LECs were seeded in 2 ml EGM 2 MV medium into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 h at 37°C. Different settings of MCF-7 spheroid/LEC inhibition or co-inhibition were used in one experiment.

- As negative control MCF-7 spheroids were treated with DMSO for 30 min at 37°C and then they were transferred to the 24 well plate containing LECs.
- MCF-7 spheroid/LEC co-inhibition: MCF-7 spheroids were treated with 10 µM Bay11-7082 for 30 min at 37°C and then they were also transferred.

- MCF-7 spheroid inhibition: MCF-7 spheroids were treated with 10 µM Bay11-7082 for 30 min at 37°C and afterwards washed with EGM 2 MV medium. Then, the spheroids were transferred to the 24 well plate.
- LEC inhibition: LECs were treated with 10 µM Bay11-7082 for 30 min at 37°C. In the next step LECs were washed with EGM 2 MV and untreated MCF-7 spheroids were transferred on the top of the cells.

After the four transfer steps of treated, or in the fourth case, untreated MCF-7 spheroids the MCF-7 spheroid/LEC co-cultures were incubated for 4 h at 37°C. Then, the size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured.

Method 3:

Lab-Tek II chambered coverslips (*155382, size 4 well) were coated with 10 µg/ml Fibronectin for 1 h at room temperature. MCF-7 spheroids were grown as describe above (MCF-7 spheroid generation). LECs were seeded in 1 ml EGM 2 MV on chambered coverslips and allowed to grow for 2 days.

Different settings of MCF-7 spheroid/LEC inhibition or co-inhibition were used in one experiment.

- As negative control MCF-7 spheroids were treated with DMSO for 30 min at 37°C and then they were transferred to the 24 well plate containing LECs.
- MCF-7 spheroid/LEC co-inhibition: MCF-7 spheroids were treated with 10 µM Bay11-7082 for 30 min at 37°C and then they were also transferred.
- MCF-7 spheroid inhibition: MCF-7 spheroids were treated with 10 µM Bay11-7082 for 30 min at 37°C and afterwards washed with EGM2MV medium. Then, the spheroids were transferred to the 24 well plate.
- LEC inhibition: LECs were treated with 10 µM Bay11-7082 for 30 min at 37°C. In the next step LECs were washed with EGM 2 MV and untreated MCF-7 spheroids were transferred on the top of the cells.

After the incubation time of 4 h the cells were washed with ice cold PBS and fixed in 4 % paraformaldehyd for 15 min at room temperature. Cells were immunostained with various antibodies and analyzed with confocal microscope.

2.2.4 Immunofluorescence

Cells were washed with ice cold PBS and fixed in 4 % paraformaldehyd for 10 min at room temperature. In the next step the cells were washed three times with PBS and permeabilised with 0.1 % Triton X-100 in 1X PBS for 30 min at room temperature. Then, cells were washed again three times with PBS and blocked for 1 hour with 10 % goat serum diluted in BSA PBS. Thereafter, the cells were incubated with the primary antibody (dilution 1:50 – 1:400 depend on the antibody in 2 % BSA/PBS) for 1 hour at room temperature. After the incubation time of 1 hour cells were washed again with PBS for three times. Then, cells were incubated with fluorescence labelled second antibody (dilution 1:1000 in 2% BSA/PBS) for 1 hour at room temperature in the dark and washed with PBS. In the last step cells were counterstained for 4 min with the nuclear stain DAPI (dilution 1:50.0000) at room temperature and afterwards cells were covered with PBS and stored at 4°C for further analysis.

2.2.5 Western Blotting

MCF7 and LECs were seeded in 6 cm dishes. (Lysate1) MCF7 cells were treated with 10 µM Bay11-7082 for 30 min at 37°C. (Lysate2) LECs were treated with 1µM 12SHETE for 0.2, 0.5, 2, 4 and 8 h. (Lysate3) LECs were pre-treated with 10 µM Bay11-7082 for 30 min and thereafter stimulated with 1 µM 12(S)-HETE for 0.2 and 0.5 h at 37°C.

Then, the cells were washed twice with ice cold PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 0,1 % Triton-X100, 1 mM phenylmethylsulfonylfluorid (PMSF) and protease inhibitor cocktail (PIC). Afterwards the lysate was centrifuged at 12000 rpm for 20 min at 4°C and the supernatant was stored at -20°C until further analysis.

Equal amounts of protein samples were separated by polyacrylamide gel electrophorese (PAGE) and electro-transferred onto Hybond PVDF-membranes (Amersham, Buckinghamshire, UK) at 100V for 1 h at 4°C. To control equal sample loading, membranes were stained with Ponceau S.

After washing with PBS/T (Phosphate Buffered Saline/Tween 20; pH: 7.2) or TBS/T (Tris Buffered Saline/Tween 20; pH: 7.6) membranes were blocked in

blocking solution (5 % non-fat dry milk in TBS containing 0.1 % Tween or in PBS containing 0.5 % Tween 20) for 1 h at room temperature.
Then membranes were washed carefully and incubated with the first antibody (in blocking solution; dilution 1:500 – 1:1000) by gently rocking at 4°C overnight or 1 h at room temperature.
Thereafter, the membranes were washed another time with PBS/T or TBS/T and continuing incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG; dilution 1:2000) for 1 h at room temperature. Chemiluminescence was detected by ECL detection Kit and the membranes were exposed to Amersham Hyperfilms.

2.2.6 Transient transfection with siRNA

LECs were grown in 6 well plates to 70 % confluence in complete medium (EGM 2 MV). Then, they were transfected using RNAiFect^{TM} (Qiagen # 301607 Hamburg, Germany). 5 µg siRNA (siZEB1 RNA and scrambled RNA were from Prof. Andreas Eger) were diluted in culture medium, containing serum and antibiotics (final volume 100 µl). 15 µl of RNAiFect transfection reagent was added to the diluted siRNA and incubated for 15 min at room temperature. Then the mix was added to the cells and incubated for 8 h at 37°C. Thereafter the medium was changed and the cells were incubated further 48 h.

2.2.7 Apoptose Assay – Hoechst 33258 and propidium iodide double staining

To measure apoptosis in LECs, cells were grown in 96 well plates (200 µl medium/well) to 40 % confluence and then treated with 100 µM Baicalein, 1 µM 12(S)-HETE and DMSO for 2, 4 and 24 h. Hoechst and propidium iodide (final concentrations 5 µg/ml and 2 µg/ml) were added to the culture medium for 1 h at 37°C. Thereafter, stained cells were examined under a fluorescence microscope with DAPI filter, photographed, analyzed and counted manually. Experiments were done in triplicate.

2.2.8 Real-time PCR

LECs were seeded in 12 well plates and then they were pre-treated with 10 µM Bay11-7082 for 30 min and thereafter stimulated with 1 µM 12(S)-HETE or with TNF-α (20ng/ml). Total RNA was isolated using the RNeasy Mini Kit 50 and QIAshredder 50 (Cat# 74104; Cat# 79654 QIAGEN, Hamburg, Germany). 1 µg of total RNA was reverse transcribed with Superscript First Strand Synthesis System (Cat.No. 11904-018; Invitrogen, Karlsruhe, Germany) and the resulting cDNA was amplified using TaqMan Universal PCR Master mix (No AmpErase UNG; PartNo 4324018; Applied Biosystems Vienna, Austria) with the E-selectin Primer (TaqMan Gene Expression Assays Part No 4331182; Applied Biosystems Vienna, Austria). PCR products were analyzed on the Abi Prism 7000 sequence detection system (Applied Biosystems). Duplicate samples were analyzed in parallel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. Relative transcript expression was calculated using the $\Delta\Delta C_T$ method.

2.2.9 Statistical analysis

Dose-response curves were analyzed using Prism 4 software (CA, USA) and significance was determined by student t-test.

3. Results

Effect of Baicalein and the structurally highly related Wogonin on inhibition of gap formation

Ductual breast cancer metastasis was shown to depend on the expression and activity of lipoxygenases in particular 12-LOX and to a minor extent 15-LOX, which generate 12(S)-HETE, a metabolite of arachidonic acid. 12(S)-HETE is produced and released by MCF-7 tumor cell spheroids and induces gaps in a LEC monolayer (Kerjaschki et al. 2010). A 3 dimensional (3D) co-culture system mimics the invading of breast cancer cells to the lymphatic vasculature and recapitulates the mechanism of metastasis (Kramer and Nicolson 1979, Uchide et al. 2007, Kerjaschki et al. 2010). Baicalein is reported to be a specific inhibitor of 12/15 lipoxygenases and is a major constituent of the plant *Scutellaria baicalensis* (Chen et al. 1999, Hsieh et al. 2007) and *Scutellaria orientalis* (Özmen et al 2009). Another constituent of these plants is Wogonin which is structurally highly related to Baicalein (figure 1). Therefore, Wogonin was also tested in the gap formation assay and compared to Baicalein. The IC_{50} of Wogonin (75 µM) is lower than the IC_{50} value of Baicalein (100 µM).

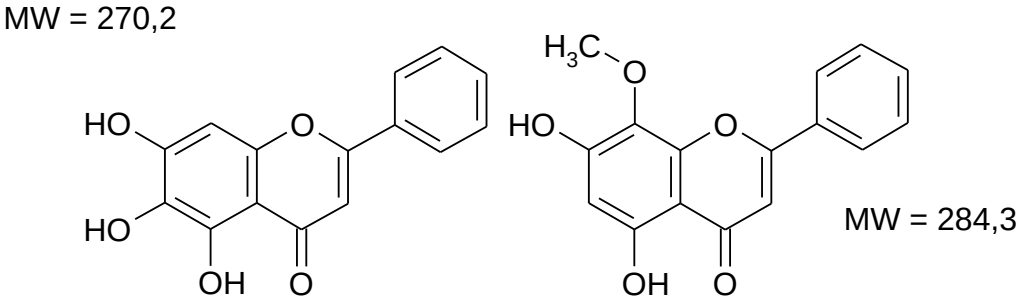


Figure 1) Chemical structure of Baicalein (left) and Wogonin (right) and molecular weight.

2)

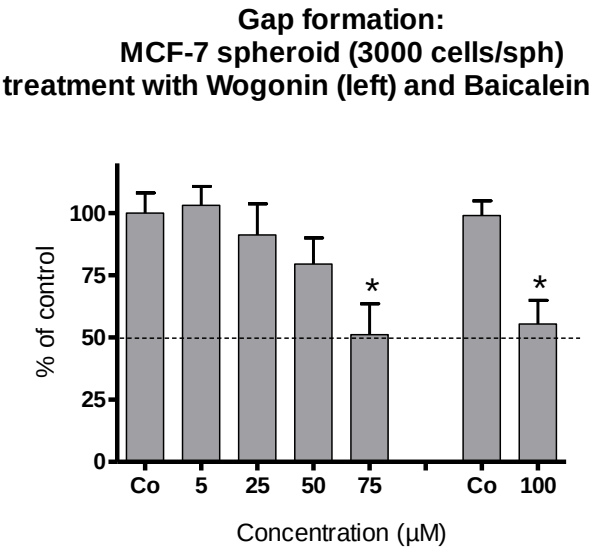


Figure 2) Gap formation inhibition assay: LECs were seeded in 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within the following two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of Wogonin and (solvent, 100 µM) Baicalein for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LECs monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

Induction of apoptosis in LECs by Baicalein

To determine the apoptotic effects of 100 µM Baicalein and 1 µM 12(S)-HETE in LECs, cells were seeded in a 96 well plate and treated as mentioned in the chapter methods. In figure 3 we see that treatment of LECs with 100 µM Baicalein for 24 h induces apoptosis in approximately 80 % of the cells. In contrast to Baicalein 1 µM 12(S)-HETE treated LECs showed no significant effect.

3)

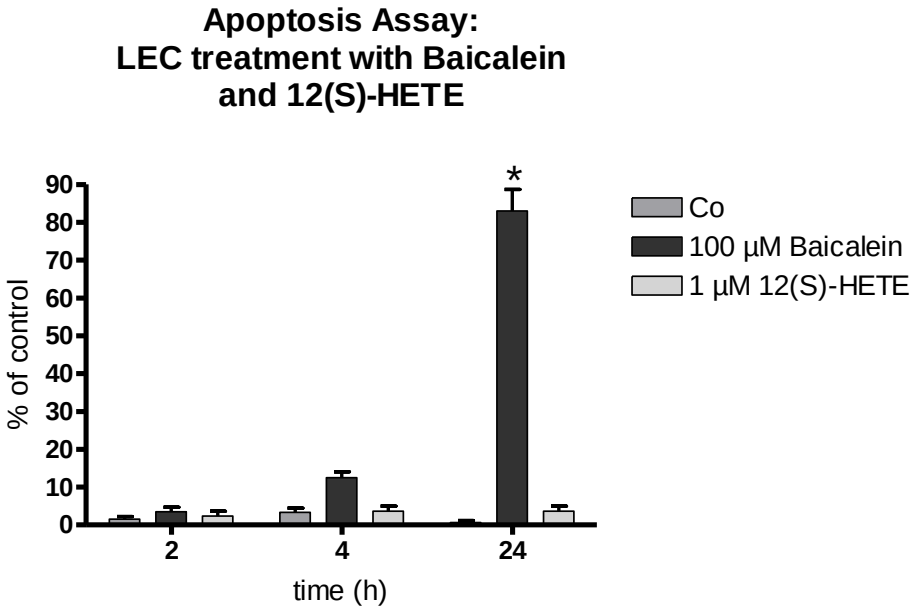


Figure 3) Apoptosis assay: cells were grown in 96 well plates (200 µl medium/well) to 40 % confluence and then treated with 100 µM Baicalein, 1 µM 12(S)-HETE and DMSO for 2, 4 and 24 h. Afterwards, cells were double stained with Hoechst and propidium iodide (final concentrations 5 µg/ml and 2 µg/ml) for 1 h at 37°C. Thereafter, cells were examined under a fluorescence microscope with DAPI filter, photographed, analyzed and counted manually. Error bars indicate SEM, asterisks significant apoptosis induction compared to control ($p<0.05$).

In preliminary experiments, besides 12(S)-HETE, we could identify also NF-κB as a major player in MCF7/LEC gap formation. Therefore, we tested compounds with putative NF-κB inhibitory properties in this 3D metastasis co-culture model.

Di-galloyl-resveratrol inhibits lymphendothelial gap formation

Earlier studies revealed that Di-galloyl-resveratrol (di-GA) (figure 4) was a strong inhibitor of gap formation and likely metastasis (Madlener et al. 2010). It was assumed that di-GA exerted the additive and synergistic effects of Resveratrol (RV) and Gallic acid (GA) (Madlener et al. 2010). Both are known to inhibit NF-κB.

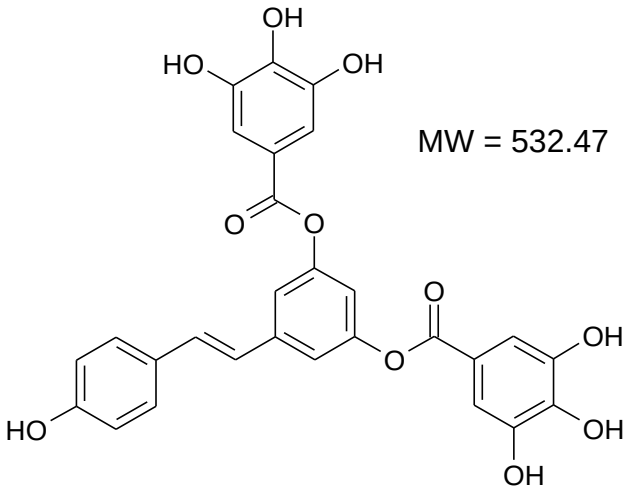


Figure 4) Chemical structure of Di-galloyl-resveratrol (di-GA) and molecular weight

Di-galloyl-resveratrol is a newly synthesized compound consisting of two GA molecules and one RV molecule (Bernhaus et al. 2009).

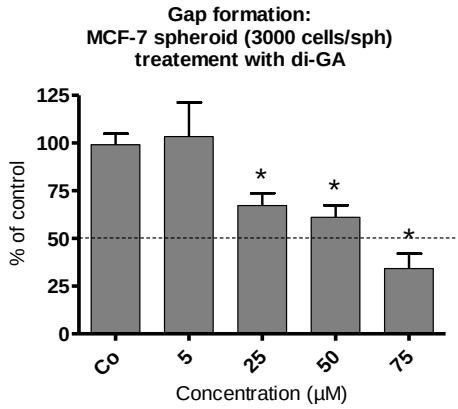
It combines the biological effects of RV and GA, which both have anti-cancer activity in a number of tumor cell lines. Madlener et al. (2010) show the proapoptotic property of 10 µM di-GA and a 10 fold stronger growth inhibitory effect of di-GA as compared to GA.

As mentioned above, di-GA is a strong inhibitor of gap formation. To investigate the contribution of the GA and RV moieties and derivatives in this assay, repetitive experiments were performed.

Lymphendothelial cells (LECs) were grown (as described in the chapter methods) and MCF-7 tumor spheroids (containing 3000 cells) were transferred on top of confluent lymphendothelial cell-monolayers. LECs were stained with cytotracker green to show the presence or absence of LECs underneath tumor spheroids.

MCF-7 spheroids were treated 30 min with increasing concentrations (5, 25, 50, 75 µM) of the different compounds before co-cultivation. The gap size underneath the spheroid was measured 4 h after co-cultivation with a Zeiss Axiovert microscope. Di-GA (figure 5a) shows a dose-depended inhibition of gap formation with a maximum inhibition of about 70 %.

5a)



5b)

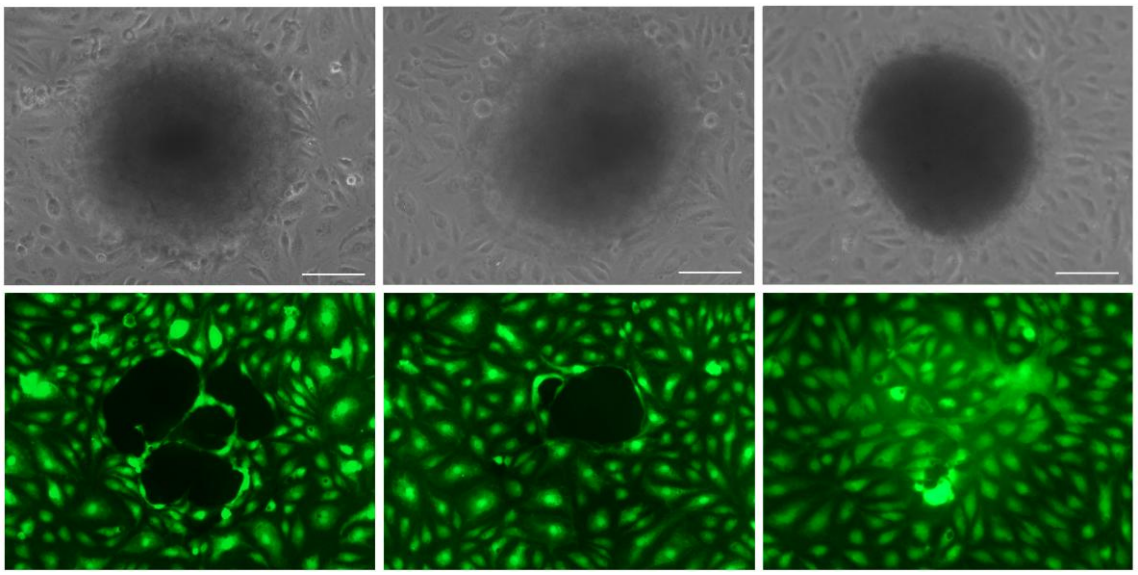


Figure 5a) Gap formation inhibition assay: LECs were seeded in 2 ml EGM 2 MV medium on 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to 30 ml MEM medium with hygromycin B containing 20 % methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within the following two days. Then, MCF-7 mock spheroids, which were treated with different concentrations of di-GA (solvent, 5, 25, 50 and 75 µM) for 30 min at 37°C, were carefully transferred to the wells of a 24 well

plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$). 5b) Upper panels are phase contrast micrographs showing the respective spheroids, the panels below show the identical power fields using FITC filter and exhibit green stained LECs underneath the respective spheroids. Scale bars: 100 µm.

Efficiency of Resveratrol and its derivatives on cell proliferation and LEC gap formation

Since RV (figure 6) is a part of the di-GA molecule, we compared the efficiency also of LEC gap formation. RV was found in grapes and red wine. It exhibits a remarkable inhibitory potential in various stages of tumor development. RV was shown to inhibit several molecular targets such as kinases and cyclooxygenases (Saiko et al. 2008). Several experiments have shown that RV suppresses the growth of cancer cells by inhibition of DNA polymerase (Locatelli 2005), ribonucleotide reductase (Fontecave et al. 1998), by inducing cell cycle arrest and apoptosis (Bourdon et al. 2007, Tanaka et al. 2000).

To prove the proliferation inhibition MCF-7 cells were seeded at a concentration of 1×10^4 cells per ml in 24 well plates and grown for 24 h. Then they were incubated with increasing concentrations (5, 25, 50, 75 µM) of RV and counted after 48 h and 72 h. 49 µM RV inhibited 50 % MCF-7 proliferation between 48 – 72 h of incubation and 50 µM inhibited LEC gap formation by ~25 % (figure 7a, 7b).

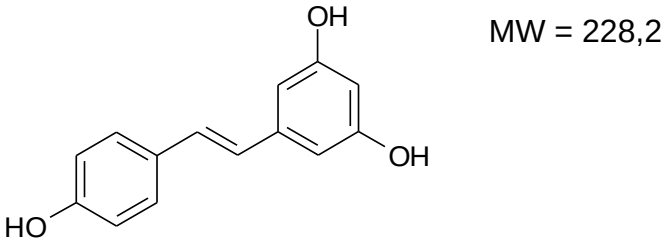
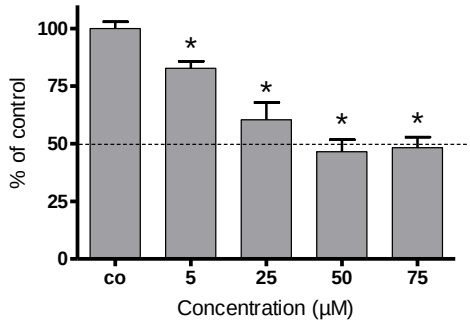


Figure 6) Chemical structure of Resveratrol (RV) and molecular weight

7a)



7b)

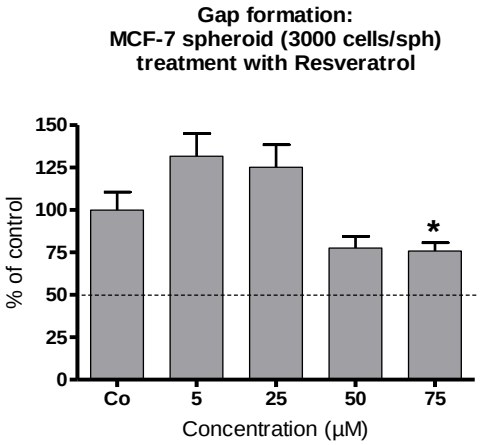


Figure 7a) Anti-proliferative effect of Resveratrol: MCF-7 cells were seeded (1 x 10^4 cells/ml) in 24 well plates and grown for 24 h. Then, they were incubated with increasing concentrations of Resveratrol (5, 25, 50, 75 µM) for 72 h. Cells were counted after 48 h and 72 h and the IC_{50} value for Resveratrol was determined. All experiments were done in triplicate. 7b) Gap formation inhibition assay: LECs were seeded in 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within the following two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of Resveratrol for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

The bioavailability of Resveratrol is very low. An intravenous dose of RV is converted within approx. 30 min to sulfate conjugates in humans. Piceatannol (PIC) is a natural occurring Resveratrol metabolite. Cytochrome P450 catalyzes the hydroxylation of RV to Piceatannol (3,5,3',4'-tetrahydroxy-trans-stilbene) (Saiko et al. 2008). PIC differs from RV by an additional hydroxyl group. Vo et al. (2009) describe the pro- and anti-carcinogenic mechanisms of Piceatannol. She shows that low PIC concentrations stimulated MCF-7 cell growth whereas very high PIC concentrations (150 µM) caused cell death. PIC exerts even more significant biochemical effects on tumor cells than RV itself. Therefore we tested PIC as an

inhibitor of gap formation. However, in the gap formation assay PIC showed no effect (data not shown).

M5 (3,4',5-trimethoxystilbene, figure 8) is the methoxylated stilbene derivative of RV. It exhibited a stronger activity than RV even in chemoresistant cancer cells (Bader et al. 2008). M5 inhibited the proliferation of MCF-7 mock cells more efficiently than RV (IC_{50}: 7,5 and 9,2 µM, respectively) (Bader et al. 2008) but on the inhibition of gap formation M5 was less efficient than RV.

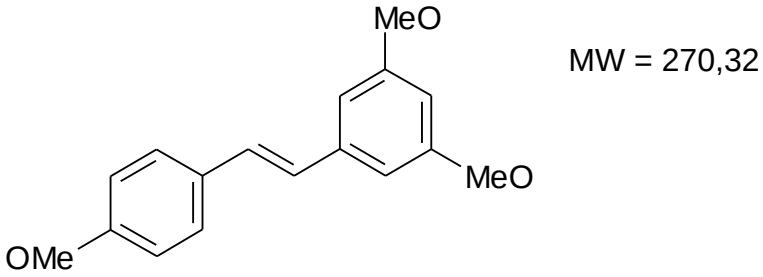


Figure 8) Chemical structure of M5 and molecular weight.

9)

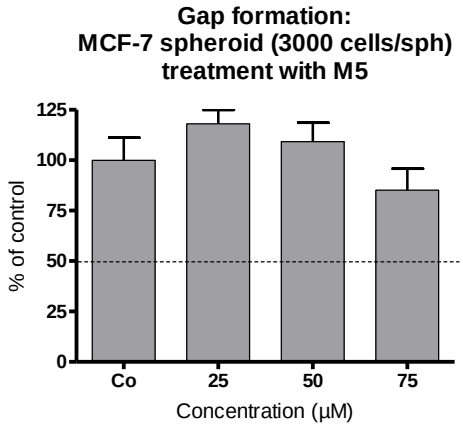


Figure 9) Gap formation inhibition assay: LECs were seeded in 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within the following two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of M5 for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each

condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

M8 (3,3',4,4',5,5'-hexahydroxystilbene) was another RV analog that was tested (Figure 9). In human promyelocytic leukemia cells (HL-60) 72 h treatment with 6,25 µM M8 inhibited 50 % cell growth (IC_{50}) (Horvath et al. 2006).
In our study 75 µM M8 inhibited 40 % of MCF-7 cell proliferation between 48-72 h of treatment and 30 % of gap formation in the 3D co-culture assay (figure 11a, 11b).

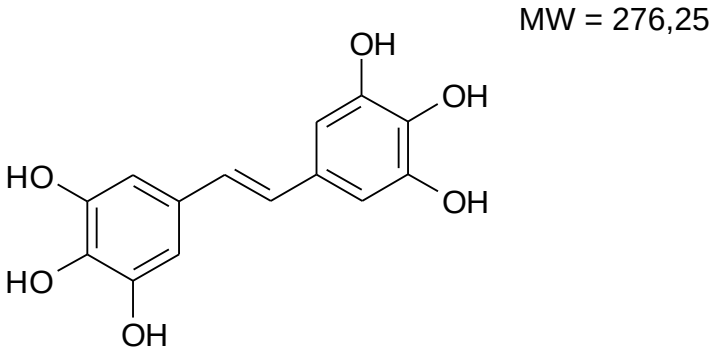
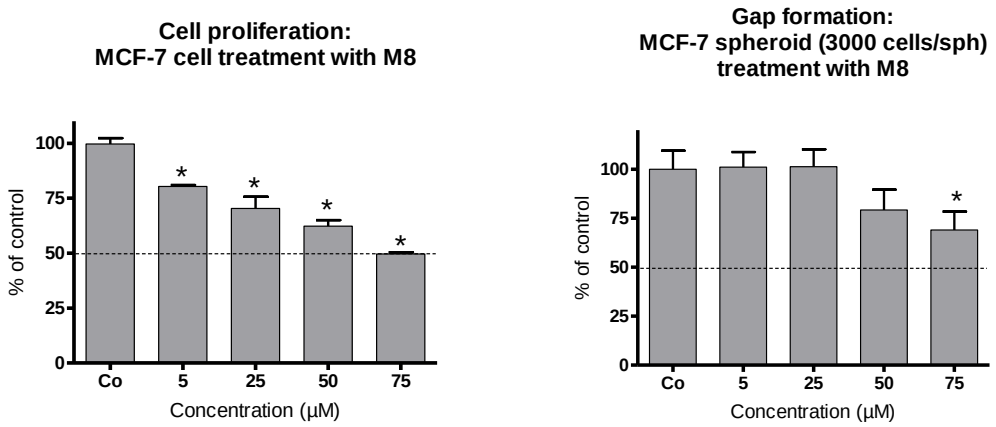


Figure 10) Chemical structure of M8 and molecular weight.

11a) 11b)



Figur 11a) Anti-proliferative effect of M8: MCF-7 cells were seeded (1 x 10^4 cells/ml) into 24 well plates, grown for 24 h to enter logarithmic growth phase. Then, they were incubated with increasing concentrations of M8 (5, 25, 50, 75 µM) for 72 h. Cells were counted after 48 h and 72 h and the IC_{50} value for M8 was determined. All experiments were done in triplicate. 11b) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose.

150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within the following two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of M8 for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroid/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

Influence of GA, Amidox, Didox and Trimidox on cell proliferation and gap formation

Two gallic acid (GA; 3,4,5-trihydroxybenzoic acid) (figure 12) moieties that are attached to the RV-backbone constitute the di-GA molecule. GA is a polyhydroxyphenolic compound that can be found in various natural products like gallnuts, tea leaves, grapes, pineapples, bananas and red wine (Madlener et al. 2007).

GA together with RV might be responsible for the “French paradox”, a 40 % lower heart infarction incidence in the French population compared with other European countries (Renaud and De Lorgeril 1992).

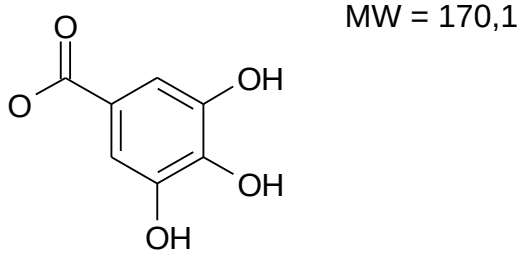


Figure 12) Chemical structure of Gallic acid and molecular weight.

GA is an excellent free radical scavenger and an inducer of differentiation and programmed cell death in a number of tumor cell lines (Salucci et al. 2002, Inoue et al. 1994, Isuzugawa et al. 2001, Sohi et al. 2003, Kawada et al. 2001). It may also play a role in the prevention of malignant transformation and cancer development.

GA had a time and dose-depended anti-proliferative effect (figure 13a) on human breast cancer cells (IC_{50} 75 µM), but on gap formation the free GA molecule showed no inhibitory effect. It even induced gap formation (figure 13b).

13a) 13b)

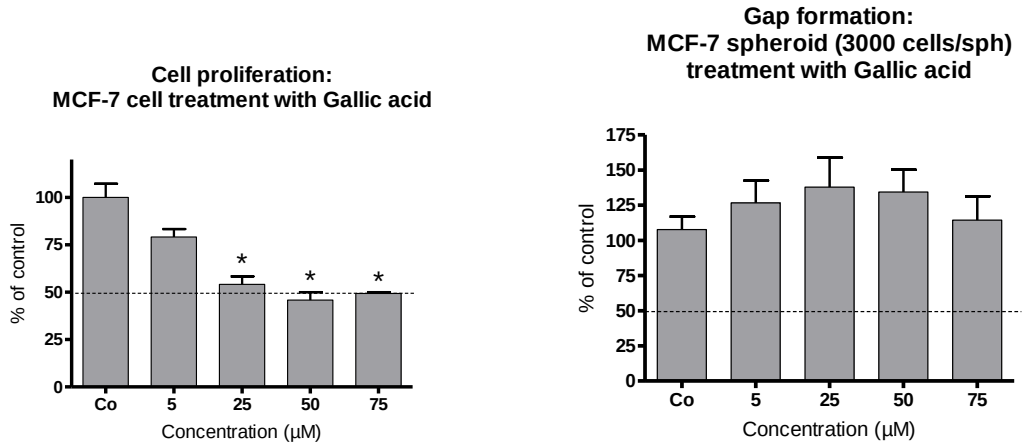


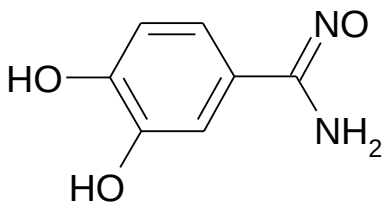
Figure 13a) Anti-proliferative effect of GA: MCF-7 were seeded at a concentration of 1 x 10^4 cells per ml in 24 well plates and grown for 24 h. Then, they were incubated with increasing concentrations of GA (5, 25, 50, 75 µM) for 72 h. Cells were counted after 48 h and 72 h and the IC_{50} value for GA was determined. All experiments were done in triplicate. 13b) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of GA for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolyer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayers were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

Amidox (3,4-dihydroxybenzamidoxime) and Didox (3,4-dihydroxybenzohydroxydroxamic acid) are two polyhydroxy-substituted benzohydroxamic acid derivatives which also act as free radical scavengers (Grusch et al. 2001).

Their chemical structures are related to GA and like RV they inhibited ribonucleotide reductase (RR) (Madlener et al. 2007). RR is the rate-limiting enzyme for de novo DNA-synthesis. The activity of RR is significantly increased in

tumor cells and therefore RR is a target for anticancer-therapy (Grusch et al. 2001).

MW = 204,61



MW = 169

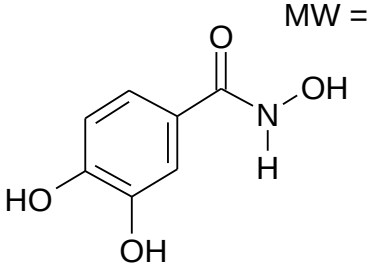
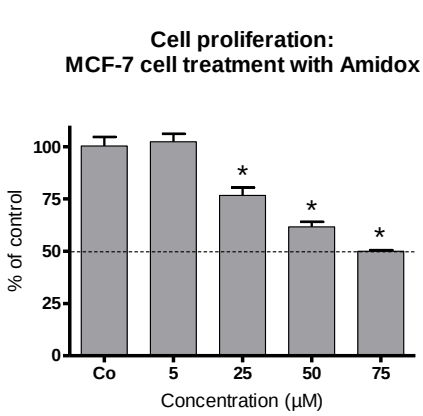


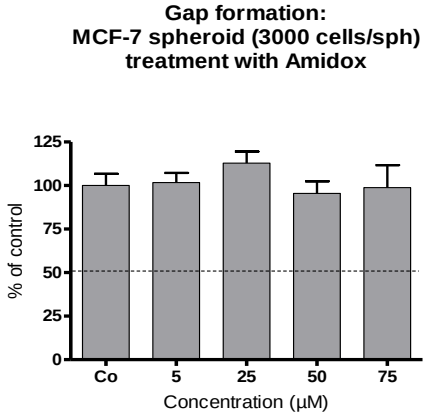
Figure 14) Chemical structure of Amidox (left) and Didox (right) and molecular weight.

Amidox and Didox show a 50 % inhibition of proliferation when treated with a concentration of 75 µM (figure 15a, 16a). As GA, the two molecules have no inhibitory effect on the gap formation (figure 15b, 16b).

15a)



15b)



16a) 16b)

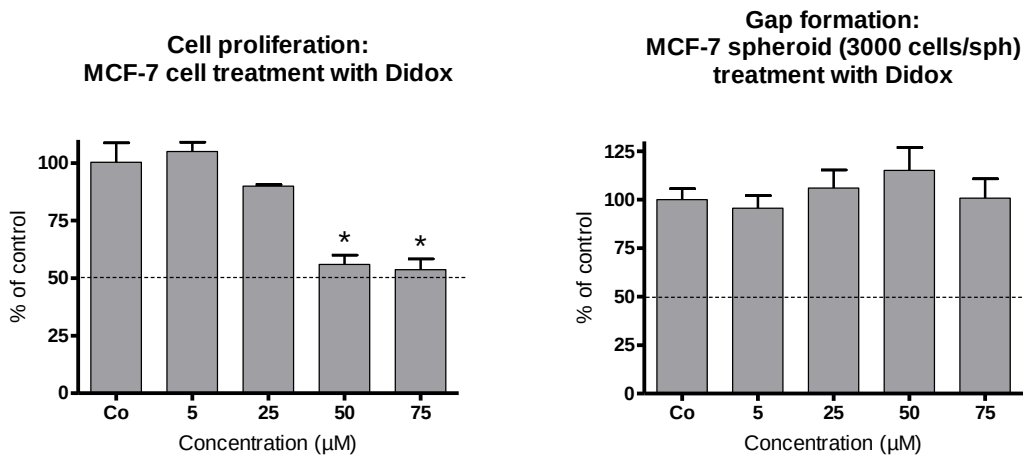
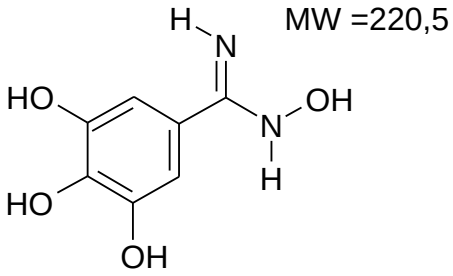


Figure 15a), 16a) Anti-proliferative effect of Amidox and Didox: MCF-7 cells were seeded (1 x 10^4 cells/ml) into 24 well plates, grown for 24 h to enter logarithmic growth phase. Then, they were incubated with increasing concentrations of Amidox or Didox (5, 25, 50, 75 µM) for 72 h. Cells were counted after 48 h and 72 h and the IC_{50} value for Amidox and Didox was determined. All experiments were done in triplicate. 15b), 16b) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of Amidox and Didox for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayer were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

Like RV, Amidox and Didox, Trimidox (3,4,5-trihydroxybenzamidoxime) is also an inhibitor of RR. Trimidox is a very effective and promising enzyme inhibitor and demonstrates excellent anticancer activity in animal tumor models (Fritzer-Szekeres et al. 1998, Szekeres et al. 1994).



Figur 17) Chemical structure of Trimidox and molecular weight.

Treatment of MCF-7 cells with Trimidox caused a time- and dose-dependent decrease of cell-proliferation. However, the treatment of the MCF-7 spheroids with Trimidox induced gap formation in LEC monolayers significantly.

18a) 18b)

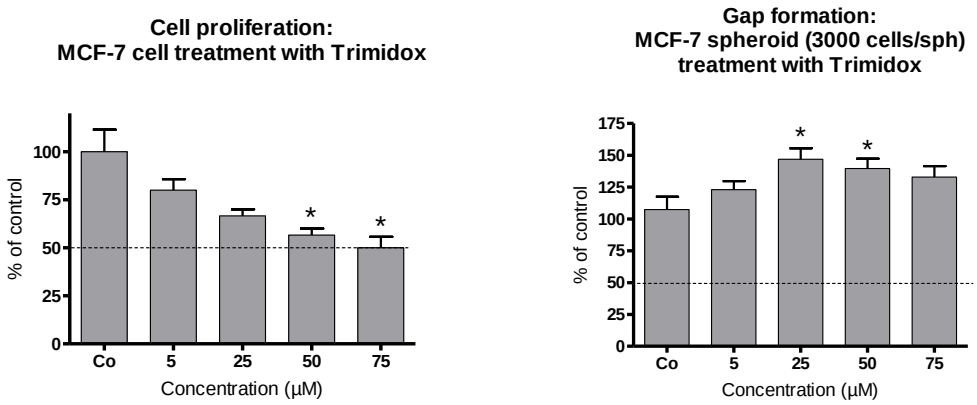


Figure 18a) Anti-proliferative effect of Trimidox: Proliferation assay: MCF-7 were seeded at a concentration of 1 x 10^4 cells per ml in 24 well plates and grown for 24 h. Then, they were incubated with increasing concentrations of Trimidox (5, 25, 50, 75 µM) for 72 h. Cells were counted after 48 h and 72 h and the IC_{50} value for Trimidox was determined. All experiments were done in triplicate. 18b) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl were transferred to each well of a 96 well plate to allow spheroid formation

within two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of Trimidox for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayer were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

In summary all of the compounds which were tested caused a time- and dose-dependent inhibition of MCF-7 cell proliferation. As mentioned above di-GA shows a dose-depended inhibition of gap formation with a maximum inhibition of about 70 %. Therefore, we anticipated that all constituents of di-GA may exhibit inhibitory effects on gap formation but the free molecule GA showed no inhibitory effect. It even induced the gap formation as well as the polyhydroxy-substituted benzohydroxamic acid derivatives Amidox, Didox and Trimidox. RV and its analog M5 and M8 showed an inhibition of gap formation but not as efficient as di-GA. The strong inhibitory effect on gap formation of di-GA is apparently not due to the GA residues and only in part to RV. Therefore, di-GA has a novel anti-metastatic property of it own.

Effect of natural compounds on gap formation

Among the synthetic drugs we also studied other natural compounds to test the inhibition of gap formation. Hyperforin, a main constituent of *Hypericum perforatum* and *Hypericum adenotrichum* is such a natural compound (Özmen et al. 2009). Hyperforin was shown to inhibit tumor growth, induce apoptosis of tumor cells and reduce tumor vascularisation (Schempp et al. 2005). It inhibited tumor invasion and metastasis as well (Dell Aica et al. 2007).

MW = 536,79

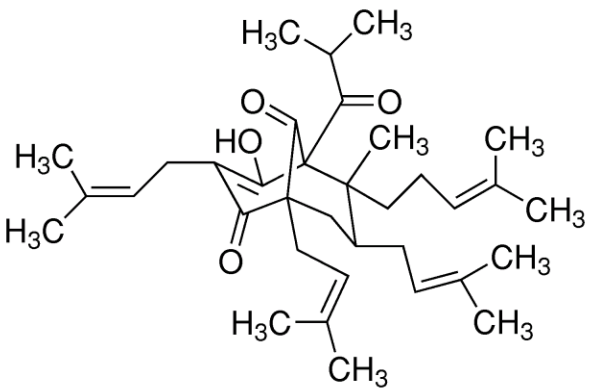


Figure 19) Picture of *Hypericum perforatum* and chemical structure of Hyperforin and molecular weight.

Treatment of MCF-7 spheroids with 75 µM Hyperforin caused a significant 30 % inhibition of gap formation (figure 20), which is consistent with its known NF-κB inhibitory property (Lorusso et al. 2009).

20)

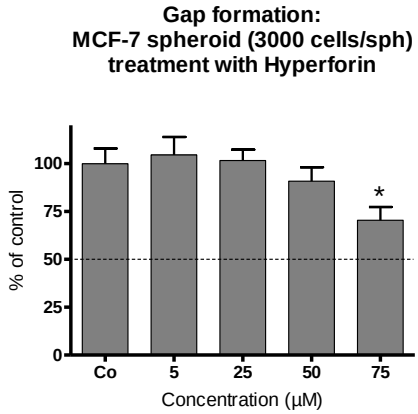


Figure 20) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of Hyperforin for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayer were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

Inhibition of NF-κB with the well accepted irreversible and specific inhibitor Bay11-7082

As shown by other groups (Pierce et al. 1997; Zhu et al. 2008) Bay11-7082 (E)-3-[(4-methylphenylsulfonyl]-2-propenenitrile (figure 21) inhibits the phosphorylation of cytokine-induced I-κBα and NF-κB translocation into the nucleus and therefore also its activity.

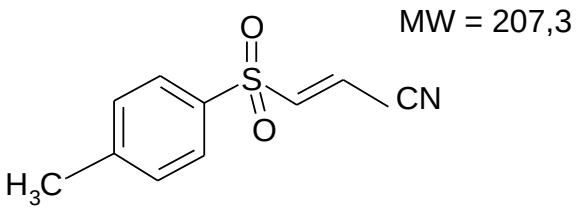


Figure 21) Chemical structure of Bay11-7082 and molecular weight.

Therefore, NF-κB inhibition through stabilization of I-κBα, blocks the expression of ICAM-1, VCAM-1 and E-selectin in TNF-α treated HUVECS (Pierce et al. 1997).

Exposure of the 3D cell culture model, with increasing concentration of Bay11-7082 caused a complete inhibition of gap formation already at 15 µM.

22)

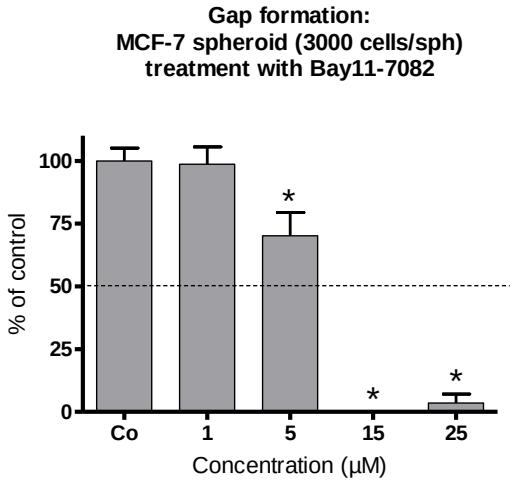


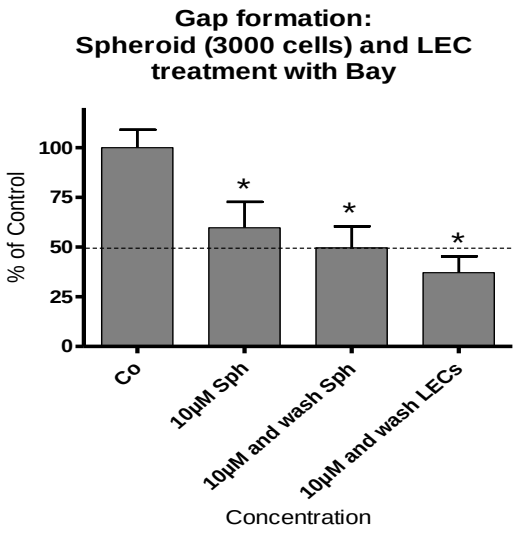
Figure 22) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B

containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within two days. Then, MCF-7 mock spheroids, which were treated with different concentrations (solvent, 5, 25, 50 and 75 µM) of Bay11-7082 for 30 min at 37°C, were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayer were measured using a Zeiss Axiovert microscope. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).

This tempted us to investigate whether this effect was due to the inhibition of NF-κB in the MCF-7 cell spheroid or in the LEC-layer. The fact that Bay11-7082 is an irreversible inhibitor and the specific design of the following experiments allowed to discriminate which of the cell type-specific NF-κB activity contributed to the gap forming effect.

Spheroids, which were treated with 10 µM Bay11-7082 for 30 min caused a gap formation of ~40 – 50 %. A similar level of inhibition was seen when the drug was washed off the MCF-7 spheroid to prevent LEC inhibition from contaminating Bay remnants (figure 22 third bar). Treatment of MCF-7 spheroids with increasing concentrations of Bay11-7082 (>10 µM) prevented a stable attachment of the spheroids to LEC-monolayers and gap formation. This let us assume that Bay11-7082 inhibited a mechanism that was necessary for the adhesion of MCF-7 cells to LECs. The Bay11-7082 treatment of LECs and subsequent washoff showed that gap formation of LECs was inhibited by ~ 60 % and LECs lost their cell-cell contacts. The loss of LEC cell-cell contacts underneath the MCF-7 spheroids and the lack of centrifugal migration gave rise to a curly cloudy pattern of LEC distribution.

23a)


Gap formation:
Spheroid (3000 cells) and LEC
treatment with Bay
% of Control
100
75
50
25
0
*
*
*
Co
10µM Sph
10µM and wash Sph
10µM and wash LECs
Concentration

23b)

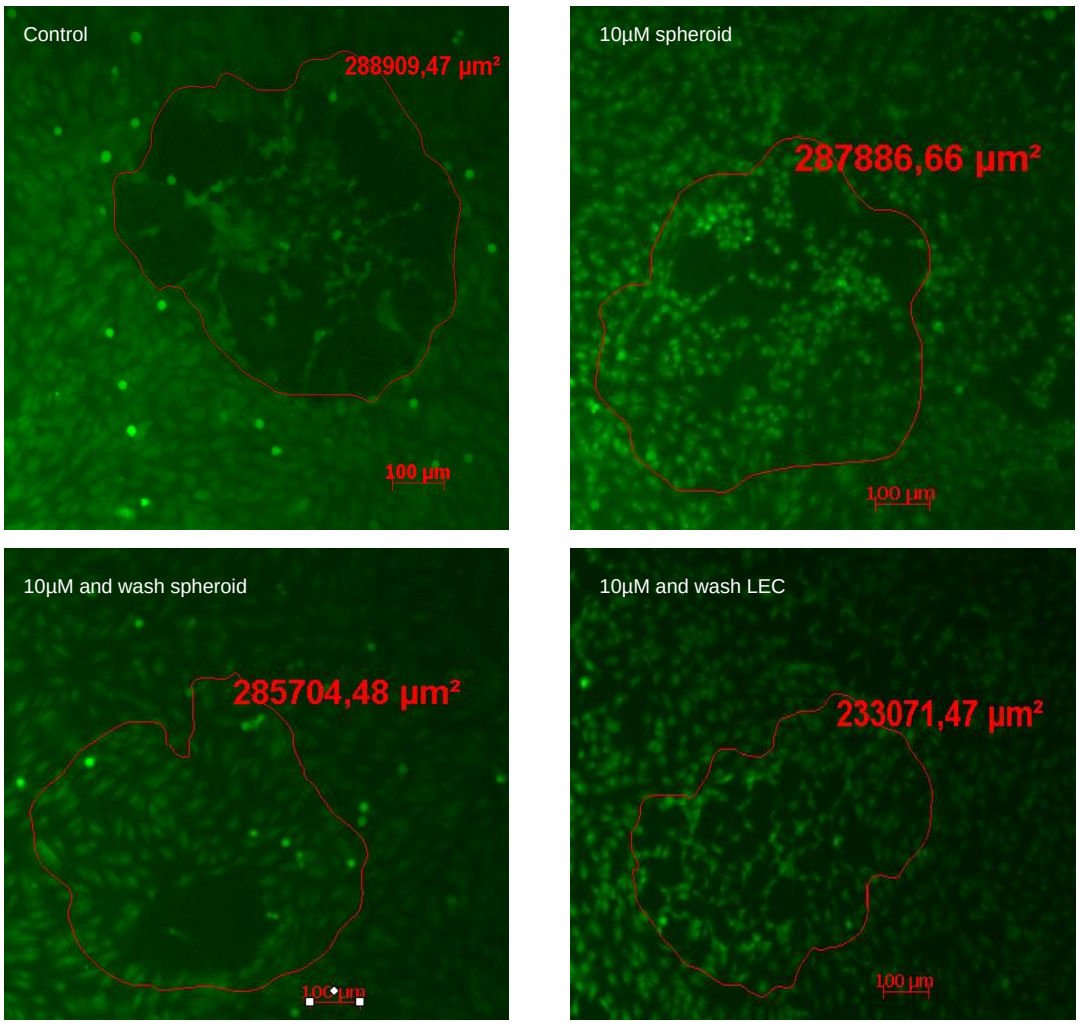

Control
288909,47 µm²
100 µm
10µM spheroid
287886,66 µm²
100 µm
10µM and wash spheroid
285704,48 µm²
100 µm
10µM and wash LEC
233071,47 µm²
100 µm

Figure 23) Gap formation inhibition assay: LECs were seeded into 24 well plates and allowed to grow for 2 days until confluence. Then, LECs were stained with cytotracker green (1:5000 dilution) for 1 hour at 37°C. MCF-7 mock cells were transferred to MEM medium with hygromycin B containing methylcellulose. 150 µl of this cell suspension were transferred to each well of a 96 well plate to allow spheroid formation within two days. Then, MCF-7 mock spheroids, which were treated in different ways (see chapter methods) were carefully transferred to the wells of a 24 well plate containing LECs. These 3D MCF-7 spheroids/LEC monolayer co-cultures were incubated for 4 h at 37°C. The size of the gaps, which were formed in the LEC monolayer were measured using a Zeiss Axiovert microscope. 23a) Bar chart which shows the inhibition of gap formation. For each condition, the gap area under at least 12 spheroids was measured. Error bars indicate SEM, asterisks significant gap formation inhibition compared to control ($p<0.05$).
23b) Pictures exhibit green stained LECs (using FITC filter) underneath the respective spheroids. The red mark shows the outline of the spheroid.

As described above treatment of LECs with Bay11-7082 results in a cloudy cell distribution (figure 23b below right panel). This cell distribution pattern suggested that LECs lost their cell-cell contact underneath the spheroid, but did not migrate thereby opening a gap. To study this effect in more detail we used immunofluorescence combined with confocal laser scanning microscopy.

NF-κB regulates LEC motility through ZEB1 dependent VE-cadherin expression

Cadherins are cell adhesion molecules which mediate adhesion via homophilic Ca^{2+}-dependent interactions. They can be devided into epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), placental cadherin (P-cadherin), muscle cadherin (M-cadherin) and vascular endothelial cadherin (VE-cadherin).
VE-cadherin is expressed specifically in endothelial cells. Vascular endothelial (VE)-cadherins are calcium-dependent adhesive proteins which are of vital importance for the maintenance and control of endothelial cell contacts. Cadherins bind directly to β-catenin (cytoplasmic protein) required for optimal adhesive function (Vestweber 2008). VE-cadherin is required for intact cell-cell interaction and is a marker for an epithelial and immotile cell phenotype.

It was shown that 12(S)-HETE induces endothelial cell retraction (Honn et al. 1994) and stimulates tumor cell spreading on matrix (Timar et al. 1992). 12(S)-HETE was produced and released from MCF-7 cells (Uchide et al. 2007). Therefore, tumor cell-induced endothelial cell retraction is a major step in breast cancer metastasis (Kerjaschki et al. 2010).

To simplify the MCF-7 spheroids/LEC co-culture system and facilitate Western blot analysis we treated LECs with 1 µM 12(S)-HETE and found a transient downregulation of VE-cadherin expression (figure 24a, 24b).

24a) 24b)

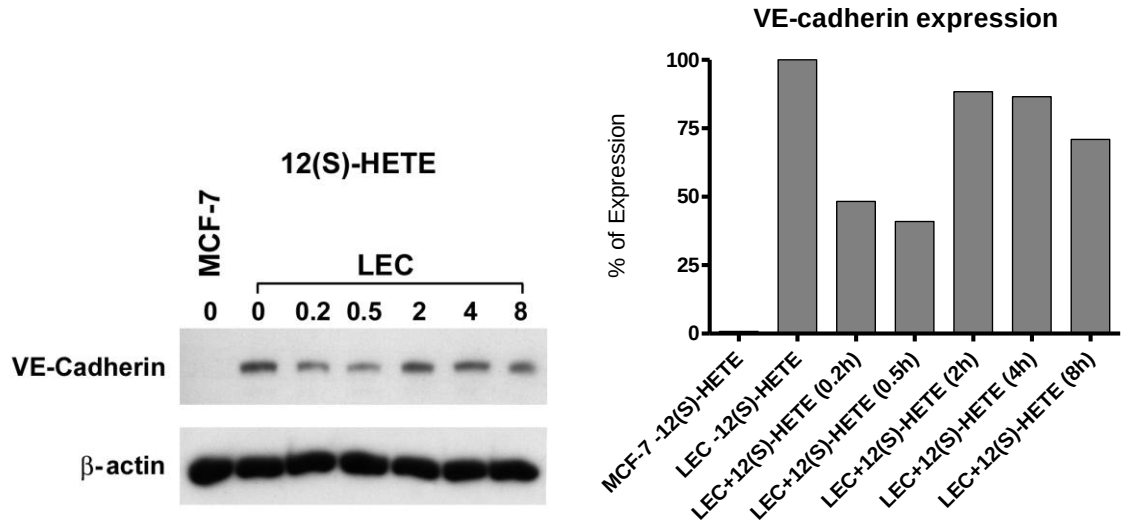


Figure 24) Analysis of VE-cadherin expression. 24a) LECs were treated with 1 µM 12(S)-HETE for 0.2, 0.5, 2, 4 and 8 h. Then, cells were harvested and protein lysates were analysed by western blotting. MCF-7 cells were used as negative control. Equal sample loading was controlled by β-actin analysis. 24b) Densitometry of VE-cadherin expression normalised by β-actin expression.

Figure 25 shows the 12(S)-HETE triggered VE-cadherin downregulation using the MCF-7 spheroid/LEC co-culture system by immunofluorescence. MCF-7 spheroids and LECs were grown as mentioned above and after an incubation time of 4 h cells were fixed with 4 % paraformaldeyhd and immunostained with the antibody against VE-cadherin (red). Figure 25a shows that in some distance to a spheroid VE-cadherin cell-cell interactions were well developed, whereas underneath a MCF-7 spheroid VE-cadherin interactions were apparently disrupted (figure 25b).

25a) 25b)

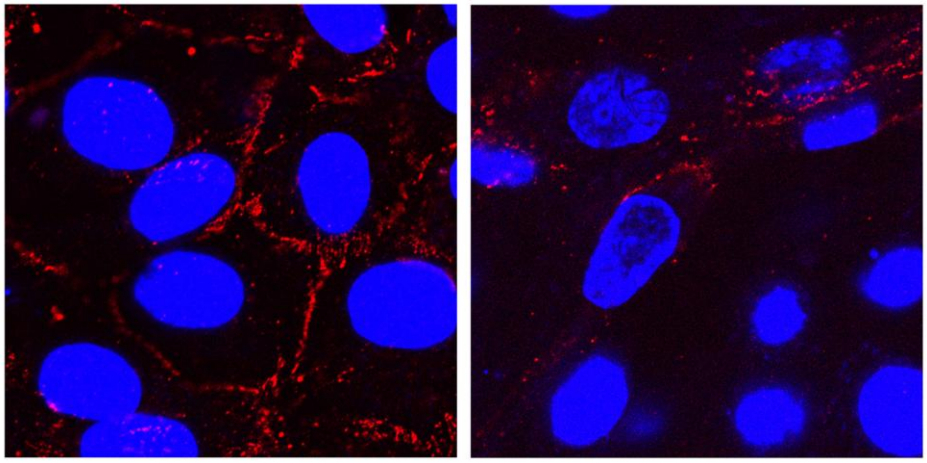


Figure 25) Confocal images of LECs a) next to a spheroid and b) under a MCF-7 spheroid. LECs were seeded on chambered coverslips and allowed to grow for 2 days until confluence. MCF-7 spheroids were treated with DMSO for 30 min at 37°C and transferred on top of LECs. MCF-7 spheroids/LECs were incubated for 4 h at 37°C to allow gap formation. Then cells were fixed for 15 min in ice cold 4 % paraformaldehyd and stained with VE-cadherin following incubation with Alexa Fluor 594-conjugated anti mouse (red) IgG and DAPI (blue).

In epithelial tumors activation of the embryonic program epithelial-mesenchymal transition is important for the dissemination and invasion of cancer cells (Yilmaz et al. 2007). Loss of epithelial differentiation and development of a mesenchymal phenotype allows separation of cancer cells from the primary tumor mass and spreading into the surrounding stroma. Loss of E-cadherin is a key initiating event in epithelial-mesenchymal transition (EMT) (Thiery 2002). It enables the first step of metastasis – local invasion and dissemination of cancer cells from the main tumor mass.

ZEB1 is a transcriptional repressor and known to downregulate the E-cadherin expression (Schmalhofer et al. 2009). High ZEB1 expression correlated with loss of E-cadherin expression and increased migratory and invasive potential (Arumugam et al. 2009). As mentioned above we found a 12(S)-HETE induced downregulation of VE-cadherin expression in LECs. Therefore we also tested the correlation of ZEB1 and VE-cadherin expression and the influence of Bay11-7082 on LEC motility.

ZEB1 was found primarily in the nucleus as a transcriptional factor and inducer of EMT (Arumugam et al. 2009). Figure 26 shows that treatment of LECs with 1 µM 12(S)-HETE for 10 min increased the ZEB1 expression and decreased the VE-cadherin expression. Pre-treatment with 10 µM Bay11-7082, an inhibitor of NF-κB, for 30 min caused a decrease of ZEB1 expression and induction of VE-cadherin expression. Therefore, NF-κB activation is associated with induction of ZEB1 expression. (Chua et al. 2007) Taken together, our results show an inverse relationship between ZEB1 and VE-cadherin. A decrease of ZEB1 and an increase of VE-cadherin expression caused by Bay11-7082 resulted in loss of MCF-7 invasiveness and LEC-migration.

26)

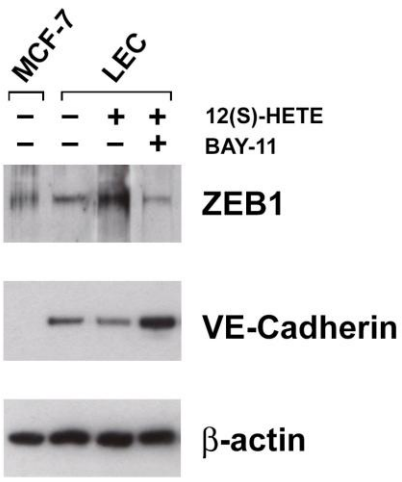
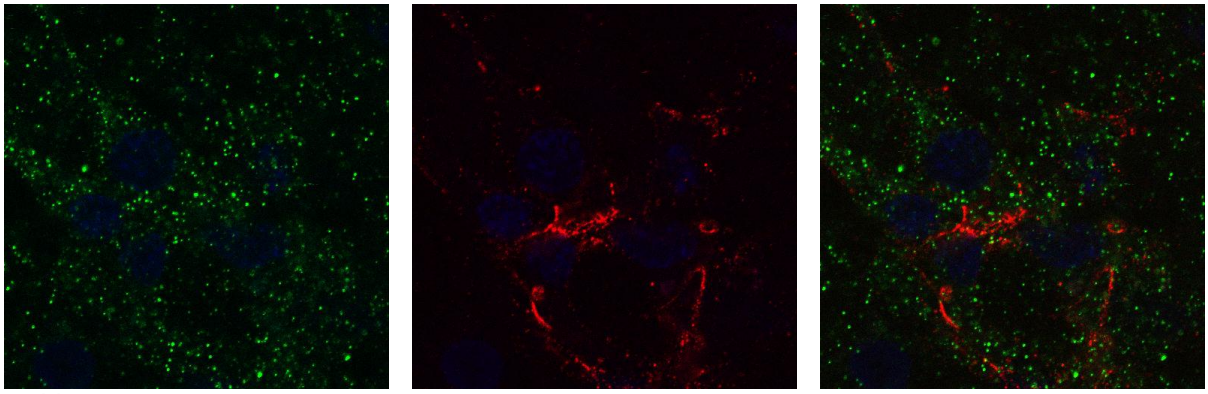


Figure 26) Analysis of ZEB1 and VE-cadherin protein expression. LECs were pre-treated with 10 µM Bay11-7082 for 30 min and stimulated with 1 µM 12(S)-HETE for 10 min. Then, cells were harvested and protein lysates were analysed by western blotting. MCF-7 cells were used as negative control. Equal sample loading was controlled by β-actin analysis.

Figure 27 shows the analysis of ZEB1 and VE-cadherin expression by immunofluorescence using a laser scanning confocal microscop. MCF-7 spheroids and LECs were grown as mentioned above and after incubation time of 4 h the system were fixed with 4 % paraformaldeyhd and immunostained with the antibody against ZEB1 (green) and VE-cadherin (red). Upper panels (figure 27a) show the high ZEB1 expression of LECs underneath a MCF-7 spheroid correlated with loss of VE-cadherin expression and consequently an increased migratory phenotype of lymphendothelial cells, which is a prerequisite for cancer cells to invade the

vasculature. The panels below (figure 27b) show the inhibition of ZEB1 expression by Bay11-7082 and as a result the increased VE-cadherin expression.

27a)



27b)

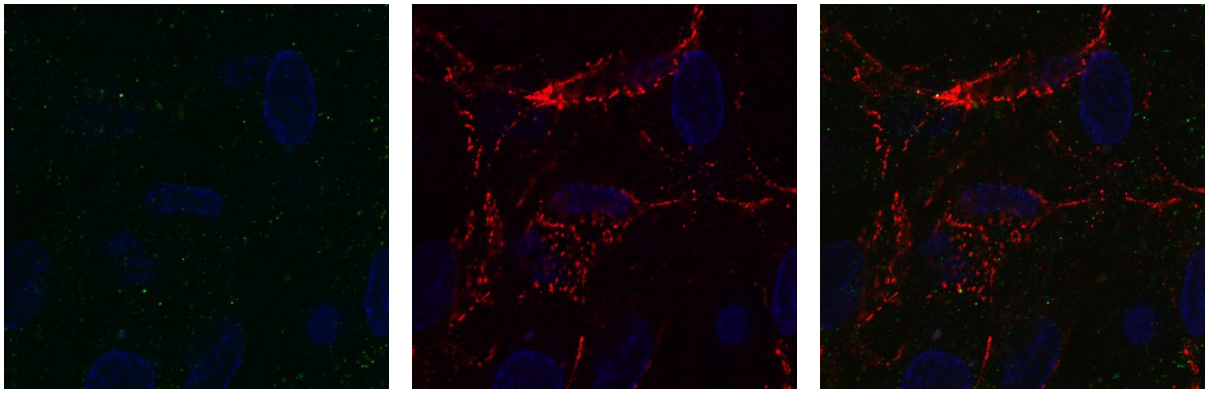
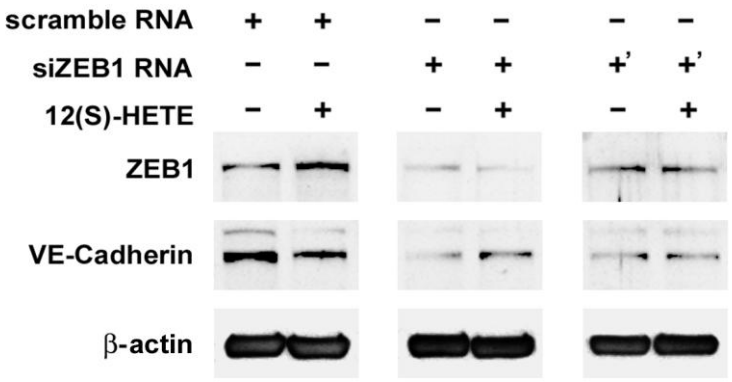


Figure 27) Confocal images of LECs under MCF-7 spheroids. LECs were seeded on chambered coverslips and allowed to grow for 2 days until confluence. MCF-7 spheroids were treated with a) DMSO or with b) 10 µM Bay11-7082 for 30 min at 37°C and transferred on top of LECs. MCF-7 spheroids/LECs were incubated for 4 h at 37°C to allow gap formation. Then cells were fixed for 15 min in ice cold 4 % paraformaldehyd and stained with ZEB1 and VE-cadherin following incubation with Alexa Fluor 488-conjugated anti-rabbit (green) and Alexa Fluor 594-conjugated anti mouse (red) IgGs and DAPI (blue).

The Western blot data (figure 26) suggested that ZEB1 seemed to negatively regulate VE-cadherin expression. To test this we transiently transfected LECs with siRNA to specifically knock down the expression of ZEB1. This resulted in a loss of VE-cadherin regulation upon 12(S)-HETE stimulation (figure 28a, 28b).

28a)



28b)

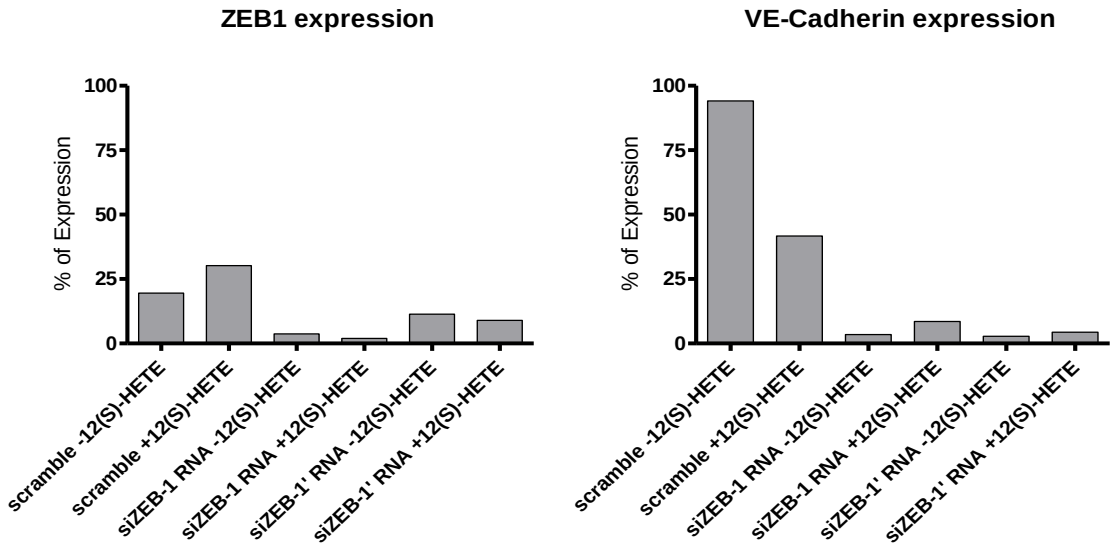


Figure 28) Analysis of ZEB1 and VE-cadherin protein expression. LECs were transiently transfected with siZEB1 RNA and scrambled RNA. Then, LECs were treated with 1 µM 12(S)-HETE. Afterwards cells were harvested and protein lysates were analysed by western blotting using antibodies against ZEB1 and VE-cadherin. Equal sample loading was controlled by β-actin analysis 28a) western blot analyses of ZEB1 and VE-cadherin expression 28b) Densitometer readings of ZEB1 and VE-cadherin western blots.

Another independent marker of motility (EMT) S100A4

To substantiate the migratory phenotype of LECs underneath the spheroids, the 3D cultures were analysed with S100A4 antibody in absence and presence of Bay11-7082 because S100A4 is a marker for cell motility and EMT.

S100A4 is a calcium-binding protein that interacts with intracellular target proteins (Mandinova et al. 1998). S100A4 is able to induce metastasis in rodent models for breast cancer. S100A4 has been associated with migratory and invasive properties (Rudland et al. 2000). mRNA for S100A4 has been shown to be at a higher level in breast carcinoma than in benign breast tumor specimens (Wang et al. 2000).

S100A4 overexpression leads to an increase of invasiveness and motility of the cancer cells. Beside that, S100A4 acts as an angiogenic factor by stimulating the motility and invasiveness of endothelial cells (Takenaga et al. 1994, Jenkinson et al. 2004, Ambartsumian et al. 2001, Schmidt-Hansen et al. 2004).

As mentioned earlier, ductal breast cancer metastasis was shown to depend on the expression and activity of lipoxygenases in particular 12-LOX, which generate 12(S)-HETE (Kerjaschki et al. 2010). To determine whether 12(S)-HETE may affect the S100A4 expression in dependence of NF-κB, we examined the levels of S100A4 in LECs. Therefore, LECs were pre-treated with 10 µM Bay11-7082 and then stimulated for 30 min with 1 µM 12(S)-HETE. Figure 29 shows that S100A4 expression was activated by treatment of LECs with 1 µM 12(S)-HETE, whereas the pre-treatment of LECs with Bay11-7082 caused an inactivation of S100A4 expression which implicates the involvement of NF-κB in S100A4 regulation.

29)

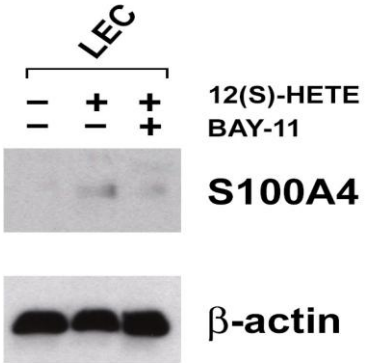
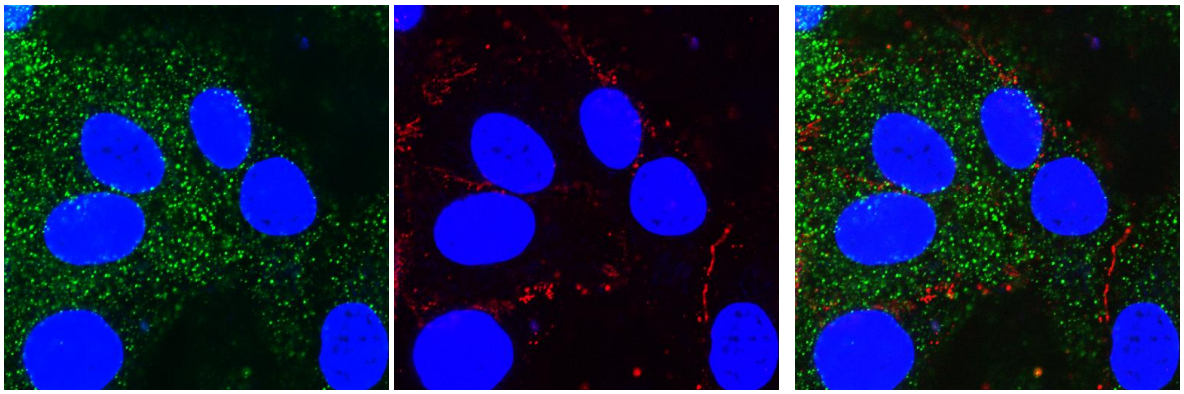


Figure 29) Western blot analysis of S100A4 expression. LECs were treated with 1µM 12(S)-HETE for 30 min and 10µM Bay11-7082 for 30 min. Then, cells were harvested and the expression of S100A4 were analysed by western blotting using an antibody against S100A4. β-actin was used as a loading control.

Figure 30 (confocal image upper panel) shows the induction of S100A4 under the spheroids and therefore, the higher motility of endothelial cells. The treatment of spheroids with Bay11-7082 inhibited the expression of S100A4.

30a)



30b)

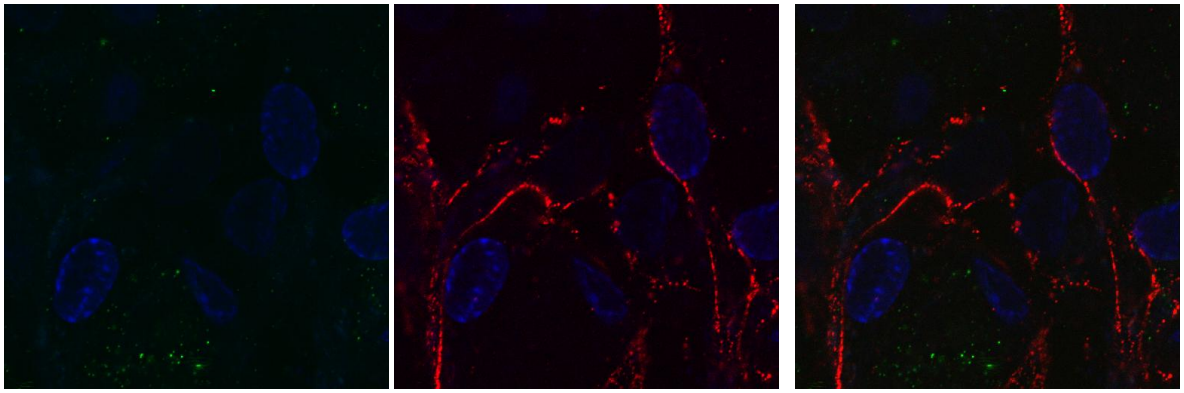


Figure 30) Confocal images of LECs under MCF-7 spheroids. LECs were seeded on chambered coverslips and allowed to grow for 2 days until confluence. MCF-7 spheroids were treated with a) DMSO or with b) 10 µM Bay11-7082 for 30 min at 37°C and transferred on top of LECs. MCF-7 spheroids/LECs were incubated for 4 h at 37°C to allow gap formation. Then cells were fixed for 15 min in ice cold 4 % paraformaldehyd and stained with S100A4 and VE-cadherin following incubation with Alexa Fluor 488-conjugated anti-rabbit (green) and Alexa Fluor 594-conjugated anti mouse (red) IgGs and DAPI (blue).

Evidence for inter-specific cell-cell adhesion

The adhesion of tumor cells to the endothelial cells represents the first important event in the process of metastasis (Honn et al. 1987). Therefore, we examined the possible role of bonafide NF-kB-regulated ligand – receptor pairs in tumor cell adhesion to the endothelium.

ICAM-1 and $\alpha_L\beta_2$ integrin

Intracellular adhesion molecule 1 (ICAM-1) is a member of the immunoglobulin gene superfamily and an inducible counter receptor for several leukocyte β_2 integrins. Endothelial cells constitutively express ICAM-1 (Collins et al. 1995). Bolick and colleagues (2005) have shown that 12(S)-HETE stimulates NF-κB activation and NF-κB dependent ICAM-1 expression through RhoA and PKCα. We chose to use 1 µM 12(S)-HETE as the concentration for our studies. We added 1µM 12(S)-HETE for 0.2, 0.5, 2, 4, and 8 h at 37°C to LEC monolayers and found a small induction of ICAM-1 expression (figure 31a). After pre-treatment of LECs with 10 µM Bay11-7082 for 30 min and stimulation with 12(S)-HETE for 10 min ICAM-1 induction was inhibited (figure 31b). The best-known ligands for ICAM-1 are the β_2 integrins (Rosenstein et al. 1991). In our study we could not detect β_2 integrin in MCF-7 cells by western blot analysis, although MCF-7 cells were described to express this integrin subtype and LFA-1 (Budinsky et al. 1997).

MUC1 (also known as tumor-associated epithelia mucin, polymorphic epithelial mucin or epithelial membrane antigen) is a 69- amino-acid long cytoplasmic domain and is well suited to support intercellular adhesion (Parry et al. 1990). It was shown that tumor cell-associated MUC1 can mediate endothelial adhesion through ICAM-1 and this suggested that also MUC1 may play a crucial role in promoting tumor cell survival and metastasis (Regimbald et al. 1996).

31a) 31b)

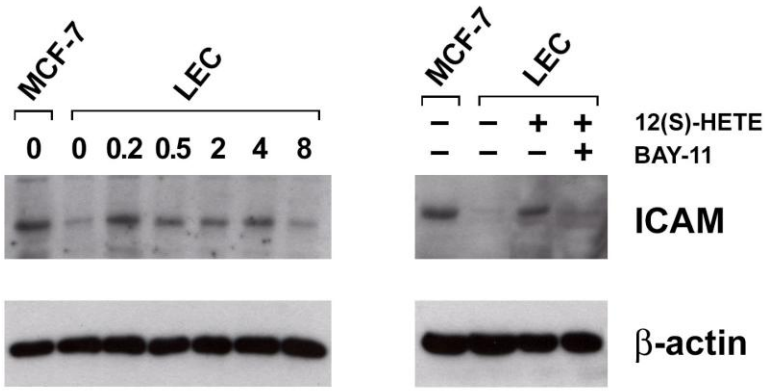


Figure 31) Analysis of ICAM-1 expression. a) LECs were treated with 1 µM 12(S)-HETE for 0.2, 0.5, 2, 4 and 8 h b) LECs were pre-treated with 10 µM Bay11-7082 for 30 min and stimulated with 1 µM 12(S)-HETE for 10 min. Then, cells were harvested and protein lysates were analysed by western blotting using an antibody against ICAM-1. MCF-7 cells were used as control. Equal sample loading was controlled by β-actin analysis.

When NF-κB is inhibited by Bay11-7082 it leads to a loss of contact and therefore to a reduction in LEC motility and gap formation.

CD31 and $\alpha_V\beta_3$ integrin

Integrins are receptors for cell-surface adhesion molecules and extracellular matrix (ECM) proteins. Integrin $\alpha_V\beta_3$ is also known as vitronectin receptor and consists of an α and a β subunit (Rupp and Little 2001).

The vitronectin receptor has been implicated in the pathophysiology and progression of malignant tumors such as breast cancer (Harms et al. 2004, Vellon et al. 2006). A positive correlation of the increased expression of $\alpha_V\beta_3$ integrin with the ability of the cancer cells to adhere to extracellular matrix and to migrate was shown in in vitro studies (Rolli et al. 2003, Felding-Habermann et al. 2001).

$\alpha_V\beta_3$ integrin characterizes the metastatic phenotype in breast cancer. It is up-regulated in invasive tumors and distant metastasis (Liapis et al. 1996). Deryungina et al. (2000) have shown the high expression of the α_V subunit and the lower expression of the β_3 subunit in MCF-7 cells.

CD31 (platelet-endothelial cell adhesion molecule 1; PECAM1) is found to be a ligand for $\alpha_V\beta_3$ integrin (Piali et al. 1995). CD31 is a member of the immunoglobulin superfamily. It is a pivotal constituent of the endothelial cell intercellular junction and because of this cellular expression pattern CD31 is implicated in several functions, including transendothelial migration of leukocytes, angiogenesis and integrin activation (Newman 1999).

Figure 32a) shows a time-dependent induction of CD31 expression with a maximum after 8 h of treatment with 1 µM 12(S)-HETE. As anticipated, MCF-7 cells showed no CD31 expression. The pre-treatment of LECs with 10 µM Bay11-7082 for 30 min and stimulation with 12(S)-HETE for 30 min caused an inhibition of CD31 expression in LECs (figure 32b).

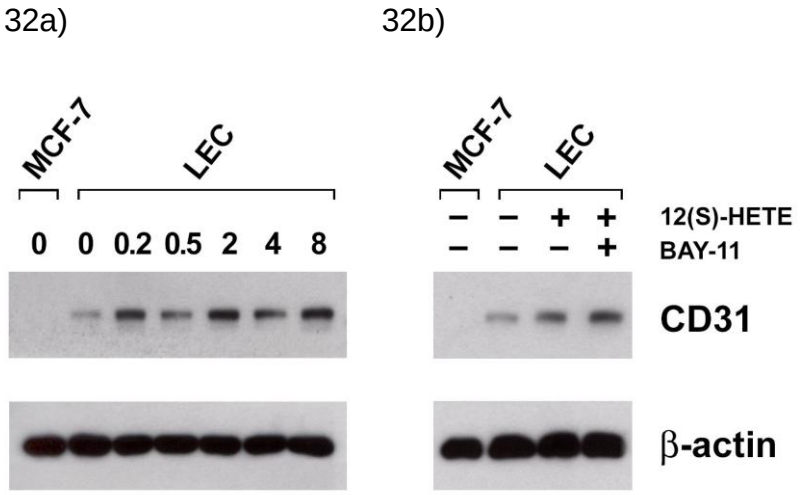


Figure 32) Analysis of CD31 expression. a) LECs were treated with 1 µM 12(S)-HETE for 0.2, 0.5, 2, 4 and 8 h b) LECs were pre-treated with 10 µM Bay11-7082 for 30 min and stimulated with 1 µM 12(S)-HETE for 30 min. Then, cells were harvested and protein lysates were analysed by western blotting using an antibody against CD31. MCF-7 cells were used as control. Equal sample loading was controlled by β-actin analysis.

We did not detect a regulation of $\alpha_V\beta_3$ integrin by Bay11-7082 in MCF-7 cells although integrin $\alpha_V\beta_3$ was described to be regulated by NF-κB (Scatena et al. 1998)

E-selectin and CD44

CD44 is a cell surface glycoprotein and acts as a adhesion molecule in cell-substrate and cell-cell interactions, in lymphocyte recruitment to inflammatory sites and tumor metastasis (Khan et al. 2004, Wang et al. 2005, Naor et al. 1997, Spessotto et al. 2002).

Endothelial leukocyte adhesion molecule-1 (E-selectin) plays a pivotal role in mediating cell-cell interactions between tumor cells and endothelial monolayer during tumor metastasis. (Laferriere et al. 2002) Several studies have demonstrated a critical role for E-selectin in regulating tumor cell transendothelial migration in breast cancer cells. (Tozeren et al. 1995, Moss et al. 2000)

Zen et al. (2008) demonstrated that the CD44 variant 4 serves as a major E-selectin ligand in mediating breast cancer cell transendothelial metastasis. We could demonstrate that CD44 is highly upregulated in MCF-7 spheroids compared to MCF-7 monolayers (Kerjaschki et al. 2010).

Figure 33 shows the expression of E-selectin in LECs by real-time quantitative PCR. Stimulation of LECs with 20ng/ml TNF-α for 60 min at 37°C show a strong induction of E-selectin expression whereas the stimulation of LECs with 1 µM 12(S)-HETE show no effect. Pre-treatment of TNF-α stimulated LECs with 10 µM Bay11-7082 show an inhibition of the E-selectin expression.

33a) 33b)

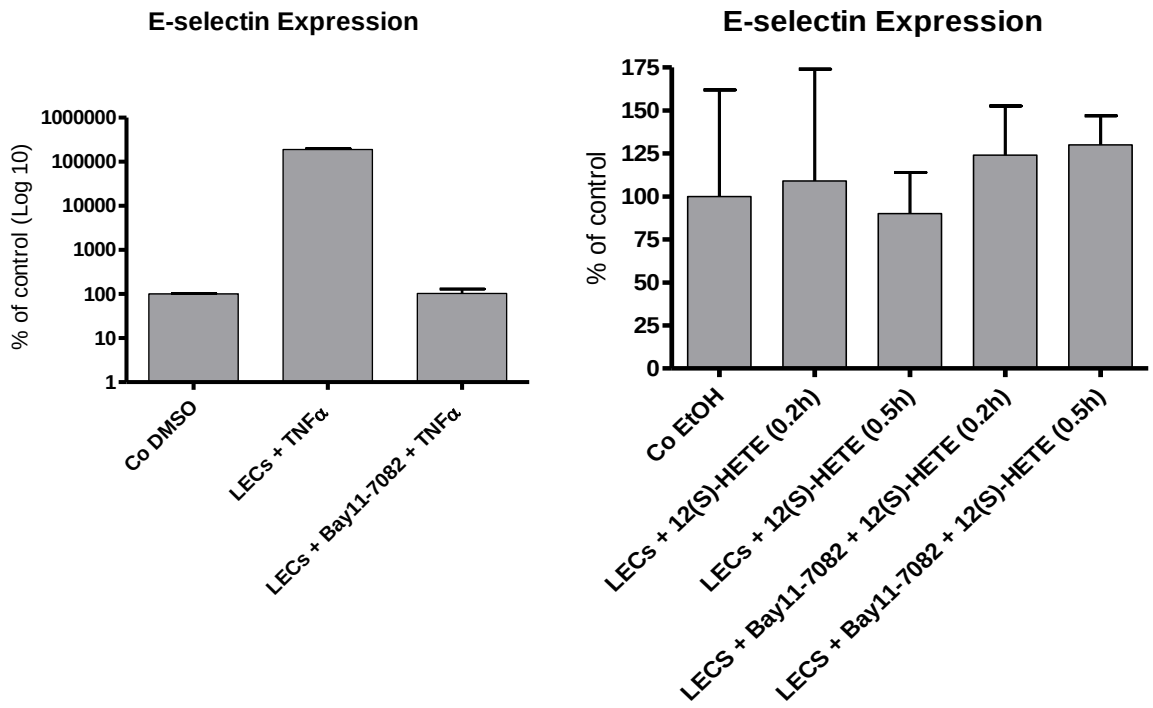


Figure 33) Analysis of E-selectin expression by real-time quantitative PCR: a) LECs were grown in 12 well plates and pre-treated with 10 µM Bay11-7082 for 30 min and thereafter stimulated with 20ng/ml TNF-α for 60 min at 37°C. b) LECs were grown in 12 well plates and pre-treated with 10 µM Bay11-7082 for 30 min and thereafter stimulated with 1 µM 12(S)-HETE for 30 min and 60 min at 37°C. PCR products were analyzed on the Abi Prism 7000 sequence detection system. Duplicate samples were analyzed in parallel. GAPDH served as internal control. Relative expression numbers calculated using the $\Delta\Delta C_T$ method.

4. Discussion

The metastatic process was found to include many steps such as dissociation of tumor cells from the bulk tumor, invasion of tumor cells, intravasation, transport through vessels, extravasation and outgrowth of secondary tumors (Geiger and Pepper 2009). Kramer and Nicolson (1979) have described the interaction of tumorigenic cells with vascular endothelial cells a long time ago. Here, we used a 3D co-culture system to mimic the invading of breast cancer cells to the lymphatic vasculature and recapitulated the mechanism of metastasis. Metastasis was shown to depend on the expression and activity of lipoxygenases (Uchide et al. 2007, Kerjaschki et al. 2010).

Lipoxygenases are enzymes which metabolize arachidonic acid to biologically active eicosanoids. Several studies have shown their involvement in tumor differentiation and progression (Jiang et al. 2006, Nithipatikom et al. 2006, Chen et al. 2005).

Targeted inhibition of LOX isoforms can inhibit the proliferation of a variety of tumor cells (Tong et al. 2002, Ma et al. 2005). Since, Baicalein was found to be a 12-LOX inhibitor (Chen et al. 1999) and in breast cancer increased levels of 12-LOX were identified (Jiang et al. 2006), we assumed that Baicalein have an inhibitory effect on the gap formation. Wang et al. (2010) described the strong anti-proliferative effect of Wogonin and Baicalein, major constituents of the plant *Scutellaria baicalensis,* on MCF-7 cells. Wogonin is structurally highly related to Baicalein. We demonstrated that Baicalein inhibits the invasivity of MCF-7 cells indicated by reduced lymphendothelial gap formation. Hence, we also expect that Wogonin might have an inhibitory effect on gap formation.

Yet, Wogonin has not been described to be a 12/15-LOX inhibitor. However, Wogonin inhibited gap formation more efficiently than Baicalein and therefore Wogonin seems to be a more potent 12/15-LOX inhibitor than Baicalein.

To confirm that the gap formation was not due to apoptosis of LECs, we performed apoptosis assay and found that treatment with 1 µM 12(S)-HETE had no effect and

treatment with 100 µM Baicalein did not induce apoptosis within the time settings of the gap formation assay.

It was reported that in cancer NF-κB activation is associated with tumor cell proliferation, survival, invasion and angiogenesis (Brown et al. 2008). This makes NF-κB an interesting target for the treatment of cancer.

Bay11-7082 is an agent that has been reported to irreversibly inhibit NF-κB triggered gene expression (Pierce et al. 1997).

In this study we found that treatment of MCF-7 spheroids with increasing concentration of Bay11-7082 caused a complete inhibition of gap formation. These data suggested that NF-κB is involved in the process of gap formation. We designed specific experiments that allowed us to discriminate whether this effect was due to the inhibition of NF-κB in the MCF-7 spheroid or in the LEC-layer. Concentrations of Bay11-7082 higher than 10 µM prevented the attachment of the spheroids to LEC-monolayers. This demonstrated that Bay11-7082 inhibited a mechanism that was necessary for the adhesion of MCF-7 cells to LECs. Another important aspect of Bay11-7082 – mediated NF-κB inhibition of LECs was the cloudy LEC distribution which suggested that LECs lost their cell-cell contact among themselves underneath the spheroid, but did not migrate and therefore failed to open a gap.
This observation rendered us to investigate the role of VE-cadherin, because cadherins are known to facilitate stable lateral cell-cell links via homophilic Ca^{2+}-dependent interactions. Different types of cadherins were described. VE-cadherin is reported to be specifically expressed in endothelial cells and it is a marker for an epithelial and immotile cell phenotype (Vestweber 2008).
To simplify the MCF-7 spheroid/LEC co-culture system we treated LEC with 1 µM 12(S)-HETE, because we could demonstate that this molecule is the gap forming factor of MCF-7 cells (Kerjaschki et al. 2010).
Here we found by western blot analysis and immunofluorescence that VE-cadherin is expressed in LECs (which to our best knowledge, was not described before) and

treatment of LECs with 12(S)-HETE caused a transient downregulation of VE-cadherin expression.
This was consistent with the fact that LECs lost their cell-cell contacts underneath spheroid because VE-cadherin interactions were indeed disrupted, and that VE-cadherin is associated with an immobile phenotype.
Furthermore we observed a 12(S)-HETE triggered increase of ZEB1 expression in LECs. ZEB1 is a transcriptional repressor and known to downregulate E-cadherin expression (Schmalhofer et al. 2009). Loss of E-cadherin is reported to be a key initiating event in epithelial-mesenchymal transition, the first step of metastasis (Thiery 2002). High ZEB1 expression was described to be associated with an increased migratory and invasive potential (Arumugam et al. 2009). Since it was not known that ZEB1 also downregulates VE-cadherin we investigated in a siRNA approach whether knockdown of ZEB1 would cause loss of VE-cadherin regulation by 12(S)-HETE, which was in fact the case. Further we observed a Bay11-7082 induced decrease of ZEB1 by western blot analysis and immunofluorescence. This implicated the involvement of NF-κB in the regulation of ZEB1, VE-cadherin and LEC motility. Also Chua et al. (2007) found that NF-κB activation is associated with induction of ZEB1 expression.

Another independent marker of motility is S100A4. Rudland et al. (2000) showed that S100A4 has been associated with migratory and invasive properties.
Extracellular addition of S100A4 activates NF-κB through induction of phosphorylation and subsequent degradation of I-κBα (Boye et al. 2008). We found that 12(S)-HETE induced S100A4 and Bay11-7082 inhibited S100A4 expression which directly correlated with LEC motility. These results propose that 12(S)-HETE induces EMT in LECs although this interpretation is somewhat problematic, because LECs are of mesenchymal origin yet with an epitheloid phenotype.

The adhesion of tumor cells to the endothelial cells represents the first important event in the process of metastasis (Honn et al. 1987). In our study we described the possible role of three bonafide NF-κB-regulated ligand – receptor pairs in tumor cell adhesion to the endothelium. Intracellular adhesion molecule 1 is a member of the immunoglobulin gene superfamily and the best-known ligands for ICAM-1 are

the β_2 integrins (Collins et al. 1995, Rosenstein et al. 1991). ICAM-1 is constitutively express in endothelial cells whereas the β_2 integrins were described to be express by MCF-7 cells (Collins et al. 1995, Budinsky et al. 1997). The contact of these two molecules allows MCF-7 cells to attach to the endothelial monolayer.
Bolick et al. (2005) demonstrated that 12(S)-HETE stimulates NF-κB activation and NF-κB dependent ICAM-1 expression through RhoA and PKCα. We found that pre-treatment of LECs with 10 µM Bay11-7082 for 30 min and stimulation with 12(S)-HETE for 10 min inhibited the ICAM-1 expression. This data suggest that MCF-7 spheroids can not attach to the LECs after treatment with Bay11-7082. In our study we could not detect β_2 integrin in MCF-7 cells by western blot analysis probably due to antibody failure.

Another NF-κB-regulated ligand – receptor pair, which is important for the adhesion of tumor cells to the endothelium, is CD31 and $\alpha_V\beta_3$ integrin. CD31 is a pivotal constituent of the endothelial cell intercellular junction and was described to be a ligand for $\alpha_V\beta_3$ integrin (Piali et al. 1995). $\alpha_V\beta_3$ integrin also known as vitronectin receptor has been implicated in the progression of malignant tumors such as breast cancer (Harms et al. 2004, Vellon et al. 2006). It was described to be regulated by NF-κB (Scatena et al. 1998). 12(S)-HETE treatment of LECs showed a time-dependent induction of CD31 expression and pre-treatment of LECs with Bay11-7082 caused its inhibition.

CD44 was described to be a major E-selectin ligand in mediating breast cancer cell transendothelial metastasis (Zen et al. 2008). CD44 was highly upregulated in MCF-7 spheroids compared to MCF-7 monolayers (Kerjaschki et al. 2010). To demonstrate the expression of E-selectin in LECs we performed real-time quantitative PCR. Here, we only found a TNF-α triggered stimulation of E-selectin expression and further a Bay11-7082 inhibition. Treatment of LECs with 12(S)-HETE showed no effect on the E-selectin expression. Never the less, constitutive expression of E-selectin could have been sufficient to facilitate E-selectin – CD44 mediated LEC- MCF-7 spheroid adhesion.

Besides 12(S)-HETE we could identify NF-κB as a major player in MCF-7 spheroid/LEC gap formation through specific inhibitor Bay11-7082 experiments. Bay11-7082 is not used in clinical therapies. Therefore, we tested the newly synthesized compound di-GA that combines the biological effects of RV and GA, which both were described to have anti-cancer activity in a number of tumor cell lines and to have health beneficial effects (RV). Madlener et al. (2010) described the strong inhibitory activity of di-GA in gap formation. We also tested the derivatives of RV and GA assuming that also these compounds as free molecules exhibited inhibitory effects on gap formation. All of the tested compounds showed strong anti-proliferative effects on MCF-7 cells which is a hallmark for anti-tumor activity. Di-GA is a compound which consist of two GA molecules and one RV molecule (Bernhaus et al. 2009).

RV was found in grapes and red wine whereas GA can be found also in grapes, red wine gallnuts, tea leaves, pineapples, bananas (Madlener et al. 2007, Saiko et al. 2008). RV modulates several biochemical processes involved in carcinogenesis (Saiko et al. 2008, Kundu and Surh 2004). It was reported to inhibit angiogenesis and metastasis, inflammation and tumor progression and the carcinogen activation and DNA-adduct formation (Dubuisson et al. 2002, Ciolino and Yeh 1999, Banerjee et al. 2002, Kimura and Okuda 2001).

The di-GA constituent RV and its analogous M5 and M8 showed an inhibition of gap formation by 20 – 30 % but that was not as efficient as di-GA.

Indeed GA and its polyhydroxy-substituted benzohydroxamic acid derivatives showed no inhibitory effects on gap formation. They even induce it.

Treatment of cancer involves several conventional modalities such as surgical resection, radiation therapy and chemotherapy. 60 % of all effective anticancer drugs used in western medicine are derived from natural compounds however there are still a lot of unexplored resources in herbal medicines which needs to be tested (Cragg et al. 2006, Balunas and Kighorn 2005).

Among the synthetic drugs we also observed the effect of natural compounds on gap formation. A main constituent of *Hypericum perforatum* and *Hypericum adenotrichum* is Hyperforin (Özmen et al. 2009).

Hyperforin was described to inhibit tumor growth, induce apoptosis of tumor cells and reduce tumor vascularisation (Schlempp et al. 2005). Dell Aica et al. (2007) reported that it inhibited tumor invasion and metastasis as well.

In the present study we found a significant 30 % inhibition of gap formation, which is may be due to its NF-κB inhibitory property.

The RV and hyperforin doses used in this study are certainly too high for in vivo studies and therefore, these natural components are unlikely to be successful in an adjuvant therapy. However, these compounds could act in synergy with standard therapies and may play a role in tumor prevention.

5. References

Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26:1324-37 (2007)

Albelda SM, Smith CW, Ward PA. Adhesion molecules and inflammatory injury. FASEB J 8:504-12 (1994)

Ambartsumian N, Klingelhofer J, Grigorian M, et al. The metastasis-associated Mts1(S100A4) protein could act as an angiogenic factor. Oncogene 20:4685-95 (2001)

Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, Gallick GE, Logsdon CD, McConkey DJ, Choi W. Epithelial to Mesenchymal Transition contributes to drug resistance in pancreatic cancer. Cancer Res 69(5):5820-9 (2009)

Bader Y, Madlener S, Strasser S, Maier S, Saiko P, Stark N, Popescu R, Huber D, Gollinger M, Erker T, Handler N, Szakmary A, Jäger W, Kopp B, Tentes I, Fritzer-Szekeres M, Krupitza G, Szekeres T, Stilbene analogues affect cell cycle progression and apoptosis independently of each other in an MCF-7 array of clones with distinct genetic and chemoresistant backgrounds. Oncol Rep 19:801-10 (2008)

Baker SJ, Markowitz S, Fearon ER, Willson JKV, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249:912-915 (1990)

Balunas MJ, Kinghorn AD. Drug discovery from medicinal plants. Life sci. 78:431-441 (2005)

Banerjee S, Bueso-Ramos C, Aggarwal BB. Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-B, cyclooxygenases 2 and matrix metalloprotease 9. Cancer Res. 62:4945-4954 (2002)

Bednar W, Holzmann K, Marian B. Assessing 12(S)-lipoxygenase inhibitory activity using colorectal cancer cells overexpressing the enzyme. Food and Chemical Toxicology 45(3):508-514 (2007)

Bernhaus A, Fritzer-Szekeres M, Grusch M, Saiko P. Krupitza G, Venkateswarlu S, Trimurtulu G, Jäger W, Szekeres T. Digalloylresveratrol, a new phenolic acid derivative induces apoptosis and cell cycle arrest in human HT-29 colon cancer cells. Cancer Letters 274:299-304 (2009)

Bishop JM. Molecular themes in oncogenesis. Cell 44:235-48 (1991)

Bolick DT, Orr AW, Whetzel A, Srinivasan S, Hatley ME, Schwartz MA, Hedrick CC. 12/15-Lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-κB. Artersioscler Thromb Vasc Biol 25(2):2301-2307 (2005)

Bourdon A, Minai L, Serre V, Jais JP, Sarzi E, Aubert S, Chretien D, De Lonlay P, Paquis-Flucklinger V, Arakawa H, Nakamura Y, Munnich A, Rotig A. Mutation of RRM2B encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. Nat. Genet 39:776-780 (2007)

Boye K, Grotterod I, Aasheim HC, Hovig E, Maelandsmo GM. Activation of NF-kappaB by extracellular S100A4: analysis of signal transduction mechansims and identification of target genes. Int J Cancer 123/6(p4):1301-10 (2008)

Brown M, Cohen J, Arun P, Chen Z, Van Waes C. NF-κB in carcinoma therapy and prevention. Expert Opin Ther Targets 12(9):1109-1122 (2008)

Budinsky AC, Brodowicz T, Wiltschke C, Czerwenka K, Michl I, Krainer M. Decreased expression of ICAM-1 and its induction by tumor necrosis factor on breast-cancer cells in vitro. Int J Cancer 71(p3):1086-1090 (1997)

Cao Y, Karin M. NF-κB in mammary gland development and breast cancer. Journal of Mammary Gland Biology and Neoplasia. Vol. 8 No.2:215-223 (2003)

Cauwe B, Van den Stehen PE, Opdenakker G. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit Rev Biochem Mol Biol 42:113-185 (2007)

Chabottaux V, Noel A. Breast cancer progression: insights into multifaceted matrix metalloproteinases. Clin Exp Metastasis 24:647-656 (2007)

Chen YQ, Duniec ZM, Liu B, Hagmann W, Gao X, Shimoji K, Marnett LJ, Johnson CR, Honn KV. Endogenous 12(S)-HETE production by tumor cells and its role in metastasis. Cancer Research 54:1574-1579 (1994)

Chen ZY, Su YL, Lau CW, Law Wi; Huang Y. Endothelium-dependent contraction and direct relaxation induced by baicalein in rat mesenteric artery. Eur J Pharmacol 374:41-47 (1999)

Chen X, Wand S, Wu N. Overexpression of 5-lipoxygenase in rat and human esophageal adenocarcinoma and inhibitory effects of zileuton and celecoxib on carcinogenesis. Clinical Cancer Research 10(19):6703-6709 (2004)

Chua HL, Bhat-Nakshatri P, Clare SE, Moimiya A, Badve S, Nakshatri H. NF-κB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene 26(p4):711-724 (2007)

Ciolino HP, Yeh GC. Inhibition of aryl hydrocarbon induced cytochrome P-4501A1 enzyme activity and CYP1A1 expression by Resveratrol. Mol. Pharmacol. 56:760-767 (1999)

Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS. Selective activation of NF-κB subunits in human breast cancer: Potential roles for NF-kB2/p52 and for Bcl-3. Oncogene 19:1123-1131 (2000)

Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. FASEB J. 9(6-7):899-909 (1995)

Cragg GM, Newman DJ, Yang SS. Natural product extracts of plant and marine origin having antileukemia potential. The NCI experience. J. Nat. Prod. 69(3):488-98 (2006)

Dehay C, Kennedy H. Cell-cycle control and cortical development. Nature Reviews Neuroscience 8:438-450 (2007)

Dell Aica I, Caniato R, Biggin S, Garbisa S. Matrix proteases, green tea and St. John´s wort: Biomedical research catches up with folk medicine. Clinica Chimica Acta 381:69-77 (2007)

Deryungina EI, Bourdon MA, Jungwirth K, Smith JW; Strongin AY. Functional activation of integrin αβ in tumor cells expressing membrane-type 1 matrix metalloproteinase. Int J. Cancer 86(1):15-23 (2000)

Dubuisson JG, Dyess DL, Gaubatz JW. Resveratrol modulates human mammary epithelial cell O-acetyltransferase, sulfotransferase, and kinase activation of the heterocyclic amine carcinogen N-hydroxyPhIP. Cancer Lett. 182:27-32 (2002)

Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. Major cell death pathways at a glance. Microbes and Infection 11:1050-1062 (2009)

Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161-174 (2002)

Epstein FH, Barnes PJ, Karin M. Nuclear Factor-kB – A pivotal transcription factor in chronic inflammatory diseases. The New England Journal of Medicine. Vol. 336 No. 15 (p2):1066-1071 (1997)

Evan G, Littlewood T. A matter of life and cell death. Science. 281:1317-1322 (1998)

Fedi P, Tronick SR and Aaronson SA (1997) Growth factors. In: Cancer Medicine, Holland JF Bast RC Morton DL Frei E Kufe DW Weichselbaum RR (eds) pp 41–64. Fedi P, Tronick SR, Aaronson SA. Growth factors. Cancer Medicine 41-64 (1997)

Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A, Mueller BM. Integrin activation controls metastasis in human breast cancer. Proc Natl Acad Sci USA 98:1853-1858 (2001)

Fontecave M, Lepoivre M, Elleingand E, Gerez C, Guittet O. Resveratrol: a remarkable inhibitor of ribonucleotide reductase, FEBS Lett. 421:277-279 (1998)

Fritzer-Szekeres M, Novotny L, Romanova D, Glöbl R, Sedlak J, Vachalkova A, Rauko P, Elford HL, Szekeres T. Enhanced effects of adriamycin by combination with a new ribonucleotide reductase inhibitor, trimidox, in murine leukaemia. Life Sci 63:545-52 (1998)

Fürstenberger G, Krieg P, Müller-Decker K, Habenicht AJ. What are cyclooxygenases and lipoxygenases doing in the driver´s seat of carcinogenesis? International Journal of Cancer 119:247-254 (2006)

Geiger TR, Peeper DS. Metastasis mechanisms. Biochimica et Bioyphysica Acta. 1796:293-308 (2009)

Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16:225-260 (1998)

Ghosh S, Karin M. Missing Pieces in the NF-κB Puzzle. Cell 109:81-96 (2002)

Goodlett CR, Horn KH. Mechanisms of alcohol-induced damage to the developing nervous system. Alcohol Res Health. 25(3):175-84 (2001)

Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiol Rev 87:69-98 (2007)

Grusch M, Fritzer-Szekeres M, Fuhrmann G, Rosenberger G, Luxbacher C, Elford HL, Smid K, Peters GJ, Szekeres T, Krupitza G. Activation of caspases and induction of apoptosis by novel ribonucleotide reductase inhibitors amidox and didox. Experimental Hematology 29: 623-632 (2001)

Haffner MC, Berlato C, Doppler W. Exploiting our knowledge of NF-κB signaling for the treatment of mammary cancer. J Mammary Gland Biol Neoplasia 11:63-73 (2006)

Hanahan D, Weinberg RA, The hallmarks of cancer. Cell. Vol. 100:57-70 (2000)

Harms JF, Welch DR, Samant RS, Shevde LA, Miele ME, Babu GR, Goldberg SF, Gilman VR, Sosnowski DM, Campo DA, Gay CV, Budgeon LR, Mercer R, Jewell J, Mastro AM, Donahue HJ, Erin N, Debies MT, Meehan WJ, Jones AL, Mbalaviele G, Nickols A, Christensen ND, Melly R, Beck LN, Kent J, Rader RK, Kotyk JJ, Pagel MD, Westlin WF, Griggs DW. A small molecule antagonist of the alpha(v)beta3 integrin suppresses MDA-MB-435 skeletal metastasis. Clin Exp Metastasis 21:119-128 (2004)

Himeji M, Ohtsuki T, Fukazawa H, Tanaka M, Yazaki SI, Ui S, Nishio K, Yamamoto H, Tasaka K, Mimura A. Difference of growth-inhibitory effect of Scutellaria baicalensis – producing flavonoid wogonin among human cancer cells and normal diploid cell. Cancer Lett. 245:269-274 (2007)

Honn KV, Steinert BW, Moin K, Onoda JM, Taylor JD and Sloane BF. The role of platelet cyclooxygenases and lipoxygenase pathways in tumor cell induced platelet aggregation. Biochem. Biophys. Res. Commun. 29:384-389 (1987)

Honn KV, Tang DG, Grossi I, Duniec ZM, Timar J, Renaud C, Leithauser M, Blair I, Johnson CR, Diglio CA, Kimler V, Taylor JD, Marnett LJ. Tumour cell derived 12(S)-hydroxyeicosatetraenoic acid induces Microvascular endothelial cell retraction. Cancer Research 54:565-574 (1994)

Horvath Z, Murias M, Saiko P, Erker T, Handler N, Madlener S, Jäger W, Grusch M, Fritzer-Szekeres M, Krupitza G, Shekeres T. Cytotoxic and biochemical effects of 3,3',4,4',5,5'-hexahydroxystilbene, a novel resveratrol analog in HL-60 human promyelocytic leukemia cells. Exp Hematol. 34(10):1377-84 (2006)

http://www.cancer.gov/cancertopics/wyntk/breast/page4 last access 10 January 2010

http://preview.cancer.gov/cancertopics/wyntk/breast/page8#f last access 10 January 2010).

Ilg EC, Schäfer BW, Heizmann CW. Expression pattern of S100 calcium-binding proteins in human tumors. Int. J. Cancer. 68(3):325-332 (1996)

Inoue M, Suzuki R, Koide T, Sakaguchi N, Ogihara Y, Yabu Y. Antioxidant gallic acid, induces apoptosis in HL-60RG cells. Biochem. Biophy. Res. Commun. 204: 898-904 (1994)

Isuzugawa K, Inoue M, Ogihara Y. Catalase contents in cells determine sensitivy to the apoptosis inducer gallic acid. Biol. Pharm. Bull. 24 1022-1026 (2001)

Jenkinson SR, Barraclough R, West CR, Rudland PS. S100A4 regulates cell motility and invasion in an in vitro model for breast cancer metastasis. Br J Cancer 90:253-62 (2004)

Jiang WG, Douglas-Jones A, Mansel RE. Levels of expression of lipoxygenases and cyclooxygenases-2 in human breast cancer. Prostaglandins, Leukotrienes and Essential Fatty Acids 69(4):275-281 (2003)

Jiang WG, Douglas-Jones AG, Mansel RE. Aberrant expression of 5-lipoxygenase-activating protein (5-LOXAP) has prognostic and survival significance in patients with breast cancer. Prostaglandins, Leukotrienes and Essential Fatty Acids 74(2):125-134 (2006)

Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J. Clin. Invest. 112:1776-1784 (2003)

Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J. Clin. Invest. 119:1420-1428 (2009)

Kawada M, Ohno Y, Ri Y, Ikoma T, Yuugetu H, Asai T et al. Anti-tumor effects of gallic acid on LL-2 lung cancer cells transplanted in mice. Anticancer Drugs 12:847-852 (2001)

Khan AI, Kerfoot SM, Heit B, Liu L, Andonegui G, et al. Role of CD44 and hyaluronan in neutrophil recruitment. J Immunol 173:7594-7601 (2004)

Kimura Y, Okuda H. Resveratrol isolated from Polygonum cuspidatum root prevents tumor growth and metastasis to lung and tumor-induced

neovascularization in Lewis lung carcinoma-bearing mice. J Nutr 131:1844-1849 (2001)

Kramer RH, Nicolson K. Interactions of tumor cells with vascular endothelial cell monolayers: A model for metastatic invasion. Proc. Natl. Acad. Sci. USA Vol. 76 11:5704-5708 (1979)

Krysan K, Reckamp KL, Sharma S, Dubinett SM. The potential and rationale for COX-2 inhibitors in lung cancer. Anitcancer Agents in Medical Chemistry 6(3):209-220 (2006)

Kundu JK, Surh YJ. Molecular basis of chemoprevention by Resveratrol: NF-κB and AP-1 as potential targets. Mutation Research 555:65-80 (2004)

Laferriere J, Houle F, Huot J. Regulation of the metastatic process by E-selectin and stress-activated protein kinase-2/p38. Ann N Y Acad Sci. 973(6):562-572 (2002)

Lee DH, Kim C, Zhang L, Lee YJ. Role of p53, PUMA and Bax in wogonin-induced apoptosis in human cancer cells. Biochem Pharmacol 75:2020-2033 (2008)

Liapis H, Flath A, Kitazawa S. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. Diagn Mol Pathol 5(2):127-35 (1996)

Lin JHC, Kobari Y, Zhu Y, Stemerman MB, Pritchard KA. Human umbilical vein endothelial cells express P450 2G8 mRNA: cloning of endothelial P450 epoxygenase. Endothelium 4:219-29 (1996)

Locatelli GA, Savio M, Forti L, Shevelev I, Ramadan K, Stivala LA, Vannini V, Hubscher U, Spadari S, Maga G. Inhibition of mammalian DNA polymerases by

Resveratrol: mechanism and structural determinants. Biochem. J. 389:259-268 (2005)

Lorusso G, Vannini N, Sogno I, Generoso L, Garbisa S, Noonan DM, Albini A. Mechanisms of Hyperforin as an anti-angiogenic angioprevention agent. European Journal of Cancer 45(p8):1474-1484 (2009)

Ma Z, Otsuyama K, Liu S, Abroun S, Ishikawa H, Tsuyama N, Obata M, Li FJ, Zheng X, Maki Y, Miyamoto K, Kawano MM. Baicalein, a component of Scutellaria radix from Huang-Lian-Jie-Du-Tang (HLJDT), leads to suppression of proliferation and induction of apoptosis in human myeloma cells. Blood 105(8):3312-3318 (2005)

Madlener S, Illmer C, Horvath Z, Saiko P, Losert A, Herbacek I, Grusch M, Elford HL, Krupitza G, Bernhaus A, Fritzer-Szekeres M, Szekeres T. Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukaemia cells. Cancer Letters 245:156-162 (2007)

Mandinova A, Atar D, Schafer BW, Spies M, Aebi U, Heizmann CW. Distinct subcellular localization of calium binding S100 proteins in human smooth muscle cells and their relocation in response to rises in intracellular calcium. J. Cell Sci. 111:2043-2054 (1998)

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat. Rev. Mol. Cell Biol. 8:741-752 (2007)
Min C, Eddy SF, Sherr DH, Sonenshein GE. NF-κB and Epithelial to mesenchymal transition of cancer. Journal of Cellular Biochemistry 104:733-744 (2008)

Meeran SM, Katiyar SK. Cell cycle control as a basis for cancer chemoprevention through dietary agents. Frontiers in Bioscience 13:2191-2202 (2008)

Moreno JJ. New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. Biochemical Pharmacology 77:1-10 (2009)

Moss MA, Zimmer S, Anderson KW. Role of metastatic potential in the adhesion of human breast cancer cells to endothelial monolayers. Anticancer Res. 20:1425-1433 (2000)

Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet Jr. RJ, Sledge Jr. GW. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol. 17:3629-3639 (1997)

Naor D, Sionov RV, Ish-Shalom D. CD44: structure, function, and association with the malignant process. Adv Cancer Res 71:241-319 (1997)

Nevins JR. The Rb/E2F pathway and cancer. Hum. Mol. Genet. 10:699-703 (2001)

Newman PJ. Switched at birth: a new family for PECAM-1. J. Clin. Invest. 103:5-9 (1999)

Nithipatikom K, Isbell MA, See WA, Campbell WB. Elevated 12- and 20-hydroxyeicosatetraenoic acid in urine of patients with prostatic diseases. Cancer Letters 233(2):219-225 (2006)

Noel A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. Semin Cell Dev Biol. doi:10.1016/j.semcdb.2077.05.011 (2007)

Overall CM, Dean RA. Degradomics: systems biology of the protease web. Pleiotropic roles of MMPs in cancer. Cancer Metastasis Rev 25:69-75 (2006)

Özmen A, Madlener S, Bauer S, Krasteva S, Vonach C, Giessrigl B, Gridling M, Viola K, Stark N, Saiko P, Michel B, Fritzer-Szekeres M, Szekeres T, Askin-Celik T, Krenn L, Krupitza G. In vitro anti-leukemic activity of the ethno-pharmacological plant Scutellaria orientalis ssp. carica endemic to western Turkey. Phytomedicine 17(1):55-62 (2009)

Özmen A, Bauer S, Gridling M, Singhuber J, Krasteva S, Madlener S, Vo TPN, Stark N, Saiko P, Fritzer-Szekeres M, Szekeres T, Askin-Celik T, Krenn L, Krupitza G. In vitro anti-neoplastic activity of the ethno-pharmaceutical plant Hypericum adenotrichum spach endemic to western Turkey. Oncol Rep. 22(4):845-52 (2009)

Parry G, Beck JC, Moss L, Bartley J, Ojakian GK. Determination of apical polarity in mammary epithelial cell cultures: the role of cell-cell, cell-substratum, and membrane-cytoskeleton interactions. Exp. Cell Res. 188:302-311 (1990)

Pecorino L. Molecular biology of cancer. Oxford University Press (2008)

Piali L, Hammel P, Uherek C, Bachmann F, Gisler RH, Dunon D, Imhof BA. CD31/PECAM-1 is a ligand for αVβ3 Integrin involved in adhesion of leukocytes to endothelium. The Journal of Cell Biology Vol. 130 2:451-460 (1995)

Pidgeon GP, Lysaght J, KRishnamoorthy S, Reynolds JV, O´Byrne K, Nie D, Honn KV. Liypoxygenase metabolism: roles in tumor progression and survival. Cancer Metastasis Rev 26:503-524 (2007)

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced IkBα phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J. Biol. Chem. Vol. 272 Nr.34, pp21096-21103 (1997)

Pietenpol JA, Stewart ZA. Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis. Toxicology 181-182:275-481 (2002)

Regimbald LH, Pilarski LM, Longenecker BM, Reddish MA, Zimmermann G, Hugh JC. The breast mucin MUC1 as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. Cancer Research 56:4244-4249 (1996)

Renaud S, De Lorgeril M. Wine, alcohol platelets, and the French paradox for coronary heart disease. Lancet 339:1523-1526 (1992)

Rolli M, Fransvea E, Pilch J, Saven A, Felding-Habermann B. Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. Proc Natl Acad Sci USA 100:9482-9487 (2003)

Rosenstein Y, Park JK, Hahn WC, Rosen FS, Bierer BE, Burakoff SJ. CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. Nature (Lond.) 354:233-235 (1991)

Rudland PS, Platt-Higgins A, Renshaw C, West CR, Winstanley JHR, Robertson L, Barraclough R. Prognostic Significance of the Metastasis-inducing Protein S100A4 (p9Ka) in Human Breast Cancer. Cancer Research 60:1595-1603 (2000)

Rupp PA, Little CD. Integrins in vascular development. Circ. Res. 89:566-572 (2001)

Saiko P, Szakmary A, Jäger W, Szekeres T. Resveratrol and its analogs: Defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutation Research 658:68-94 (2008)

Salucci M, Stivala LA, Maiani G, Bugianesi R, Vannini V, Flavonoids uptake and their effects on cell cycle of human colon adenocarcinoma cells (Caco2), Br. J. Cancer 86:1645-1651 (2002)

Scarborough PE, Ma J, Qu W, Zeldin DC. P450 subfamily CYP2J and their role in the bioactivation of arachidonic acid in extrahepatic tissues. Drug Metab Rev 31:205-34 (1999)

Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM. NF-κB mediates $\alpha_V\beta_3$ integrin-induced endothelial cell survival. The Journal of Cell Biology Vol 141 No 4 1083-1093 (1998)

Schempp CM, Kiss J, Kirkin V, Averbeck M, Haarhaus BS; Kremer B, Termeer CC, Sleeman J, Simon JC. Hyperforin acts as an Angiogenesis Inhibitor in vitro and in vivo. Planta Med 71:999-1004 (2005)

Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, β-catenin, and ZEB1 in malignant progressio of cancer. Cancer Metastasis Rev 28(p5-6):151-166 (2009)

Schmidt-Hansen B, Ornas D, Grigorian M, et al. Extracellular S100A4(mts1) stimulates invasive growth of mouse endothelial cells and modulates MMP-13 matrix metalloproteinase activity. Oncogene 23:5487-95 (2004)

Sherr CJ. Principles of tumor suppression. Cell 116:235-246 (2004)

Sohi KK, Mittal N, Hundal MK, Khanduja KL, Gallic acid, an antioxidant, exhibits antiapoptotic potential in normal human lymphocytes: a Bcl-2 independent mechanism, J. Nutr. Sci. Vitaminol. (Tokio) 49:221-227 (2003)

Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM et al. Aberrant nuclear factor-kB/Rel expression and the pathogenesis of breast cancer. J. Clin. Invest. 100:2952-2960 (1997)

Spessotto P, Rossi FM, Degan M, Di Francia R, Perris R, et al. Hyaluronan-CD44 interaction hampers migration of osteoclast-like cells by down-regulating MMP-9. J Cell Biol 158:1133-1144 (2002)

Sporn MB. The war on cancer. Lancet. 347(9012):1377-81 (1996)

Sun Z, Andersson R. NF-κB activation and inhibition: a review. Shock 18(2):99-106 (2002)

Szekeres T, Gharehbaghi K, Fritzer M, Woody M, Srivastava A, van´t Riet B, Jayaram HN, Elford HL. Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase. Cancer Chemother Pharmacol 34:63-6 (1994)

Takenaga K, Nakamura Y, Endo H, Sakiyama S. Involvement of S100-related calcium-binding protein pEL98 (or mts1) in cell motility and tumor cell invasion. Jpn J Cancer Res. 85:831-9 (1994)

Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, Takei Y, Nakamura Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 404:42-49 (2000)

Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nature Reviews Cancer 2:442-454 (2002)

Timar J, Chen YQ, Liu B, Bazaz R, Taylor JD, Honn KV. The lipoxyenase metabolite 12(S)-HETE promotes αIIbβ3 integrin mediated tumor cell spreading on fibronectin. Int. J. Cancer 52:594-603 (1992)

Tong WG, Ding XZ, Adrian TE. The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells. Biochemical and Biophysical Research Communications 296:942-948 (2002)

Tozeren A, Kleinman HK, Grant DS, Morales D, Mercurio AM, et al. E-selectin-mediated dynamic interactions of breast- and colon-cancer cells with endothelial-cell monolayers. Int J Cancer 60:426-431 (1995)

Tran K, Merika M, Thanos D. Distinct functional properties of I-kBα and I-kBβ. Mol Cell Biol. 17:5386-5399 (1997)

Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. Nat. Rev. Mol. Cell Biol. 3:11-20 (2002)

Uchide K, Sakon M, Ariyoshi H, Nakamori S, Tokunaga M, Monden M. Cancer cells cause vascular endothelial cell retraction via 12(S)HETE secretion; The possible role of cancer cell derived microparticle. Annals of Surgical Oncology 14(2):862-868 (2007)

Van den Heuvel S, Dyson NJ. Conserved functions of the pRB and E2F families. Nature Reviews Molecular Cell Biology 9:713-724 (2008)

Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. Semin. Cancer Biol. 9:211-220 (1999)

Vellon L, Menendez JA, Lupu R: A bidirectional "alpha(v)beta(3) integrin-ERK1/ERK2 MAPK" connection regulates the proliferation of breast cancer cells. Mol Carcinog 45:795-804 (2006)

Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 36:131-149 (2003)

Vestweber D. VE-cadherin: The major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler. Thromb. Vasc. Biol. 28(p1-2):223-232 (2008)

Vo TP, Madlener S, Bago-Horvath Z, Herbacek I, Stark N, Gridling M, Probst P, Giessrigl B, Bauer S, Vonach C, Saiko P, Grusch M, Szekeres T, Fritzer-Szekeres M, Jäger W, Krupitza G, Soleiman A, Pro- and anti-carcinogenic mechanisms of piceatannol are activated dose-dependently in MCF-7 breast cancer cells. Carcinogenesis.(2009)

Vogelstein, B. et al. Surfing the p53 network. Nature 408:307–310 (2000)

Wang CZ, Li XL, Wang QF, Mehendale SR, Yuan CS. Selective fraction of *Scutellaria baicalensis* and its chemopreventive effects on MCF-7 human breast cancer cells. Phytomedicine 17:63-68 (2010)

Wang G, Rudland PS, White MR, Barraclough R. Interaction in Vivo and in Vitro of the Metastasis-inducing S100 Protein, S100A4 (p9Ka) with S100A1. The Journal of biological chemistry; Vol 275, No 15, 11141-11146 (2000)

Wang HS, Hung Y, Su CH, Peng ST, Guo YJ, et al. CD44 cross-linking induce integrin-mediated adhesion and transendothelial migration in breast cancer cell line by up-regulation of LFA-1 (alpha L beta2) and VLA-4 (alpha4beta1). Exp Cell Res 304:116-126 (2005)

WHO The global burden of disease 2004: http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html

Willis RA. The spread of tumors in the human body, 3rd ed. London. Butterworth 1-282 (1973)

Yilmaz M, Christofori G, Lehembre F. Distinct mechanisms of tumor invasion and metastasis. Trends in Molecular Medicine 13:535-541 (2007)

Yin X, Zhou J, Jie C, Xing D, Zhang Y. Anticancer activity and mechanism of Scutellaria barbata extract on human lung cancer cell line A459. Life Sci. 75:2233-2244 (2004)

Zandi E, Rothwarf DM, Delhase M, et al. The IkB kinase complex (IKK) contains two kinase subunits, IKKα and IKK-β, necessary for IkB phosphorylation and NF-κB activation. Cell 91:243-252 (1997)

Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. J Clin Invest 119(p3):1429-1437 (2009)

Zen K, Liu DQ, Guo YL, Wang C, Shan J, Fang M, Zhang CY, Liu Y. CD44v4 is a major E-selectin ligand that mediates breast cancer cell transendothelial migration. PLoS ONE Vol 3 Issue 3 e1826 (2008)

Zhu B, Liu Z, Wang P, Wu C, Xu H. A Nuclear Factor-kB Inhibitor Bay11-7082-7082-7082 Inhibits Interactions between Human Endothelial Cells, T Cells, and Monocytes. Transplantation Proceeding 40:2724-2728 (2008)

Zucker S, Pei D, Cao J, Lopez-Otin C. Membrane typematrix metalloproteinases (MT-MMP). Curr Top Dev Biol 54:1-74 (2003)

Lebenslauf

Name:	Caroline Vonach
Geburtsdatum:	25. Februar 1984
Geburtsort:	Bregenz
Eltern:	Edgar Vonach
	Siegrid Vonach

Schulbildung:

1990 – 1994 Volksschule Dorf / Lauterach

1994 – 1998 Sacré Coeur Riedenburg Gymnasium / Bregenz

1998 – 2003 Handelsakademie / Bregenz

Studium:

ab Okt. 2003:	Studium der Biologie / Anthropolgie an der Universität Wien
Sept. 08 – Nov. 09 :	Diplomarbeit am Institut für Klinische Pathologie, Medizinische Universität Wien
	Titel: “Mechanism of breast cancer in vitro metastasis”

Scientific Publications

Khan M, Giessrigl B, **Vonach C**, Madlener S, Prinz S, Herbaceck I, Hölzl C, Bauer S, Viola K, Mikulits W, Quereshi RA, Knasmüller S, Grusch M, Kopp B, Krupitza G, Berberine and a Berberis lyceum extract inactivate Cdc25A and induce alpha-tubulin acetylation that correlate with HL-60 cell cycle inhibition and apoptosis. Mutat Res. 683(1-2):123-30 (2010)

Stark N, Gridling M, Madlener S, Bauer S, Lackner A, Popescu R, Diaz R, Tut FM, Vo TP, **Vonach C**, Giessrigl B, Saiko P, Grusch M, Fritzer-Szekeres M, Szekeres T, Kopp B, Frisch R, Krupitza G, A polar extract of the Maya healing plant Anthurium schlechtendalii (Aracea) exhibits strong in vitro anticancer activity. Int J Mol Med. 24(4):513-21 (2009)

Vo TP, Madlener S, Bago-Horvath Z, Herbacek I, Stark N, Gridling M, Probst P, Giessrigl B, Bauer S, **Vonach C**, Saiko P, Grusch M, Szekeres T, Fritzer-Szekeres M, Jäger W, Krupitza G, Soleiman A, Pro- and anti-carcinogenic mechanisms of piceatannol are activated dose-dependently in MCF-7 breast cancer cells. Carcinogenesis (2010)

Özmen A, Madlener S, Bauer S, Krasteva S, **Vonach C**, Giessrigl B, Gridling M, Viola K, Stark N, Saiko P, Michel B, Fritzer-Szekeres M, Szekeres T, Askin-Celik T, Krenn L, Krupitza G, In vitro anti-leukemic activity of the ethno-pharmacological plant Scutellaria orientalis ssp. Carica endemic to western Turkey. Phytomedicine. 17(1):55-62 (2009)

Madlener S, Saiko P, **Vonach C**, Viola K, Huttary N, Stark N, Popescu R, Gridling M, Vo N, Herbacek I, Davidovits A, Giessrigl B, Venkateswarlu S, Geleff S, Jäger W, Grusch M, Kerjaschki D, Mikulits W, Trimurtulu G, Fritzer-Szekeres M, Szekeres T, Krupitza G, Multifactorial anti-cancer effects of di-galloyl resveratrol encompass apoptosis, cell cycle arrest, and inhibition of lymphendothelial gap formation in vitro. British Journal of Cancer (2010 in print)

Kerjaschki D, Rudas M, Bartel G, Bago-Horvath Z, Sexl V, Wolbank S, Schneckenleithner Ch, Dolznig H, Krieger S, Hantusch B, Nagy-Bojarszky K, Huttary N, Raab I, Kalt R, Lackner K, Gnant M, Krautgasser K, Schachner H, Kaserer K, Rezar S, Madlener S, **Vonach C**, Davidovits A, Nosaka H, Hirakawa S, Detmar M, Alitalo K, Nijman S, Offner F, Maier TJ, Geisslinger G, Krupitza G, Tumour invasion into intrametastatic lymphatic causes lymph node metastasis. Nature (2010 in revision)

Paper in preparation

Vonach C, Viola K, Giessrigl B, Hämmerle M, Huttary N, Raab I, Kalt R, Krieger S, Vo TP, Madlener S, Bauer S, Unger C, Seeliger M, Marian B, Hantusch B, Stary S, Kerjaschki D, Dolznig H, Jäger W, Szekeres T, Eger A, Mikulits W, Krupitza G. Mechanism of 12(S)-HETE induced lymphendothelial cell migration