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The inhibition of 12(S)-HETE stimulated mobility and gap formation of lymphendothelial cells

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Abbreviations

AA	Arachidonic acid
ABP	Actin-binding proteins
AP	Activator protein
ARF	Alternative reading frame
Bay-11	Bay-11-7082
BEC	Blood vessel endothelial cell
CAM	Cell adhesion molecule
CDK	Cyclin dependent kinase
CKI	Cyclin-dependent kinase inhibitor
COX	Cyclooxygenases
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated protein kinase
FGF	Fibroblast growth factor
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GF	Growth Factor
GS	Growth Signals
GTP	Guanosine triphosphate
HDMVEC	Human dermal microvascular endothelial cells
HETE	Hydroxyeicosatetraenoic acid
HGF	Hepatocyte growth factor
HLF	Human lung fibroblasts
HUVECs	Human umbilical vein endothelial cell
IC_{50}	Concentration which inhibits by 50 %
ICAM	Intracellular adhesion molecule 1
IL-1	Interleukin 1
ILK	Integrin-linked kinase
I-κB	Inhibitor kappa B
INK	Inhibitor of kinase

LEC	Lymph endothelial cell
LO or LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MPF	Mitosis promoting factor
MPO	Myeloperoxidase
MT-MMP	Membrane-type matrix metalloproteinase
MYPT	Myosin phosphatase target
MYPT1	Myosin-binding subunit of myosin phosphatase
NF-κB	Nuclear factor kappa B
NLS	Nuclear localisation signals
PAK	p21-activated kinase
PDGF	Platelet-derived growth factor
PIC	Piceatannol
Rb	Retinoblastoma
RNA	Ribonucleic acid
RHD	Rel homology domain
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
siRNA	small interfering RNA
TCM	Traditional Chinese medicine
TIMP	Tissue inhibitors of metalloproteinases
TGF	Tumour/transforming growth factor
TNF	Tumour necrosis factor
TSG	Tumour suppressor gene
u-PA	urokinase-plasminogen activator
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

Zusammenfassung

Das Eindringen von Brustkrebs erfolgt stets in der Form von Zellclustern, die sich durch Lücken in den Lymphendothelien, in axillare “gate keeping” Wächter-Lymphknoten ausbreiten um schließlich weitere Lymphknoten und entfernte Organe zu befallen. Aus diesem Grund ist die Invasion von Krebszellclustern in das Lymphgefäßsystem ein Hauptziel pharmakologischer Eingriffe. Durch den Einsatz eines 3D Co-cultur *in vitro* Systems, bestehend aus einer T1S1 (telomerase-immortalised human lymphendothelial) Zellschicht und menschlichen MCF-7 Brustkrebszellspheroiden, wurde versucht die Krebszellinduktion von Lücken in der Lymphendothelzell/LEC-schicht nachzuahmen. Bisherige Studien haben zwei Mechanismen identifiziert, die für die Induktion von Lücken in Lymphendothelien verantwortlich sind: die Aktivität der 12/15 Lipoxygenase und ihres Produkts 12(S)-HETE, das von MCF-7 Zellen ausgeschieden wird; und Matrix Metallo Proteinasen (MMPs). Zusätzlich spielt auch NF-κB eine entscheidende Rolle in der Tumorcluster Transmigration. Die vorliegende Diplomarbeit versucht die Beteiligung dieses Transkriptionsfaktors zu untersuchen, und weiters die hemmende Wirkung unterschiedlicher natürlich vorkommender und synthetischer Substanzen auf die Bildung von Lücken in Lymphendothelien zu erforschen. Aus diesem Grund wurden „gap formation assays“ als Eingangsexperimente durchgeführt, wobei die Fähigkeit von MCF-7 Zellspheroiden zur Lückeninduktion in LEC-Schichten durch die Behandlung der Krebsspheroide mit Bay-11, Baicalein, Heteronemin, Xanthohumol, Berberine, und GM6001 versucht wurde zu inhibieren. Die Resultate zeigen, dass alle gestesteten Substanzen, bereits in geringen Mengen (~ 5µM), eine starke Lücken-hemmende Wirkung besitzen. Mit weiterführenden Experimenten wurde versucht die molekularen Mechanismen dieser Substanzen auf die Krebsspheroid-veranlasste Lückenbildung in den LEC-Schichten und den Lückenbildungsfaktor 12(S)-HETE zu untersuchen. Wobei unsere Daten zeigen, dass NF-κB nötig ist für 1) die Expression von spezifischen Adhesionsmolekülen, die zusammen mit 12(S)-HETE eine Voraussetzung für die Induzierung von LEC Mobilität darstellen; 2) die Aktivierung der Migrationsmaschinerie in LECs wie die 12(S)-HETE induzierte S100A Mobilität, sowie das Mobilitätspotential durch Paxillin-, ROCK1-, MLC2-, Mypt1-, ILK-, Erk1/2-, Arp2/3- und Akt-Expression. Die Resultate zeigen die hemmende Wirkung der NF-κB Hemmer auf die Mobilität der LECs. Auch die Tests mit dem synthetischen MMP Hemmer GM6001 zeigten eine Dosis-abhängige Lückenhemmung, wobei MMP-11 eine Hauptrolle in MMP-ausgelösten LEC Lücken zu spielen scheint.

Summary

Ductal breast cancer seems to invade the lymphatic vasculature always as a cell bulk through a gap in the lymphendothelial wall, followed by a spread into the axillary "gate keeping" sentinel lymph node and colonisation of "post-sentinel" nodes and distand organs. The bulky invasion of cancer cells into the lymphatic vasculature is therefore a target for pharmacological intervention, but the cellular and molecular basis of this invasion is largely unknown. By using a 3D co-culture *in vitro* array consisting of T1S1 telomerase-immortalised human lymphendothelial cell layers and human MCF-7 breast cancer spheroids we mimick the induction of gap formation into the lymphendothelial cell layer. Thereby two mechanisms were already identified to be responsible for the induction of gaps. The mechanisms of 12/15 lipoxygenase and its product 12(S)-HETE, secreted by MCF-7 cells, as well as matrix metallo proteinases (MMPs), generate lymphendothelial gap formation. Preliminary experiments suggest that also NF-κB may play a significant role in tumour bulk transmigration through lymphendothelial borders.
Therfore this diploma thesis proposes to examine the contribution of this transcription factor. Furthermore, a subset of natural and synthetic substances was tested on their ability to inhibit gap induction by MCF-7 cell spheroids. As starting experiments we performed gap formation assays, whereby the gap induction by MCF-7 cell spheroids into lymphendothelial cell (LEC) layers was attempted to become inhibited by the pre-treatment of the spheroids with different substances that were selected on their specific reported bioactivities. All tested substances (bay-11, baicalein, heteronemin, xanthohumol, berberine, GM6001) did show a strong ability to inhibit gap formation, even at low concentrations (~ 5μM). Further experiments aimed to elucidate the molecular mechanism of the compounds that interfered with spheroid-triggered gap-formation of LEC layers, and of the gap forming factor 12(S)-HETE and the downstream effects in LECs. The data indicate that NF-κB was required for 1) adhesion of MCF-7 spheroids to LECs, which was a prerequisite together with 12(S)-HETE to induce the mobility of LECs; 2) the activation of the migratory machinery in LECs i.e. 12(S)-HETE-induced S100A expression, as well as the constitutive mobile potential provided by paxillin, ROCK-1, MLC2, Mypt1, ILK, Erk1/2, Arp2/3, and Akt expression. Also tests with the synthetic MMP inhibitor GM6001 revealed its dose dependet ability to inhibit gap formation, whereby MMP-11 seems to play a major role in MMP-triggered gaps.

1 Introduction

The origin of cancer are mutations in genes, which regulate essential pathways of cell function, leading to uncontrolled outgrowth of tissue cells (Hanahan and Weinber 2000). Angiogenesis, tumour invasion and metastasis include the attachment of tumour cells to the basement membrane, the degradation of the local connective tissue, and penetration and migration through proteolysed stroma. To invade the blood- and lymphatic circulation is thereby a major ability of cancer cells. (Roeb and Matern 2003)

All these processes are shown to include the LOX- and the MMP system (Kerjaschki et al. 2010). MMPs, and the 15-lipoxygenase arachidonic acid metabolite 12(S)-HETE were both expressed by mammary carcinoma cell lines, and by metastatic tumour cells in human sentinel lymph nodes (Kerjaschki et al. 2010). In addition, we investigated other mechanisms, which as well may contribute to LEC gap formation. Therefore we tested the contribution of NF-κB, and other molecules (bay-11, heteronemin, xanthohumol, berberine, GM6001), that play a role in cell mobility.

1.1 Cell cycle of eukaryotic cells

Tumours are characterised by abnormal cell proliferation. Cell proliferation involves the reproduction of a cell to form two daughter cells. In eukaryotes the cell cycle can be devided into two periods, the interphase, during which the cell grows before it can enter cell division; and the mitosis (M) phase, during which the cell splits itself into two distinct cells. The interphase is again devided into

- the G_1-phase (growth phase, or G indicating gap), which starts at the end of the previous mitosis, and during which biosynthetic activites reach high rates to prepare the cell for synthesis (Kiaris 2006).
- the S-phase (synthesis phase), starting the DNA synthesis leading to a replication of all chromosomes (amount of DNA doubles).
- the G_2-phase (gap- or praemitotic phase), involving high biosynthesis rates (e.g. microtubules production) to prepare the cell for mitosis (e.g. cell-cell contacts get lost). (Table 1; Figure 1).

The mitosis or M-phase is composed of two coupled processes: mitosis, which occurs only in eukaryotic cells and during which the chromosomes are divided in the cell nucleus into two identical sets in two daughter nuclei (karyokinesis); and cytokinesis, during which the cytoplams is divided in forming two distinct cells (Pecorino 2008) (Table 1; Figure 1).

Errors during the mitosis can either lead to apoptosis (programmed cell death), or cause mutations, which may lead to cancer.

Cells that are fully grown and have temporarily or reversibly stopped dividing, have reached the state of quiescence called G_0-phase (Table 1; Figure 1). Most cells in an adult are not in the process of cell division. Such cells are quiescent and enter this inactive period that is outside the cell cycle. Mitogens or growth factors can induce such cells to re-enter the cell cycle. (Kiaris 2006)

Table 1: Cell cycle phases

State	Phase	Description
quiescent/senescent	Gap 0 (G_0)	cell has left the cycle and has stopped dividing (resting phase)
Interphase	Gap 1 (G_1)	size increase; preparatory phase for S
	Synthesis (S)	DNA replication
	Gap 2 (G_2)	cell continues to grow; preparatory phase for M
Cell division	Mitosis (M)	cell growth stops and the mother cell is divided into two daughter cells, genetically identical to each other and to their parent cell

After the cell division, each daughter cell starts the interphase of a new cycle.

The average length of the cycle is 16 hours (15 hours for interphase and 1 hour for mitosis), but this can vary depending on the cell type (Pecorino 2008). Each phase of the cycle shows its distinct set of specialised biochemical processes, which prepare the cell for initiation of the cell division. The molecular events that control the cell cycle are ordered and directional; that is, each process occurs in a sequential fashion and it is impossible to "reverse" the cycle. (Smith and Martin 1973; Kiaris 2006; Pecorino 2008)

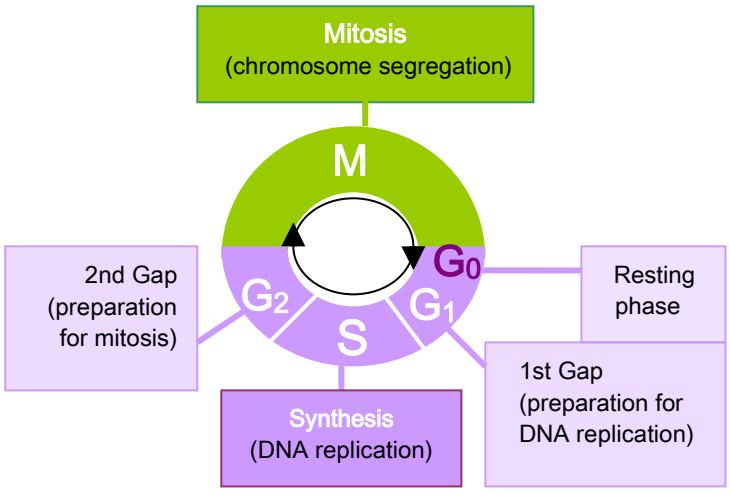


Figure 1: Cell cycle phases

Disregulation and tumour formation

The cell cycle of tumour cells is not regulated by the organism. The cells start to replicate autonomously. Therefore disregulation of the cell cycle components may lead to uncontrolled cell- and tissue growth and tumour formation. Important regulatory genes, such as p53 (TSG), may cause the cell to multiply uncontrollably or get lost due to mutations. The cell cycle in tumour cells is equal to that of normal cells, but the share of cells, which are in active cell division (versus cells in G_0-phase) in tumours, is higher than that in normal tissue. During cell division the DNA is relatively exposed and hence sensible for damage by drugs or radiation, which is made use of in cancer treatment. (Picorino 2008)

1.2 Cancer

Cancer can be defined as a group of diseases (> 100 types of cancer) that are characterised by unregulated cell growth and the invasion and spread of cells from their site of origin, to other sites in the body. One can specify tumours into benign tumours, which do not metastasise; and malignant tumours, which invade and metastasise. Carcinomas, cancers that occur in epithelial cells, make up approximately 85% of all cancers. Sarcomas on the other hand, are cancers that are derived from mesoderm cells (e.g. bone, muscle); whereas adenocarcinomas are cancers of glandular tissue (e.g. breast). Depending on the origin, different cancers show different features and different major factors for their occurrences, e.g. UV radiation targets the skin, and smoke targets the lungs. Factors that may influence human carcinogenesis are e.g. the environment (exposure to carcinogens), the reproductive life (hormonal modifications), diet, and smoking. Most carcinogens (agents that cause cancer) cause alterations in the DNA sequence or mutations (mutagens). Such mutations accumulate in cells over time, which explains the increased risk of cancer with age. All cancer cells in one patient arise from one single cell that contains accumulated mutations, therefore the development of cancer is clonal. (Pecorino 2008) In general three stages of cancer development can be identified: During the initiation stage cells become cancerous when their DNA is damaged i.e. by radiation, oxidative stress, or specific toxins. Due to cellular repair mechanisms, only few of these mutations remain that may produce cancer. Even initiated cells, cells that survive mutations and potentially become cancerous, rarely become cancerous, as cells in most tissues have lost the ability to replicate themselves. Additionally apoptosis (programmed cell death) prevents such potentially cancerous cells from passing the damaged DNA to future generations of cells. However, under certain

circumstances, initiated cells regain the ability to replicate. Such “immortalised” cells have therefore undergone the second stage of cancer development, promotion. Even if collections of initiated cells that have undergone promotion occur, the immune system normally destroys these cells. If such defense mechanisms have been overcome, cancerous tissues are in the final stage of cancer development, progression. In such cases three therapeutic strategies are used to prevent proliferation (cytostatic effect) and to kill the cancer cells (cytotoxic effect): surgically removing as much of the cancer as possible, and chemotherapy and radiotherapy are used to inhibit or eradicate metastasised cells (Pecorino 2008). Whereby chemotherapy uses chemicals that target DNA, RNA, and proteins to disrupt the cell cycle in rapidly dividing cancer cells, by causing severe DNA damage and triggering apoptosis (Pecorino 2008).

1.2.1 Hallmarks of cancer

As already mentioned cancer is a disease involving dynamic changes in the genome (genome instability). Malfunctions of the complex array of DNA monitoring and repair enzymes explain this increased mutability (Lengauer et al. 1998). Tumourigenesis is a multi-step process, whereby these steps reflect multiple genetic alterations (point mutations or deletions and chromosomal translocations), which lead to the progressive transformation of normal cells into highly malignant ones (Hanahan and Weinberg 2000). Six essential alterations in cell physiology dictate malignant growth (Figure 2) (Hanahan and Weinberg 2000): self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

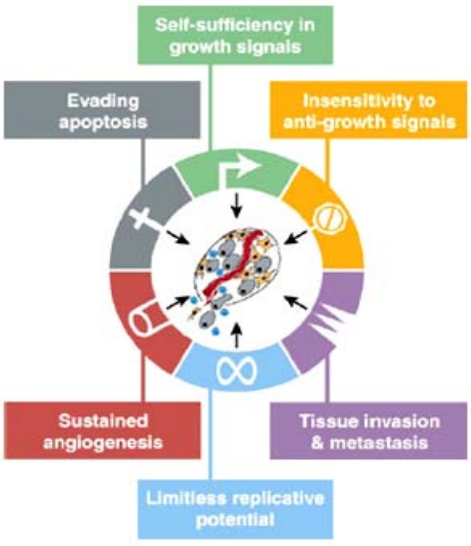


Figure 2: The six hallmarks of cancer (Hanahan and Weinberg 2000)

As the pathways of cancer are highly variable, the sequence in the acquisition of the biological capabilities mentioned above can vary widely, both among tumours of the same type and between tumours of different types. Nevertheless the hallmark capabilities of cancer are shared by all types of tumours. (Hanahan and Weinberg 2000)

1.2.1.1 Self-sufficiency in growth signals – growth signal autonomy

To proliferate normal cells need mitogenic growth signals (GS), which are transmitted by transmembrane receptors that bind signalling molecules (diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules). Tumour cells show a reduced dependence on exogenous growth stimulation, as they generate many growth signals by themselves (many oncogenes mimic normal growth signalling), and reduce thereby their dependence on stimulation from their tissue microenvironment. (Hanahan and Weinberg 2000) Many cancer cells show the ability to produce growth factors (GF) to which they are responsive, and creating that way a positive feedback signalling loop (autocrine stimulation) (Fedi et al. 1997). Additionally, the cell surface receptors, which transduce stimulation signals into the cell, are over expressed in many cancers, therefore cancer cells become hyper responsive to low levels of GFs (Fedi et al. 1997), and/or the extracellular matrix receptors (integrins) can be switched by cancer cells, favouring ones, that transmit pro-growth signals (Lukashev and Werb 1998; Giancotti and Ruoslahti 1999).

Furthermore, successful tumour cells acquire the ability to co-opt normal cells by inducing them to release abundant fluxes of growth-stimulating signals (Skobe and Fusenig 1998)

1.2.1.2 Insensitivity to growth-inhibitory (antigrowth) signals

Diverse antiproliferative signals (soluble growth inhibitors and immobilized inhibitors in the ECM and on the surfaces of nearby cells) are present in a normal tissue, to maintain cellular quiescence and tissue homeostasis. Growth-inhibitory signals are received by transmembrane cell surface receptors coupled to intracellular signalling circuits. (Hanahan and Weinberg 2000)

Triggered by growth-inhibitory signals, proliferation can be blocked by two mechanisms: cells can be forced to leave the active proliferate cycle and to enter the G_0 raste state (re-emerging is possible), or cells are induced to permanently stop their proliferative potential by entering into post mitotic stages (Hanahan and Weinberg 2000). Cancer cells have to evade such antiproliferative signals in order to develop.

1.2.1.3 Evasion of programmed cell death (apoptosis)

The apoptotic program is present in almost all cell types. The cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extruded, the chromosomes are degraded, and the nucleus is fragmented (30-120 minutes), followed by the engulfment of the cell corpse by nearby cells. The dead cell disappears normally within 24 hours. (Wyllie et al. 1980) To achieve homeostasis the rate of cell proliferation and the rate of cell attrition (apoptosis represents a major source of cell attrition) should be in balance, as uncontrolled cell proliferation respectively insufficient rate of apoptosis results in cancer (Hanahan and Weinberg 2000). Two components, sensors and effectors, are important for the apoptotic machinery. Sensors monitor the extracellular and the intracellular environment for conditions that influence whether a cell should live or die, and effectors of the apoptotic machinery are regulated by these intracellular sensors and activate the death pathway in response to detected abnormalities (e.g. DNA damage, signalling imbalance provoked by oncogenes action, survival factor insufficiency, or hypoxia) (Evan and Littlewood 1998). The resistance toward apoptosis is a characteristic of most and perhaps all types of cancer (Hanahan and Weinberg 2000).

1.2.1.4 Limitless replicative potential

The three above mentioned capabilities of cancer cells – growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis – lead the independence of the cell's growth program from signals in its environment (Hanahan and Weinberg 2000). Nevertheless an intrinsic program limits the multiplication of many and perhaps of all types of mammalian cells. Cells do have a finite replicative potential, as after a certain number of doublings, they stop growing (process of senescence). (Hayflick 1997) Cancer cells have a limitless replicative potential, they are immortalised, as they e.g. maintain the telomeric DNA, which normally reduces by 50-100bp at each replication. The telomere maintenance above a certain threshold, through up regulated expression of the telomerase enzyme, permits unlimited multiplication of cancer cells. (Shay and Bacchetti 1997)

1.2.1.5 Sustained angiogenesis

The supply with oxygen and nutrients is essential for cell function and survival. Therefore cells in a tissue are obligated to reside within 100µm of a capillary blood vessel (Hanahan and Weinberg 2000). Angiogenesis – the growth of new vessels – is an invasive process

that requires proteolysis of the extracellular matrix, and proliferation and migration of endothelial cells, as well as synthesis of new matrix components. (Stetler-Stevenson 1999)
The vasculogenesis and angiogenesis contribute both during embryonic development to the formation of the circulatory system. In the adult, angiogenesis is initiated in response to a pathologic condition, as inflammation or hypoxia. Therefore the angiogenic response is necessary for progression of e.g. wound healing. (Stetler-Stevenson 1999) But also for the rapid clonal expansion associated with the formation of macroscopic tumours, neovascularisation is necessary. Angiogenesis starts with the local degradation of the basement membranes (surrounding capillaries), followed by invasion of the surrounding stroma by the underlying endothelial cells in the direction of the angiogenic signal. Endothelial cells migrate by cell growth at the leading edge of the migrating column, and then organise themselves into three-dimensional structures to form new capillary tubes. (Zetter 1998)
During tumour development, cancer cells acquire the ability to induce and sustain angiogenesis, by changing the balance of angiogenesis inducers and countervailing inhibitors. Therefore either the expression of vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2), which both bind to transmembrane tyrosine kinase receptors displayed by endothelial cells (Fedi et al. 1997; Veikkola and Alitalo 1999) is increased (Rak et al. 1995; Maxwell et al. 1999), or the expression of endogenous inhibitors (e.g. thrombospondin-1 or β-interferon) is down regulated. (Singh et al. 1995; Volpert et al. 1997; Hanahan and Weinberg 2000)

1.2.1.6 Tissue invasion and metastasis

The majority of human cancer deaths (90%) is caused by the capability of tumours for invasion and metastasis (Sporn 1996). The invasion can occur either across blood vessels (haematogenous spread) or lymphatic vessels (lymphogenous spread) to access the general circulation, or by invasion of body cavities such as the pleura or peritoneum (Figure 3) (Stacker et al. 2002).

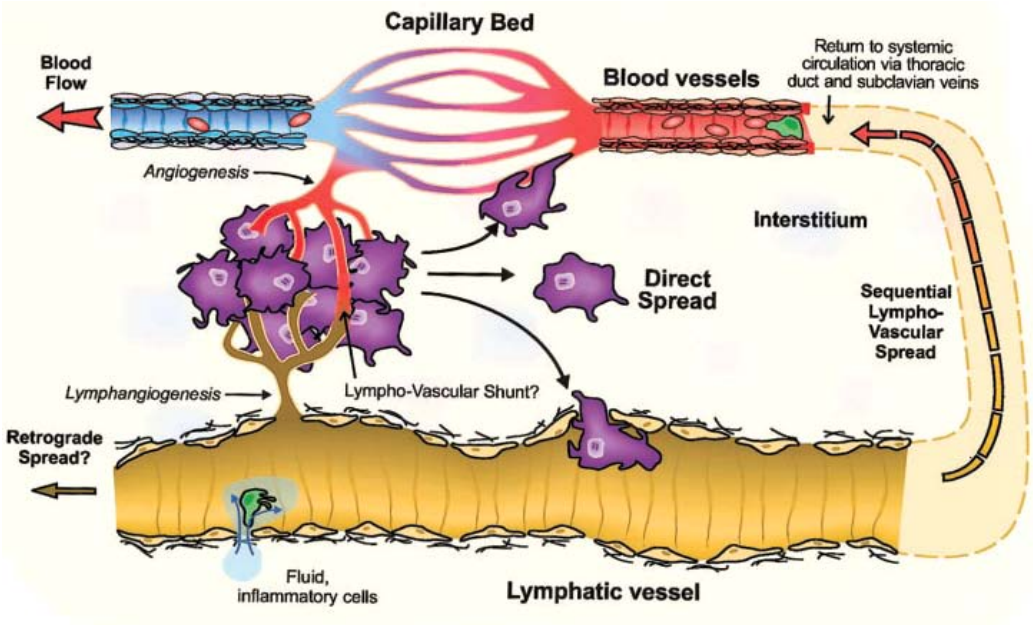


Figure 3: Lymphatic vessel structure and potential modes of tumour cell dissemination. (Stacker et al. 2002)
Lymphatics are vessels with thin walls and low pressure, which collect fluid and cells from the interstitium and return it to the circulation via the thoracic duct. **Blood vessels** are subject to high pressure and have a more robust structure with a well-defined basement membrane and supporting cells. **Tumour cells** spread from the primary tumour into the surrounding tissue, from which they subsequently invade preexisting blood vessels or lymphatic vessels. Alternatively, angiogenesis or lymphangiogenesis, induced by tumour associated factors, could promote the growth of new vessels into the tumour. Cells that invade the lymphatics could find their way into the bloodstream via the thoracic duct (sequential lymphovascular spread) or by the formation of shunts, speculated to exist in tumours, between the vascular system and the lymphatic system. (Stacker et al. 2002)

Pioneer cells move out of the primary tumour to invade adjacent tissues, and reach distant sites to found new colonies (metastases) where nutrients and space are not limiting. The invasion and metastasis involves changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. (Hanahan and Weinberg 2000) Protein classes which are altered in cells possessing invasive or metastatic capabilities include cell-cell adhesion molecules (CAMs) – which mediate cell-to-cell interactions – and integrins, which link cells to extracellular matrix substrates (Hanahan and Weinberg 2000). The most common alteration in cell-to-environment interactions in cancer involves E-cadherin, a cell-to-cell interaction molecule expressed on epithelial cells, which act as a suppressor of invasion and metastasis by epithelial cancers. Therefore its function is lost in a majority of epithelial cancers (Christofori and Semb 1999). The change of integrin expression is necessary, as invading and metastasising cancer cells experience changing tissue microenvironments (e.g. novel matrix components). Thus adaptation in the spectrum of integrins is for a successful colonisation necessary. Carcinoma cells shift their

expression of integrins from those that favour the ECM present in normal epithelium to other integrins (Varner and Cheresh 1996; Lukashev and Werb 1998).

Before human carcinoma cells disseminate to distant organs (e.g. lung, brain and bones), they initially metastasise to regional lymph nodes. The before mentioned acquired biological capabilities of tumour cells enable them to emigrate from their primary site, and to form premetastatic niches in target organs (Fidler 2003). Additionally, tumours, which are programmed for lymph node metastasis, show the strategy for pre-metastatic adaption of their regional lymph node (Mumprecht and Detmar 2009), which includes the expansion of lymph node sinus and the transformation of their lining cells into lymphatic endothelial cells. This "pre-metastatic lymph node lymphangiogenesis" (Mumprecht and Detmar 2009) is an unspecific response to diverse stimuli (e.g. chemokines, growth factors, mechanical obliteration of efferent lymphatic vessels) (Steinmann et al. 1982; Angeli et al. 2006).
The factors that regulate initial lymph node colonisation and dissemination into distant organs remain unclear. One theory state that metastatic tumour cells colonise distant organs via the blood stream either from lymph nodes ("metastasis from metastasis") (Tait et al. 2004), or by cross seeding from the primary tumour by recirculation (Norton and Massagué 2006). Another theory state that clonogenic tumour cells with stem cell-like characteristics are able to disseminate from primary tumours into both the blood and the lymphatic vasculature, and develop into metastases in both compartments (Ben-Porath et al. 2008).

The classical metastatic cascade (Figure 4) (Geiger and Peeper 2009) starts with cellular transformation and growth of the primary tumour, followed by extensive vascularisation through the synthesis and secretion of angiogenic factors. After EMT and breach of the basement membrane barrier (Geiger and Peeper 2009), detached and embolised tumour cells or aggregates enter the circulation. The tumour cells that survived circulation now exit the circulation (extravasation) into an organ where they proliferate within the parenchyma and develop again a vascular network for further growth. After angiogenesis and proliferation, blood vessels are invaded and the circulation is re-entered to produce additional metastases. (Fidler 2003)

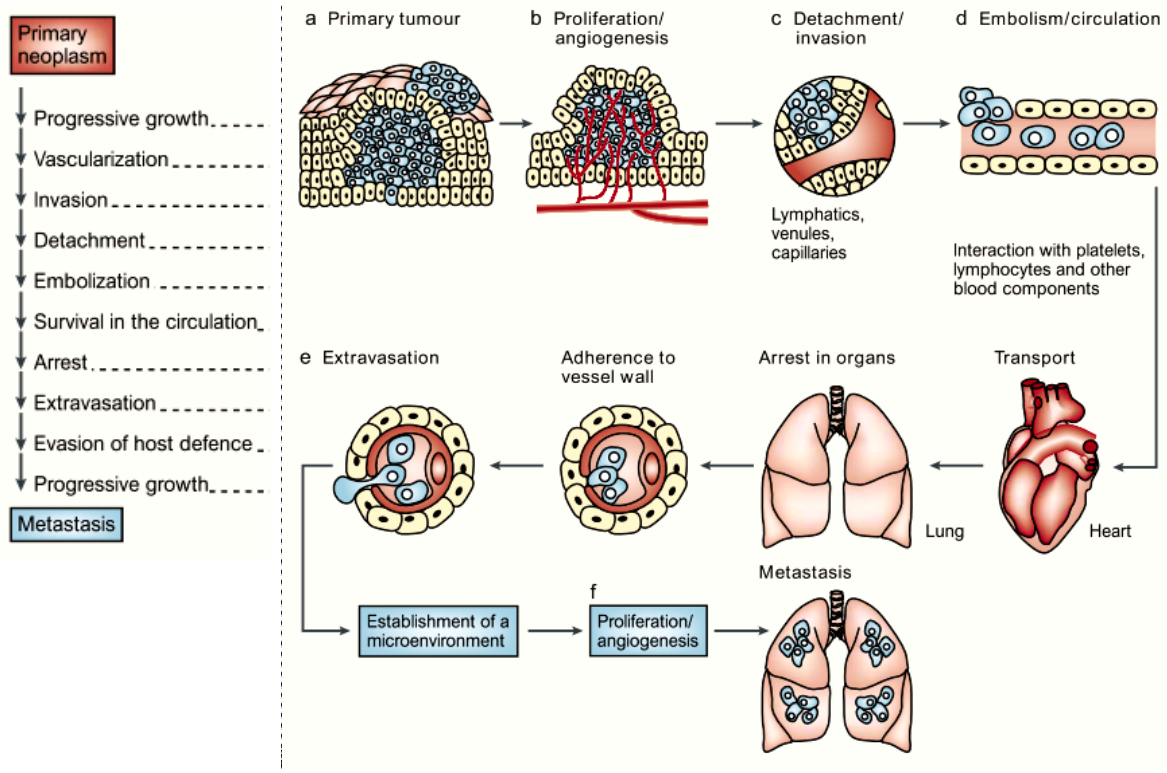


Figure 4: Metastatic cascade (Fidler 2003)

Primary tumours show a tendency to metastasise to particular secondary sites, which implies that the location of metastatic growth is governed by specific characteristics, such as the selective growth of the cancer cells in the tissue that favours their proliferation, the selective adhesion onto the endothelium similar to the tissue of metastatic growth, and the attraction of the cancer cells by factors produced by the tissue. (Kiaris 2006)

The fact that tumour cells colonise selectively distant organs with favourable environment enabling the survival of tumour cells was already hypothesised 1889 by Stephen Paget's "seed and soil" theory (Fokas et al. 2007), which indicates that certain organs provide appropriate conditions for the development of metastases, leading to organ selecitivity (Paget 1889). Recent studies have identified the molecular interactions between tumour cells (seeds) and stromal environment (soil), leading to the tissue-specificity of metastasis formation (Fokas et al. 2007). Additionally for many cancer types studies indicate that metastases originate from a single proliferating cell. This clonality of metastases was also reported for breast cancer and fibrosarcoma, as well as melanoma. (Fidler 2003)

1.2.2 Cancer modifier genes, oncogenes and TSGs

Two major types of mutated genes involved in growth, differentiation, or death, are oncogenes, and tumour suppressor genes (Duffy et al. 2000). Oncogenes are genes mutated such that its protein product is produced in higher quantities, or shows increased activity (Pecorino 2008). On the other hand tumour suppressor genes (TSGs) code for proteins, which are important in inhibiting growth and tumour formation (Pecorino 2008).

1.2.2.1 Growth factor signalling and oncogenes

As mentioned above unregulated growth is an essential characteristic of cancer. Four types of proteins are involved in the transduction of growth factor signals: growth factors, growth factor receptors, intracellular signal transducers, and nuclear transcription factors (Pecorino 2008).

The epidermal growth factor (EGF) (member of the family of receptor tyrosine kinases) serves as a model for how a signal from an extracellular growth factor can be transduced through the cell. To get the extracellular growth signal into the cell, where gene expression is regulated, several steps are required: binding of the growth factor (EGF) to the receptor (EGFR), which causes a conformational change, whereby members of the receptor tyrosine kinase receptor family (e.g. EGFR) contain an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic protein tyrosine kinase domain; receptor dimerisation, (two EGFR monomers interacting to form a dimer); autophosphorylation of one EGFR because of the close proximity of two receptors, which causes a conformational change; leading to an activation of intracellular transducers and a cascade of kinases; and the regulation of transcription factors for gene expression. (Pecorino 2008)

The effects of growth factor cell signalling are manifold; beside cell proliferation it may have effects on cell behaviour and motility (Pecorino 2008).

Oncogenes are genes mutated – mutations in one allele are sufficient for an effect – such that its protein product is produced in higher quantities, or shows increased activity (Pecorino 2008). For example many cyclins have been identified as potential oncogenes, as they are capable of inducing tumour growth and are up regulated in various primary human cancers (Kiaris 2006). Oncogenes can be nuclear (e.g. various transcription factors), cytoplasmic (e.g. soluble enzymes), membrane proteins (e.g. receptors), or secreted proteins that operate as growth factors (Kiaris 2006).

Almost all known oncogenes are altered forms of normal genes or proto-oncogenes (altered genes transduced by retroviruses), which are activated through point mutations and

deletions. For each type of protein involved in a growth factor signal transduction pathway there are oncogenes known. (Pecorino 2008) Major oncogenes is the *ras* family of oncogenes, as they control proliferation, angiogenesis, malignant transformation, and can affect the metastatic ability of cancer cells (Kiaris 2006). They initiate diverse signalling cascades, which result in the regulation of growth, development and oncogenesis. For example by one *ras* initiated signalling cascade, NF-κB (see chapter: Nuclear factor kappa B (NF-κB)) is activated, by suppressing the activity of its inhibitor I-κB. The product of the *ras* family genes is $p21^{ras}$ is situated at the inner surface of the plasma membrane and operates as a switch between GTP and GDP (Ras-GTP as the active form; Ras-GDP as the inactive form). Because of the wide variety of *ras* effector proteins it shows a huge diversity in its effects. (Kiaris 2006)

1.2.3 Cell migration

In order to metastasise, cancer cells must be able to acquire properties that permit their movement. A common manner of cell movement is the so called collective movement. When ECM is proteolysed by cancer cell enzymes, ECM proteins such as fibronectin, laminin, thrombospondin, induce the cancer cells to migrate. Chemotaxis and aptotaxis correspond to movement towards a chemical gradient or a substrate, which are the major driving forces for the specific movement of cancer cells during local invasion. Also factors from the stroma (e.g. IGF and HGF) are involved in the motility of cancer cells. (Kiaris 2006) Single cancer cells which detach from the tumour may migrate either mesenchymal or amoeboid. For the mesenchymal migration the remodelling of the ECM is required. In the presence of protease inhibitors cancer cells show an amoeboid migration, and migrate through the ECM. Therefore to control cancer cell migration and thereby metastasis, both migration types have to be repressed simultaneously. (Paulitschke et al. 2009)

1.2.3.1 Epithelial-mesenchymal transition

During development cells can assume various phenotypic states – process of differentiation. Thus cells in certain epithelia are able to switch between epithelial and mesenchymal states during embryogenesis and organ development. But also in terminally differentiated epithelium this switch occurs, which enables transdifferentiation. (Kalluri and Weinberg 2009) Epithelial cells are characterised by cell polarity, and multiple adhesive interactions, which serve as tumour and/or metastasis suppressors (Kiemer et al. 2001). Thus the loss of this epithelial phenotype is necessary for tumour progression.

Polarised epithelial cells do normally interact with basement membrane via its basal surface (Kalluri and Weinberg 2009). Due to the reversible process of epithelial-mesenchymal transition (EMT) biochemical changes that affect e.g. oncogenes and tumour suppressor genes (e.g. E-cadherin) (Kalluri 2009) are performed, which enable epithelial cells to gain a mesenchymal cell phenotype (Figure 5) that includes an increased migratory capacity, invasiveness, increased resistance to apoptosis, and an increased production of ECM components (Kalluri and Neilson 2003; Zeisberg and Neilson 2009). The definition of EMT in vitro involves the loss of epithelial cell polarity, the separation into individual cells and the dispersion after the acquisition of cell motility (Vincent-Salomon and Thiery 2003). The EMT process involves the disassembly of tight junctions, adherens junctions and desmosomes, and the reorganisation of cell substrate adhesion complexes (Vincent-Salomon and Thiery 2003). The cytoskeleton is remodelled after the loss of cell polarity to acquire a more spindle-like morphology with increased motility and invasiveness, involving dynamic actin microfilament networks (Tomaskovic-Crook et al. 2009). Thus the epithelial and mesenchymal phenotypes show specific transcription profiles including cytoskeletal- and ECM components (Vincent-Salomon and Thiery 2003). The EMT is completed with the degradation of the basement membrane and with the formation of a spindle-shaped mesenchymal cell that exhibit end-to-end polarity (Kalluri 2009), and which can migrate away from its epithelial layer (Kalluri and Weinberg 2009). EMT can be initiated by diverse molecular processes interacting, such as the activation of transcription factors, expression of specific cell-surface proteins, reorganisation and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs (Kalluri and Weinberg 2009). EMT can also be activated in association with tissue repair and pathological stress due to inflammation and carcinomas (Kalluri and Weinberg 2009). In response to such stimuli, mesenchymal cells are dispersed in injured tissues, and initiate the invasive and metastatic behaviour of epithelial cancers (Kalluri and Weinberg 2009).

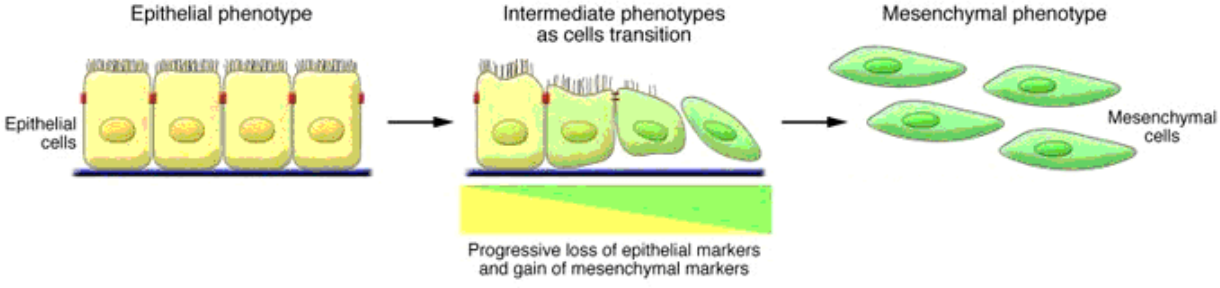


Figure 5: Epithelial-mesenchymal transition (EMT) (Kalluri and Weinberg 2009)
EMT is a functional transition of polarised epithelial cells into mobile mesenchymal cells.

EMTs can be classified into three biological subtypes. Type 1 EMTs are associated with implantation, embryo formation and organ development; and generate cells with the same mesenchymal phenotype, which is not invasive. Type 2 EMTs are associated with wound healing, tissue regeneration, and organ fibrosis. Among others fibroblasts are generated to reconstruct tissues. The induction of type 3 EMTs is facilitated by genomic alterations in neoplastic cells. Type 3 EMTs produce cells with invasive properties, and enable them to move into the blood stream and spread to other organs. Which signals induce type 3 EMTs in carcinoma cells is still unclear, but such signals may originate from the tumour stroma associated with primary carcinomas. (Kalluri and Weinberg 2009)

The activation of EMTs seems to be a critical mechanism for the acquisition of malignant phenotypes by epithelial cancer cells, and for tumour progression (Thiery 2002) (Figure 7). Carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers (α-SMA, FSP1, vimentin, and desmin) (Yang and Weinberg 2008). Such cells are seen at the invasive front of primary tumours and enter into the invasion-metastasis cascade (Figure 6), i.e. entry into the circulation (intravasation), transport through the circulation, exit from the circulation (extravasation), forming micrometastases, and colonisation (Thiery 2002; Fidler and Poste 2008; Brabletz et al. 2001). In order to build a secondary tumour, metastasising cancer cells have to undergo MET (mesenchymal-epithelial transition) (Figure 7) (Zeisberg et al. 2005), which is likely to occur when the local microenvironment and its signals encountered after extravasation into distant organs, is similar to the one experienced in the primary tumour (Thiery 2002; Jechlinger et al. 2002; Bissell et al. 2002).

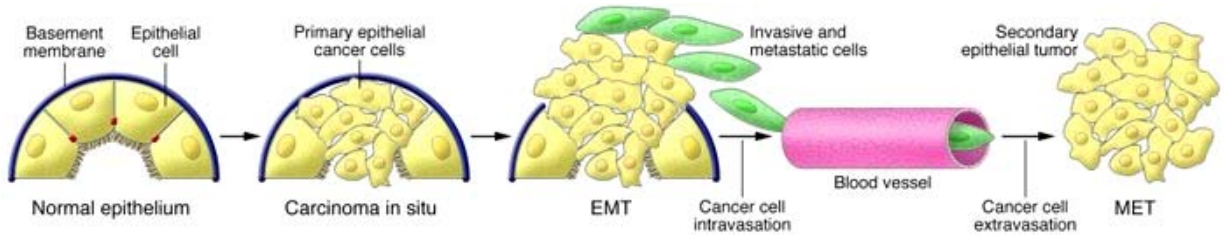


Figure 6: Contribution of EMT to cancer progression (Kalluri and Weinberg 2009)
Several stages have to be passed in the progression from normal epithelium to invasive carcinoma cells. Epithelial cells lose their polarity and detach from the basement membrane. The basement membrane undergoes also changes, altering cell-ECM interactions and signalling networks. EMT enables cancer cells to enter circulation and exit the blood stream at a remote site, where these cells can form metastases (mesenchymal-epithelial transitions – MET – may be involved). (Kalluri and Weinberg 2009)

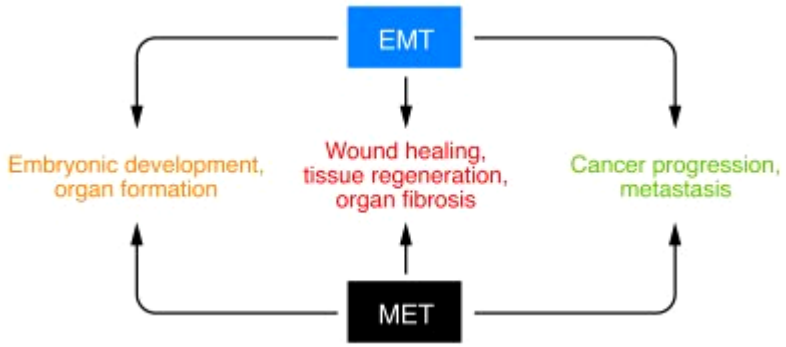


Figure 7: EMT and MET in health and disease (Kalluri 2009)
There is evidence for the role of EMT in embryonic development, organ formation, wound healing, tissue regeneration, organ fibrosis, cancer progression, and metastasis. The role for MET in wound healing, tissue regeneration, organ fibrosis, cancer progression, and metastasis is only speculative and evidence is still lacking. (Kalluri 2009)

In many carcinomas, EMT-inducing signals are produced from the tumour-associated stroma. HGF, EGF, PDGF, and TGF-β, seem to be responsible for the induction and activation of diverse EMT-inducing transcription factors (Snail, Slug, ZEB1, etc.) in cancer cells (Thiery 2002; Jechlinger et al. 2002). Signal-transducing proteins like ERK, MAPK, Akt, RhoB, Ras, and cell surface proteins, are necessary to implement the EMT program (Tse and Kalluri 2007), whereby the Ras/mitogen-activated protein kinase (MAPK) pathway plays a major role in inducing EMT (Vincent-Salomon and Thiery 2003). EMT is furthermore enhanced by the disruption of cell-cell adhesion and the cell-ECM adhesion, mediated by integrins (Yang and Weinberg 2008; Weinberg 2008).

1.2.3.2 Motility proteins

The expression of different proteins marks the specific cell phenotype. In the following chapter selected proteins are described, marking either the epithelial cell phenotype or the mobile/mesenchymal one. Later on, the expression levels of these proteins in LECs and their alterations by the treatment with diverse anti-cancer substances will be further investigated by western blotting (see chapter “results”).

VE-Cadherin (CD144): The family of cadherins (“Calcium adhering” proteins) are calcium-dependent cell-cell adhesion glycoproteins, responsible for cell-cell adhesions in diverse tissues, and located in desmosomes and adherens junctions. They play a major role in stabilising of cell-cell contacts, the embryonic morphogenesis, cell polarity, and signal transduction. (Takeichi 1990) VE- Cadherin (vascular endothelial) is a member of the cadherin family, and is located at the intercellular cleft sites of the junction within endothelial tissue (Leach et al. 1993).

Through the formation of dimers between VE-Cadherins in two neighbour cells, it bridges the extracellular space between cells, and with its cytoplasmic tail VE-Cadherin interacts via diverse interaction partner proteins with the actin cytoskeleton. Thus VE-Cadherin supports the stability of tissues, and because of the intercellular junctions organised by VE-Cadherin it plays a major role in the permeability properties of vascular endothelium (Takeichi 1990). VE-Cadherin is a reported marker of the epithelial phenotype, and is therefore down regulated specifically at sites of EMT. (Vincent-Salomon and Thiery 2003; Zeisberg and Neilson 2009)

ERK1/2: Mitogen-activated protein kinases (MAPKs) are a family of protein kinases, and their signalling cascades are involved in many cellular programs, as cell proliferation, survival and differentiation, motility, and death (Roux and Blenis 2004). Misregulations of MAPK cascades may lead to cancer and other human diseases (Roberts and Der 2007). Erk1/2 (p44/42 MAPK) (extracellular signal-regulated protein kinase) is activated by diverse intracellular stimuli such as mitogens, growth factors, and cytokines (Roux and Blenis 2004). Activated ERKs phosphorylate and regulate the activities of a variety of proteins (~ 160) (Yoon and Seger, 2006), like nuclear proteins, or others that are found in the cytoplasm (Roberts and Der 2007). Erk1/2 is a target in the diagnosis and treatment of cancer, as its signalling pathways acitivate beneath others the *ras* small GTPase, which is a frequently mutated oncogene in human cancers and is a downstream effector of EGFR (Roberts and Der 2007). An increase of phosphorylated Erk1/2 (12(S)-HETE induced) leads to an increased cyclin D expression and therefore enhanced cell growth/proliferation in cancer cells (Roberts and Der 2007). Studies showed additionally that the disruption of microtubule networks may lead to the activation of MAPkinases (Erk1/2, p38 MAPK) (Stone and Chambers 2000). Therefore the activation of Erk1/2 may contribute to cytoskeletal remodeling and permeability changes in cancer cells (Birukova et al. 2004b). The role in endothelial cells remains to be established.

Paxillin: Paxillin, a focal adhesion phosphoprotein localised in the cytoskeleton, to the intracellular surface of sites of cell adhesion to the ECM (Deakin and Turner 2008), is the key regulator of cell migration (Paulitschke et al. 2009), signal transduction, regulation of cell morphology, and in the recruitment of structural and signalling molecules to focal adhesions (Salgia et al. 1995). Paxillin provides many protein-binding modules, and serves therefore as a linkage for regulatory and structural proteins, which together control changes in cell adhesion and cytoskeletal reorganisation (Deakin and Turner 2008). Paxillin is a key

coordinator of proteins of the Rho GTPase family (Rho, Rac, Cdc42), and their signalling cascades leading to cell spread and migration (Paulitschke et al. 2009). Paxillin is necessary for the turnover during cell migration and directly interacts with ILK (Paulitschke et al. 2009). Due to its interaction with ILK and actin-binding proteins, it can provide a linkage to actin filaments (Wu and Dedhar 2001). In absence of Erk1/2 a decrease in the expression of paxillin could be revealed *in vivo* and *in vitro*, resulting in reduced endothelial cell motility (Roll et al. 2000), as paxillin has proven to be a marker of adhesion sites (Ross et al. 2000). Previous experiments showed a 12(S)-HETE-induced loss of paxillin from adhesion sites in pseudopods of carcinoma cells (Ross et al. 2000).

S100A: S100A is a member of the s100 family of proteins containing two calcium-binding motifs. S100 proteins are situated in the cytoplasm and/or nucleus of a wide range of cells. S100 is involved in the regulation of cellular processes, as cell cycle progression and differentiation. The protein may be involved in motility, invasion and tubulin polymerisation, whereby the altered expression of the gene is involved in tumour metastasis. (Human Protein Atlas 2010) S100A4 is beside others a marker of the mesenchymal phenotype (Zeisberg and Neilson 2009).

α-tubulin: Tubulin is one major component of the cytoskeleton, and is a heterodimer that consists of α and β tubulin (Weisenberg 1981). Such heterodimers multimerise to build up a microtubule filament, which is a cylindrical structure present in almost all eukaryotic cells. Microtubules are structural and mobile elements in mitosis and cytokinesis, and play a role in intracellular transport, flagellar movement and in the cytoskeleton (maintenance of cell shape) (Piperno and Fuller 1985). The different α and β isoforms show a variety of posttranslational modifications that affect the stability and the interactions of the microtubule, therefore the acetylation of α tubulin may play a major role in the differentiation of microtubule structure and function (Piperno and Fuller 1985; Weisenber 1981).

Rho GTPases: GTPases are a large family of hydrolase enzymes, which are able to bind and hydrolyze guanosine triphosphate (GTP). The familiy of Rho GTPases (member of the super family of *ras* proteins; see chapter: Growth factor signalling and oncogenes) are small signalling G proteins, that regulate many aspects of intracellular actin dynamics (Boureux et al. 2007; Bustelo et al. 2007). There are three main classes of Rho GTPases – Rac1, Cdc42, and RhoA – controlling cell protrusions during migration (Paulitschke et al. 2009). The GTPase Rho controls various cellular processes, such as cell adhesion and

motility, enhancement of contractile responses, cytokinesis and transcriptional regulation (Nakagawa et al. 1996). Rho GTPases activate two kinases, p21-activated kinase (PAK) and ROCK (Rho-associated kinase). The promotion of the formation of actin-based structures as lamellopodia and filopodia is promoted through Rac (Sahai and Marshall 2003). Cdc42 activation is required for the initiation of filopodia, to sense the surrounding extracellular environment, and blocks actin depolymerisation (Sakamura et al. 2005). Additionally Rac blocks the depolymerisation of actin, and RhoA encourages the retraction of actin (Sakamura et al. 2005). Active RhoA increases the stability of actin-based structures like stress fibres and focal adhesions. ROCKs are the key effectors of RhoA and RhoC, and cause the formation of actin stress fibres and focal adhesions generating an amoeboid movement (Sahai and Marshall 2003). ROCK-1 for example shows a direct link from RhoA to cell morphology through the phosphorylation of MLC.
The tumour suppressor p53 was shown to be a negative regulator of ROCK, inhibiting cell migration (Qin et al. 2009). In multiple types of cancer an over expression of Rho, Rac, and Cdc42 could be observed (Ellenbroek and Collard 2007). Thus these proteins promote cellular functions, which become overly active in cancerous cells (Ellenbroek and Collard 2007). Rho activity can suppress apoptosis, contribute to the loss of cell polarity, and alter adhesion proteins; contributing to EMT of cancerous cells (Ellenbroek and Collard 2007). For example Rho proteins are essential in the formation and the disassembly of E-cadherin-mediated cell-cell adhesion, and are involved in the regulation of the cytoskeleton remodelling, and therefore may contribute to EMT and metastasis (Ellenbroek and Collard 2007).

ROCK-1: Rho-associated kinase (ROCKs) are key effectors of Rho (GTPase) generating an amoeboid movement (Sahai and Marshall 2003). ROCK may be present in intracellular compartments and be involved in Rho-mediated cytoskeletal regulation (Nakagawa et al. 1996). Two isoforms of ROCK – ROCK-1 and ROCK-2 – have been identified (Nakagawa et al. 1996).

ARP2/3: The activation of the Rho GTPase family member Cdc42 recruits and activates the Arp2/3 complex (Welch and Mullins 2002; Svitkina et al. 2003). The Arp2/3 protein complex, which is situated in the cytoplasm and cytoskeleton, consists of seven subunits, such as Arp2/3 subunit 1B, and has been implicated in the control of actin polymerisation in cells, as the Arp2/3 complex binds G-actin, generating a stable trimer and nucleus for the

growth of a filament (Winder et al. 2005). The Arp2/3 activity regulates the mesenchymal invasion, whereas ROCK-1 is crucial for amoeboid invasion (Paulitschke et al. 2009).

Actin: Actin is a major cytoskeletal protein, which is involved in many cellular functions such as cell polarity, endocytosis and intracellular trafficking, contractility, motility, adhesion, cell division, and generation and maintenance of cell morphology (Winder et al. 2005). In vertebrates six actin isoforms have been identified – four α isoforms (skeletal, cardiac, and two smooth muscle actins), and two cytoplasmic actins (β and γ). There exist two principal forms of actin – globular monomeric (G) actin, and filamentous polymeric (F) actin. Diverse actin-binding proteins (ABPs) (e.g. Arp2/3) coordinate the assembly and disassembly of actin filaments into functional networks. Actin essembly and interaction with ABPs is regulated amongst others by phosphorylation. Upon stimulation with epidermal growth factor (EGF), mammalian actin is phosphorylated *in vitro* at tyrosine residues and *in vivo* at serine residues (Jungbluth et al. 1995). The phosphorylation of Tyr-53 of actin is of interest because this modification occurs in vivo; it is induced upon re-initiation of growth, ATP-depletion, or other stresses; and it is reversible (Jungbluth et al. 1995).

Myosin light chain 2 (MLC2): Myosins can be described as actin-dependent molecular motors, which produce movement through the hydrolysis of ATP (Winder et al. 2005). Therefore actin is used by myosins as a track to move along (Winder et al. 2005). There are more than 17 different classes of myosins with various functions (Hodge and Cope 2000). Myosin consists of six polypeptide chains – two identical heavy chains and two pairs of light chains. Depending on its distribution, Myosin light chain 2 (MLC2) has many isoforms. In smooth muscle, myosin light chain kinase (MLCK) phosphorylates MLC2 at Thr18 and Ser19 (Ikebe and Hartshorne 1985), which is correlated with myosin ATPase activity and smooth muscle contraction (Tan et al. 1992). Furthermore ROCK phosphorylates Ser19 of smooth muscle MLC2, which regulates the assembly of stress fibres (Totsukawa et al. 2000)
When stimulating LEC monolayer with synthetic 12(S)-HETE, the phosphorylation of MLC increased with time evidencing the mobile phenotype (Figure 29).

Mypt1: MYPT1 (myosin-binding subunit of myosin phosphatase) is the regulatory/targeting subunit of the myosin phosphatase, which regulates the interaction of actin and myosin in response to signalling through the GTPase Rho (Feng et al. 1999). Rho inhibits myosin phosphatase via ROCK. The phosphorylation of Mypt1 at the rim of the

"gap" (Kerjaschki et al. 2010) leads to its inhibition and the cytoskeletal reorganisation (Birukova et al. 2004a; Birukova et al. 2004b). Mypt shows a mitosis-specific phosphorylation and that the phosphorylation is reversed during cytokinesis (Totsukawa et al. 1999).

Akt: Akt is an important component in migratory and prosurvival signalling pathways (Franke et al. 1997; Burgering and Coffer 1995; Franke et al. 1995; Albini et al. 2005). It is a serine/threonine protein kinase, activated by insulin and growth and survival factors, like PDGF (Burgering and Coffer 1995; Franke et al. 1995); and lies at a key junction in cellular signalling pathways leading to NF-κB activation and cell motility (Albini et al. 2005). Akt is involved in cell cycle regulation by preventing the phosphorylation and degradation of cyclin D1 (Diehl et al. 1998) and by negatively regulating the Cdk inhibitors p27Kip1 (Gesbert et al. 2000) and p21 $^{CIP1/Waf1}$ (Zhou et al. 2001).

ILK1: ILKs (integrin-linked kinase) couple integrins and growth factors to activate pathways involved in cell survival, cell cycle control, cell-cell adhesion and cell motility (Wu and Dedhar 2001). ILK is a key component of cell–ECM adhesion structures (Wu and Dedhar 2000), as ILK bridges the ECM and growth factor receptors to the actin cytoskeleton through interactions with e.g. integrin (Wu and Dedhar 2000). Paxillin, and probably other ILK-binding proteins can bind to actin filaments, coupling integrins to the actin cytoskeleton via ILK (Winder and Ayscough 2005). The interactions between ILK and the actin cytoskeleton allow cells to modulate the strength of the connection at the cell matrix contact sites, and could facilitate signal transduction and regulation (Winder and Ayscough 2005). ILK is stimulated through cell-ECM interactions and by certain growth factors (Winder and Ayscough 2005).
Additionally ILK is required for the activation of Rac and Cdc42, and is thus essential in Rac- and Cdc42-mediated actin cytoskeleton reorganisation (Paulitschke et al. 2009). Therefore ILK regulates cancer motility and invasiveness (Filipenko et al. 2005). Furthermore ILK is involved in regulating Erk, as over expression of ILK prevents Erk inactivation, and ILK can directly phosphorylate Akt, and MLC in a calcium-independent manner, regulating smooth muscle contraction and possibly cell motility in non-muscle cells (Wu and Dedhar 2001; Persad et al. 2001). When ILK is over expressed in normal epithelial cells cell-cell adhesion is lost, due to the down regulation of E-cadherin expression; and apoptosis is suppressed (Winder and Ayscough 2005).

1.2.4 Breast Cancer – mammary carcinoma

The initial metastasis of most mammary carcinomas is formed in the axillary lymph node most proximal to the primary tumour, the "sentinel lymph node" (Figure 8), as it receives its afferent lymph from the tumour and peritumoural tissue. (Kerjaschki et al. 2010) Progression of the metastasis occurs by the colonisation of the post-sentinel lymph nodes in the axillary basin. The number of such post-sentinel lymph nodes is the strongest clinical predictor for the development of distant metastases and patient outcome (Carlson et al. 2009), thus their colonisation is a key event in metastatic tumour progression (Kerjaschki et al. 2010).

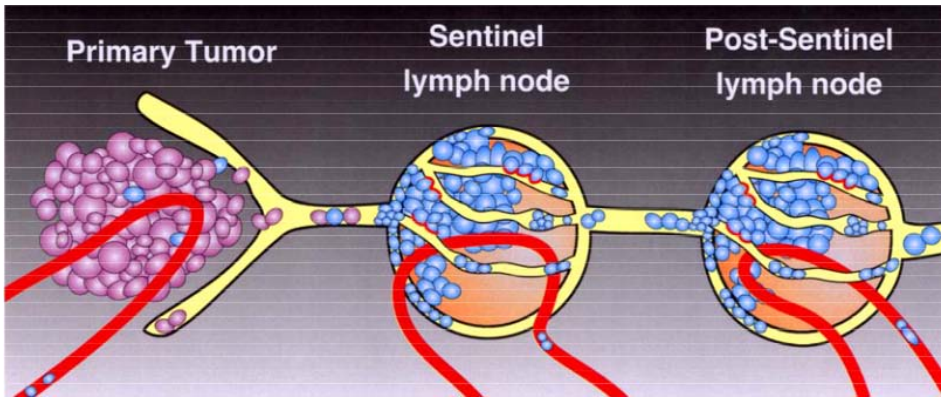


Figure 8: Overview of the development of lymph node metastases (Kerjaschki et al. 2010)
Metastatic spreading of carcinoma cells (blue) from the primary tumour (left) to the sentinel (center), and post-sentinel (right) lymph nodes; **Primary tumour**: the tumour is surrounded by lymphatic vessels (yellow), which are invaded by detached potentially metastatic tumour cells (peritumoural carcinosis). **Sentinel lymph node**: by expansion of lymph node sinus and conversion of their lining cells into lymphatic endothelial cells a pre-metastatic niche is formed („lymph node lymphangiogenesis"). Tumour cells from the metastatic colony invade intrametastatic lymphatic vessels (red caps on tumour cells), and in the lymphatic vessels tumour emboli migrate towards the subsequent lymph nodes. **Post-sentinel lymph node(s):** Repetition of events in the sentinel lymph nodes. It is currently not clear if tumour cells enter blood vessels (red) in primary or metastatic tumours. (Kerjaschki et al. 2010)

For our experiments human MCF-7 adenocarcinoma cells were used, which originate from the mammary gland. This ephithelial breast cancer cell line was isolated in 1970 from a 69-year-old Caucasian woman. (Soule et al. 1973) Several characteristics of differentiated mammary epithelium were observed in this cell line, as the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes (LGC/ATCC 2009).
In our experiments we used on the one hand empty vector transfectant cells (MCF-7^{mock}), and on the other hand transfected MCF-7 cells over expressing VEGF-C (MCF-7^{VEGFC}). The expression of VEGF-C by metastatic tumour cells exceeds that of the corresponding primary tumours; therefore we used the MCF-7^{VEGFC} cells to mimic metastatic tumour cells (Kerjaschki et al. 2010; Karpanen et al. 2001). VEGF-C is active as an endothelial-cell-specific mitogen, is induced by a range of growth factors and proinflammatory

cytokines (Ristimaki et al. 1998), is a regulator of lymphangiogenesis in mammals (Oh et al. 1997; Mäkinen et al. 2001), and can induce vascular permeability (Joukov et al. 1996; Joukov et al. 1998).

1.3 LEC gap assay – in vitro co-culture

We demonstrate the bulk invasion of metastatic tumour cells through large gaps of the lymphatic vessel wall, by performing *in vitro* experiments to mimic the *in vivo* situation. The analysis of the mechanisms of tumour cell mediated disruption of the lymphatic vascular walls was performed using an *in vitro* co-culture system. MCF-7 mammary carcinoma cell spheroids (Offner et al. 1992; Uchide et al. 2007) were used to imitate the clusters of cells seen in tumour emboli *in vivo* (Kerjaschki et al. 2010). The advantages of MCF-7 cells are that they remain stable for > 6 hours, and that their gene expression patterns are changed when compared with monolayers (Table 2). Telomerase "immortalised" human dermal lymphatic endothelial cells (LECs) (Schoppmann et al. 2004) in the form of confluent monolayers were taken to replace intrametastatic lymphatic vessels.

Table 2: Over expression of genes inMCF-7 spheroids vs. monolayers (Kerjaschki et al. 2010)

Description	Gene	Spheroid/monolayer	p-value
Gain			
CD44 (Indian blood group)	*CD44*	**3.45**	0.0039
Intercellular adhesion molecule 1 (CD54)	*ICAM 1*	**2.67**	0.0012
Vascular endothelial growth factor (VEGF A)	*VEGFA*	**2.73**	0.0100
Selectin L (lymphocyte adhesion molecule 1)	*SELL*	**2.45**	0.0055
Thrombospondin 2	*THBS2*	**2.30**	0.0125
Matrix metallopeptidase 11 (stromelysin 3)	*MMP11*	**2,20**	0,0271
Cadherin 1 type 1, E-cadherin (epithelial)	*CDH 1*	**1.80**	0.0139
Integrin alpha 5 (fibronectin receptor)	*ITGA 5*	**1.76**	0.0370
Arachidonate 15-lipoxygenase	*ALOX 15*	**1.53**	0.0397
Loss			
Laminin beta 1	*LAMB 1*	**-3.87**	0.0133
Collagen type XII, alpha 1	*COL 12A1*	**-4.21**	0.0016
Platelet/endothelial cell adhesion molecule (CD31)	*PECAM 1*	**-1.95**	0.0041
Thrombospondin 1	*THBS 1*	**-1.91**	0.0008
Vascular endothelial growthfactor C (VEGF C)	*VEGFC*	**-1.81**	0.0295
Vitronectin	*VTN*	**-1.45**	0.0559

MCF7 cells were grown as spheroids or as monolayers, lysed to extract and reverse transcribe RNA for low density arrays (Human Extracellular Matrix and Adhesion Molecules PCR Array; SABiosciences). Genes were identified which are differentially induced or repressed by MCF7 cell spheroid formation and could be related to bulk-like invasion through the lymphatic vasculature. (Kerjaschki et al. 2010)

The MCF-7 cell spheroids (3000 cells/spheroid, average diameter ~ 300nm) were placed onto the LEC monolayer and after few hours of co-incubation (4 h.), the formation of circular gaps in the LEC monolayer underneath the MCF-7 spheroids could be observed (Figure 9). This gap formation was similar to the defects seen in lympho-vascular walls at sites of tumour cell invasion *in vivo* (Kerjaschki et al. 2010).

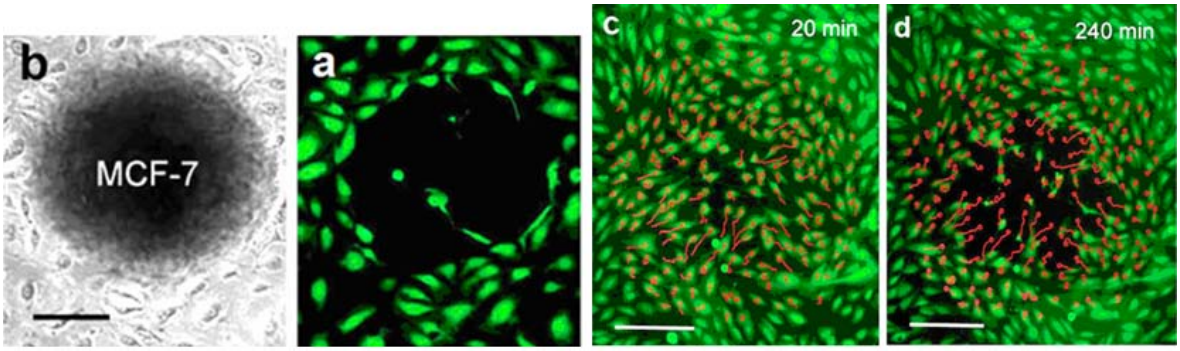


Figure 9: MCF-7 cell spheroids induce gaps in LEC monolayers (Kerjaschki et al. 2010).
a, and **b**, Spheroids made from MCF7 mammary carcinoma cells induce circular gaps in LEC monolayers (cyto-tracker tagged in green). **c,** and **d**, Tracings of lymphatic endothelial cell migration (red lines, starting positions are marked by circles) beneath a spheroid during a 4 hr. co-incubation. Scale bars: a, b, c, d 100 µm, in c and d 25 µm. (Kerjaschki et al. 2010)

We performed several experiments to proof the specificity of the used gap formation assay, and the results are all in accordance with our expectations: As MCF-7 cells migrate across LECs, MCF-7 spheroids induced much bigger gaps in LEC monolayers than in blood endothelial cell (BEC) monolayers (Figure 10: c) (Kerjaschki et al. 2010). Compared to MCF-7 spheroids inducing gaps in monolayers of LECs, spheroids of non-malignant human breast epithelial cells (MCF-10A), and human lung fibroblasts (HLF) show an extremely marginal gap formation induction ability (Figure 10 d) (Kerjaschki et al. 2010). Human lung fibroblast (HLF) spheroids failed to induce any defects in human LEC monolayers, whereas MCF-7 spheroids induced circular gaps in the same time horizon (4 h of co-incubation) (Figure 10 a, b) (Kerjaschki et al. 2010).

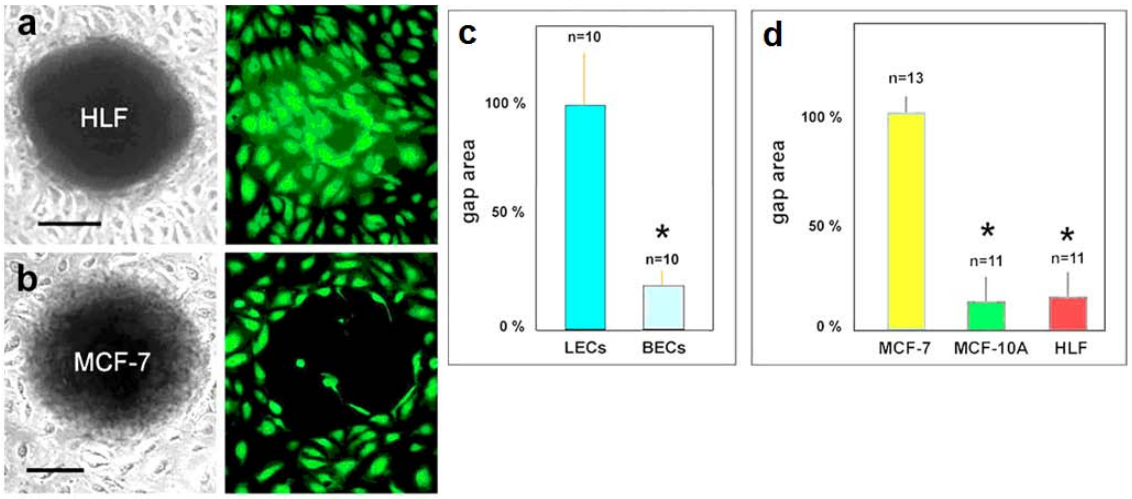


Figure 10: HLF spheroids induce reduced gaps in LEC monolayers (Kerjaschki et al. 2010).
a, Spheroids of human lung fibroblasts (HLF) fail to induce any defects in a monolayer of human lymphatic endothelial cells (cyto-tracker tagged in green) after 4 h of co-incubation. **b**, By contrast, spheroids made from MCF7 mammary carcinoma cells induce circular gaps. **c**, MCF7 cell spheroids induce preferentially gaps in monolayers of lymphatic endothelial cells (LECs, left bar), but significantly less (14.3% of lymphatics, p=0.0047) in monolayers of microvascular blood endothelial cells (BECs, right bar). **d**, When compared to spheroids of MCF7 cells (left bar, 100%), gap formation in lymphatic monolayers is marginally induced by non-malignant human breast epithelial cells MCF-10A (9.6% of MCF7 spheroids, $p < 0.0001$), and of human lung fibroblasts (HLF) (11,3%, $p < 0.0001$). Scale bars: a, b, 100 µm, in c and d 25 µm. (Kerjaschki et al. 2010)

The induction of gaps by cancer cells into lymphendothelial cell walls depends on two main mechanisms – the LOX system and the acivity of MMPs. Both mechanisms are functional in MCF-7 spheroids and support the centrifugal migration of lymphatic endothelial cells away from the sites of contact with tumour cells (Kerjaschki et al. 2010).
The third mechanism involved in gap induction by cancer cells may be the mechanism of NF-κB.

1.4 Lipoxygenases (LOX)

Many lipids and their metabolites are important second messengers in cell signal transduction, whereby the disruption of this signal transduction cascade may lead to cell transformation. Arachidonic acid (AA) and its metabolites are produced in many cells in response to extracellular signals (Chen et al. 1994). Phospholipids release AA by actions of phospholipases, followed by the metabolism of AA via the lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P-450-dependent monooxygenase (epoxygenase) pathways (Chen et al. 1992).
Compared to MCF-7 monolayers, the specific induction of several genes can be observed in MCF-7 cell spheroids, including the enzyme 15-LOX (Table 2) that metabolises AA to 12- and 15-hydroperoxyeicosatetraenoic acid, which is then reduced to 12(S)- and 15(S)-

HETE, that are bioactive lipids implicated in tumour angiogenesis, growth and metastasis (Nie et al. 2003; Matsuyama et al. 2004).

1.4.1 Arachidonic acid and 12(S)-HETE

The ability of tumour cells to produce 12(S)-HETE is positively correlated to their metastatic potential, as 12(S)-HETE is a crucial intracellular signalling molecule, which activates protein kinase C and mediates biological functions of many growth factors and cytokines (Honn et al. 1994b). Two enzymes in humans produce 12(S)-HETE, 12-LOX and 15-LOX (products of human *ALOX12* and *ALOX15* genes) (Funk 1996). 12-LOX may play a major role in tumour promotion, progression and metastasis and has been implicated in the pathogenesis of cancer (Nie et al. 2003; Nie and Honn 2004; Yoshimura et al. 2004). The blockade of the 12-LOX pathway leads to antiproliferative effects and apoptosis in a variety of human cancers e.g. breast cancer cells, through cytochrome *c* release; caspase-9 (cysteine protease) activation, which acitivate caspase-3 (marker of apoptosis) (Zou et al. 1997; Srinivasula et al. 1998); and changes in the levels of Bcl-2 family proteins (e.g. Bcl-2 and Bax), which are regulators of apoptosis (Kluck et al. 1997; Tong et al. 2002a, b).

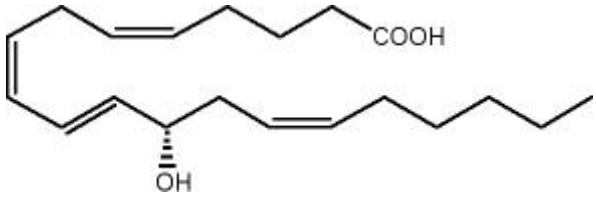


Figure 11: Chemical structure of 12(S)-hydroxyeicosatetraenoic acid

12(S)-HETE (mainly produced by platelets, leukocytes, smooth muscle, epithelium, neuron, and fibroblast cells) (Spector et al. 1988) induces a protein kinase C-dependent cytoskeletal rearrangement and retraction of microvascular endothelial cells, and an increased tumour cell adhesion to exposed ECM (Tang et al. 1993; Honn et al. 1994a; Kerjaschki et al. 2010). Together with metalloproteases (MMPs), that loosen inter-endothelial junctions and matrix attachment (Deryugina and Quigley 2006), 12(S)-HETE supports the centrifugal migration of lymphatic endothelial cells away from the sites of contact with tumour cells (Figure 12) (Kerjaschki et al. 2010); as both – MMPs and 12(S)-HETE – are released by MCF-7 tumour spheroids (Uchide et al. 2007). MMPs and LOX affect different cellular mechanisms, therefore the co-incubation with GM6001 (MMP inhibitor) and baicalein (LOX inhibitor), or LOX15-knock down cells, resulted in additive effects in the reduction of gap size by over 60% (Figure 14 e) (Kerjaschki et al. 2010).

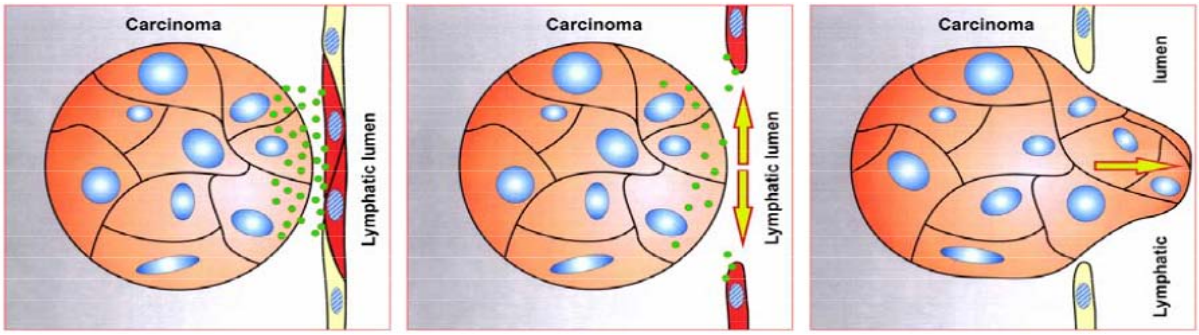


Figure 12: Mechanisms of tumour cell invasion into lymphatic vessels (Kerjaschki et al. 2010).
Bulk tumour cell invasion through LEC monolayers; Clusters of tumour cells *in vivo* (or tumour spheroids *in vitro*) release 12(S)-HETE in membrane microvesicles (left figure, green circles). This induces a migratory response (green arrows, middle figure) in nearby lymphatic endothelial cells (red), causing a gap in the endothelial monolayer, through which the tumour cell cluster invades into the lymphatic lumen (green arrow, right figure). (Kerjaschki et al. 2010)

In order to proof the increasing impact of 12-, and 15-LOX on the induction of gaps in LEC monolayers, we performed a gap formation assay with MCF-7 spheroids and HTB-26 breast adenocarcinomas cell spheroids. In contrast to MCF-7 cells, HTB-26 cells do not express any LOX. In accordance with this fact, HTB-26 cell spheroids induced smaller gaps (~ 40%) in the underlaying LEC monolayer then MCF-7 spheroids did (Figure 13).

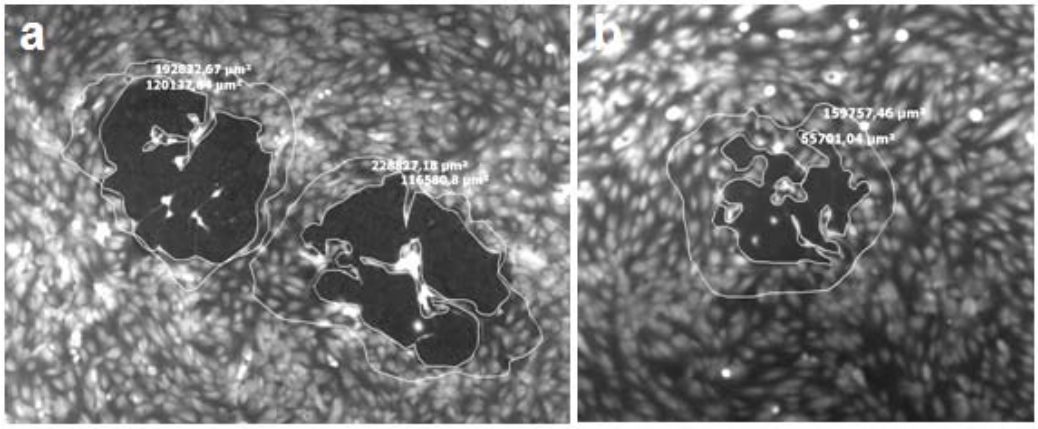


Figure 13: Impact of LOX on gap formation
a. Untreated MCF-7 cell spheroids were seeded onto untreated LEC monolayers. **b**. Untreated HTB-26 cell spheroids were seeded onto untreated LEC monolayer. Pictures were taken after 4 hr of incubating the co-cultures at 37°C.

When placing fibroblast spheroids, treated with synthetic 12(S)-HETE, onto LEC monolayers, the induced gap formation is similar to that induced by MCF-7 spheroids (Figure 14 a) (Kerjaschki et al. 2010). In contrast, when blocking 12(S)-HETE in MCF-7 spheroids, the formation of gaps is inhibited (Figure 14 b) (Kerjaschki et al. 2010). When treating MCF-7 spheroids with the LOX inhibitor baicalein (Li-Weber 2009), the gap formation was dramatically reduced (Figure 14 c and d) (Kerjaschki et al. 2010). By shRNA mediated knock down of 15-LOX in MCF-7 cells (MCF-7/15LO$^-$), the induction

of gap formation is similar to baicalein treated MCF-7 spheroids (Figure 14 e) (Kerjaschki et al. 2010).

In previous experiments 12(S)-HETE showed little or no effects on actin-binding proteins (filamin and tropomyosin) or on microtubules (Tang et al. 1993), as 12(S)-HETE induces a protein kinase C-dependent cytoskeletal rearrangement of microvascular endothelial cells, which seems to be mediated through enhancing phosphorylation of proteins that comigrated with myosin light chain, actin, and vimentin (Tang et al. 1993).

Experiments showed that the induction of fibroblast and pancreatic cancer cells with 12(S)-HETE resulted in an increase of phosphorylated Erk1/2, leading to an increased cyclin D expression and therefore enhanced cell growth/proliferation. Therefore 12(S)-HETE activates the Erk1/2 pathway (Roberts and Der 2007).

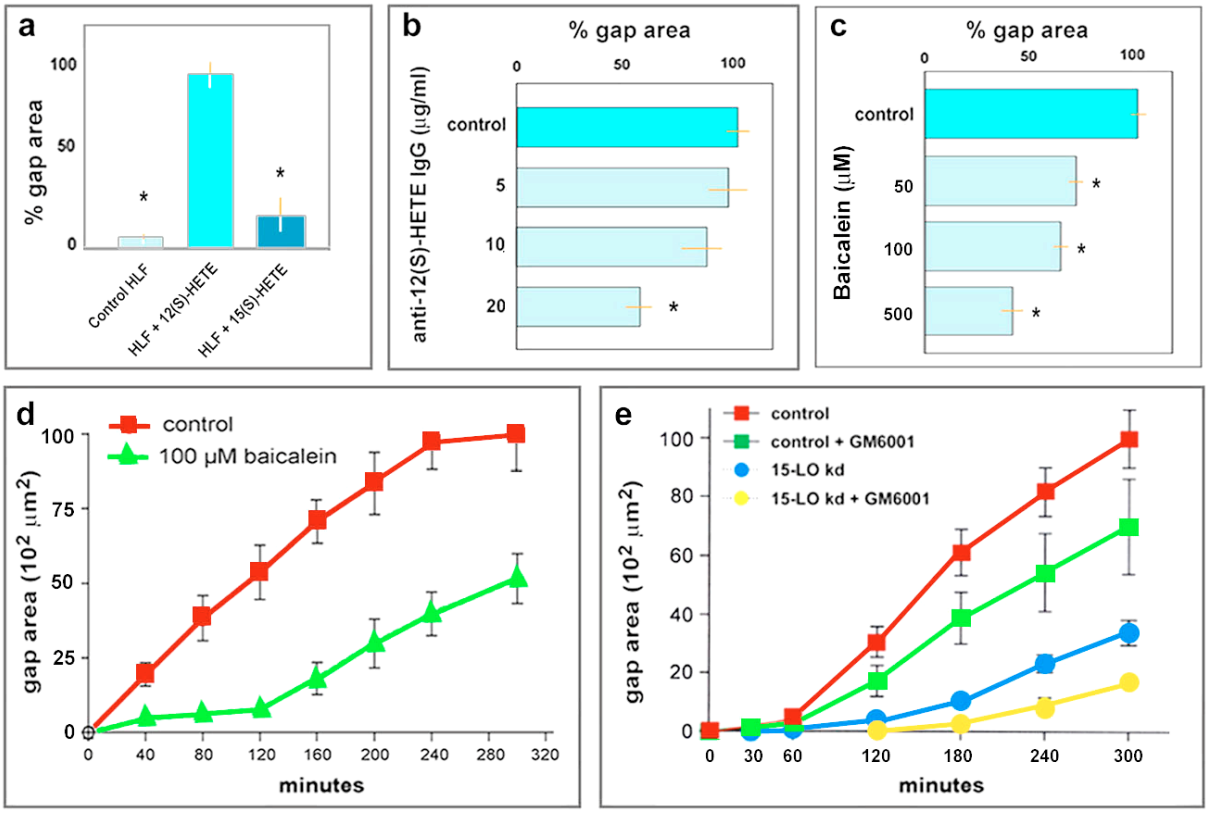


Figure 14: 12(S)-HETE causes gaps in LEC monolayers (Kerjaschki et al. 2010).
a, Gap area in LEC monolayers induced by HLF cell spheroids that were presoaked with synthetic 12(S)-HETE (n=11), 15(S)-HETE (n=12), or with solvent alone (n=21). 12(S)-HETE induced a ~ 20 times lager gap than the controls ($p < 0.0001$) after 4 h of co-incubation. **b**, Antibody against 12(S)-HETE reduces the gap area by ~50% ($p=0.0020$). **c**, The LO-inhibitor baicalein reduces gap area in a dose-response fashion, with the highest dose of 500 µM (n=11) causing reduction to 38% ($p< 0.0001$) of controls (n=20). **d**, Time course incubation over 4 h with 100 µM baicalein in the media results in ~ 90% reduction of gap size in the first 2 h, and a gradual increase in gap formation to > 50% after 4 h. **e**, shRNA mediated knock down of 15LO in MCF7 cells (blue line) causes a similar reduction of gap size as baicalein. This is further aggravated by co-incubation with 20 µM of the pan-metalloprotease inhibitor GM6001 (yellow line) that had a similar effect (green line) on controls (MCF7 cells transduced with scrambled shRNA, red line). (Kerjaschki et al. 2010)

1.4.2 LOX inhibition via Baicalein

Baicalein is a bioactive flavonoid constituent originally isolated from e.g. *Scutellaria baicalensis*, a commonly used plant in the traditional Chinese medicine (TCM). Especially the roots of the plant are used to treat various inflammatory conditions (Ma et al. 2005). Anticancer effects in human hepatoma cell lines were already shown (Himeji et al. 2007). Baicalein induces antiproliferative effects against cancer cells, via the inhibition of 12/15-LOX and their metabolite 12(S)-HETE; and of reverse transcriptases (Tong et al. 2002a, b). It further inhibits

- LPS induces nitric oxide production in murine macrophages (Wakabayashi 1999),
- chemokines (fibroblastic eotaxin) (Nakajima et al. 2001),
- protein tyrosine kinase and PMA-stimulated protein kinase C (Huang et al. 1994),
- IL-1β-induced IL-6 and -8 protein production (Nakamura et al. 2003), and
- induces cell death in hepatocellular carcinoma cell lines (Leung et al. 2007).

As 12-LOX plays a major role in tumour promotion, progression, and metastasis (Nie et al., 2003; Nie and Honn, 2004; Yoshimura et al., 2004), baicalein shows promise in the fight against cancer.

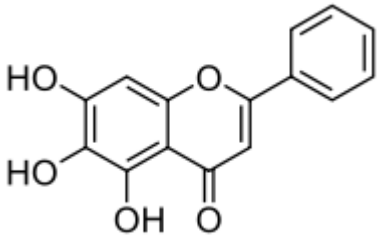


Figure 15: Structure of baicalein (Vonach 2010)
Chemical class: cell-permeable flavone; molecular formula: $C_{15}H_{10}O_5$; molecular weight: 270.3 g/Mol

Baicalein induces apoptosis partially dependent on caspase-3 pathway in human lung nonsmall carcinoma H460 cells and human leukaemia HL-60 cells (Leung et al. 2007). It inhibits the proliferation of H460 cells and HepG2 cells via S-phase arrest, as it increases p53 and therefore decreases the protein levels of Cdk1 and cyclin B1, which are the regulating proteins of S-phase transition to G_2/M-phase (Leung et al. 2007). Exogenous 12(S)-HETE did not reverse the effect of baicalein on the induction of apoptosis (H460 cells; Leung et al. 2007). This indicates that the cell death once triggered by baicalein might be irreversible (Leung et al. 2007). Previous gap formation assays showed a maximum inhibition in LEC gaps underneath MCF-7 cancer cells of ~ 50% through the treatment with baicalein (100µM) (Vonach 2010).

1.5 Matrix metalloproteinases (MMPs)

The invasion and metastasis involve attachment of tumour cells to the basement membrane, degradation of the local connective tissue, and penetration and migration through proteolysed stroma (Roeb and Matern 2003). MMPs are the key enzymes responsible for ECM breakdown (Roeb and Matern 2003), and are key regulators for creating and maintaining an environment that supports tumour growth (Chambers and Matrisian 1997). MMPs are zinc-dependent extracellular membrane associated neutral endopeptidases, which mediate physiological and pathological processes, like matrix degradation, tissue remodelling, inflammation, and tumour metastasis (Duffy et al. 2000), either directly or by release of growth factors sequestered in the extracellular matrix (Stetler-Stevenson 1999). MMPs play a role in normal tissue remodelling during embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing (Duffy et al. 2000) (Figure 16). Abnormal expression contributes to various pathological processes including rheumatoid arthritis and osteoarthritis, pulmonary emphysema, and tumour growth, invasion and metastasis (Duffy et al. 2000; Chambers and Matrisian 1997). MMPs have been associated with the malignant phenotype in a wide variety of human tissues, including lung, prostate, stomach, colon, breast, ovaries and thyroid, as well as squamous carcinoma of the head and neck (Ray and Stetler-Stevenson 1994).

MMPs include four main groups according to their substrate specificity and domain structure – the interstitial collagenases, gelatinases, stromelysins, and membrane-type MMPs (possessing a transmembrane domain) (Duffy et al. 2000). At least 19 members of this family are known to exist, as MMP-1 (interstitial collagenase), MMP-7 (matrilysin), MMP-3, -10, -11, -13 (stromelysins), MMP-14 (membrane-type MMP, MT-MMP), and MMP-2 and MMP-9 (gelatinase A and B) (Rosenberg et al. 1996; Mun-Bryce and Rosenberg 1998).

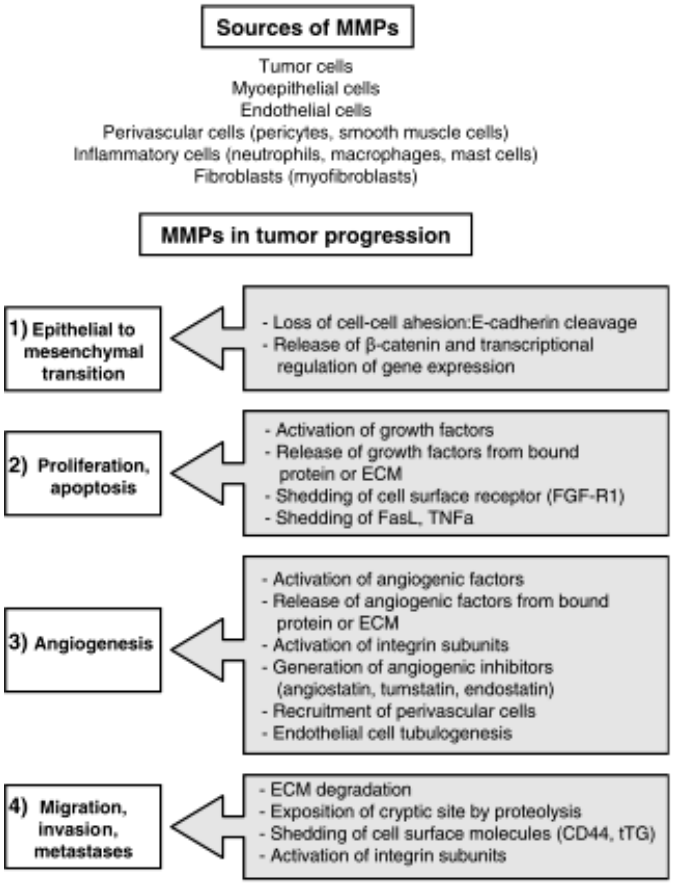


Figure 16: Sources and implications of MMPs (Chabottaux and Noel 2007)

1.5.1 Activation and inhibition of MMPs

MMPs are produced by a variety of cell types, including epithelial cells, fibroblasts and inflammatory cells (Stetler-Stevenson 1999). Additionally oncogenes may contribute to tumourgenesis by regulating the expression of MMPs (Duffy et al. 2000). MMPs consist of five modular domains, including a signal sequence; a profragment activation locus; a zinc binding, catalytic domain; a proline-rich hinge region; and a haemopexin- or vitronectin-like C-terminal domain, which is connected with the catalytic domain via the hinge region (Figure 17) (Ray and Stetler-Stevenson 1994). MT-MMPs show an additional transmembrane domain at their C-termini. (Figure 17) (Roeb and Matern 2003)

MMPs are secreted as latent precursors (zymogens) and are activated in the extracellular space (Ray and Stetler-Stevenson 1994). Pro-MMPs are inactive as long as a cysteine residue located in the propeptide portion of the pro-MMP interacts with the catalytic zinc atom, and therefore blocks the access of substrates to the catalytic pocket of the enzyme (Hidalgo and Eckhardt 2001). The proteoloytic cleavage of the propeptide leads to the dissociation of the cysteine residue from the catalytic site, which is now exposed to the

substrate (Hidalgo and Eckhardt 2001). MMP-11 differs from other MMPs, as it is processed intracellularly by furin (intracellular activation) (Pei and Weiss 1995), and can therefore be secreted as a potentially acitve protease.

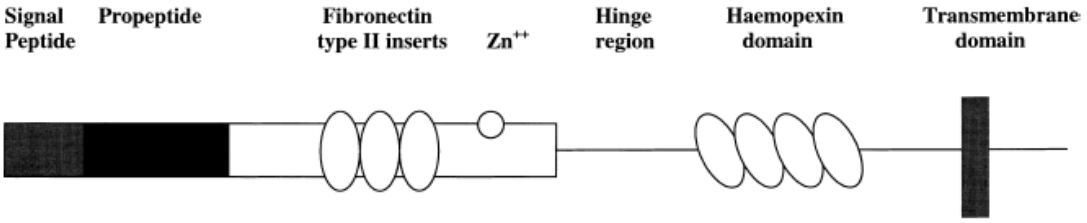


Figure 17: General structure of the matrix metalloproteinases (MMPs) (Hidalgo and Eckhardt 2001)
The general structure of the MMPs includes a signal peptide, a propeptide domain, a catalytic domain with a highly conserved zinc-binding site, and a haemopexin-like domain linked to the catalytic domain by a hinge region. MMP-2 and MMP-9 contain fibronectin type II inserts within the catalytic domain. The membrane-type MMPs (MT-MMPs) contain a transmembrane domain at the C-terminal end of the haemopexin-like domain. The haemopoxin domain is absent in the smallest MMP, matrilysin (MMP-7). (Hidalgo and Eckhardt 2001) [Modified with permission from Kahari and Saarialho-Kere 1999]

The MMP gene transcription is induced by a variety of extracellular stimuli, such as cytokines (IL-4 and IL-10), growth factors (EGF, TGF-α, bFGF, TGF- β, PDGF), TNFα, hormones, and cell-cell or cell-matrix interactions (Ray and Stetler-Stevenson 1994; Westermarck and Kähäri 1999). All these stimuli trigger a cascade of intracellular reactions, which are mediated through at least three different classes of mitogen-activated protein kinases (MAPK): extracellular signal-regulated kinase, stress activated protein kinase/Jun N-terminal kinases and p38 (Reddy et al. 1999). The activation of these kinases leads to the activation of a nuclear AP-1 transcription factor, which binds to the AP-1 cis element and activates the transcription of the corresponding MMP gene (Sato et al. 1997).
The activity of MMPs is down regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs), which consists of four members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Stetler-Stevenson 1999). TIMPs inhibit the active form of the MMPs and their activation process by forming high-affinity complexes with the zinc region, and the haemopexin domain of active MMPs (Duffy et al. 2000). TIMPs are soluble proteins, with the exception of TIMP-3, which is associated with the ECM (Roeb and Matern 2003). Additionally TIMPs induce changes in cell morphology, stimulate growth of several cell types, promote steroidogenesis and germ cell development, and inhibit angiogenesis (Hidalgo and Eckhardt 2001). As endothelial cells come in contact with different extracellular matrix environments, the type of extracellular matrix may alter the profile of MMP expression (Stetler-Stevenson 1999). The activity of MMPs is determined by the balance between the levels of activated MMPs and free TIMPs. Changing the equilibrium affects the process of cellular invasion. (Ray and Stetler-Stevenson 1994)

1.5.2 MMPs and angiogenesis

In general MMPs are implicated in angiogenesis (Stetler-Stevenson 1999), and are able to promote tissue invasion of neutrophils and macrophages (Mun-Bryce and Rosenberg 1998; Heo et al., 1999). MMPs promote angiogenesis by at least two different mechanisms: by degrading barriers and thereby facilitating endothelial cell invasion; and by initiating signalling pathways and liberating factors (through extracellular matrix degradation) that promote or support the angiogenic phenotype (Stetler-Stevenson 1999). Collagenases catalyse the degradation of fibrillar forms of collagen, whereas gelatinases degrade gelatine (denatured collagen), which is present in basement membranes (membranes that separate organ parenchyma from the underlaying stroma (Duffy et al. 2000). Stromelysins show broad substrate specificity, catalysing degradation of many different substrates in the ECM, such as laminin, and fibronectin (Chambers and Matrisian 1997; Duffy and McCarthy 1998). Nevertheless, MMPs may also produce or release angiogenesis inhibitors such as angiostatin from the extracellular matrix (Stetler-Stevenson 1999).

1.5.3 MMPs and cancer

MMPs contribute to cancer initiation or to tumour cell growth, by promoting angiogenesis, activating stimulating growth factors or their receptors, and inactivating inhibiting growth factors (Duffy et al. 2000). MMPs activity is an early event in the angiogenic response, and this activity may directly influence endothelial cell behaviour (Stetler-Stevenson 1999). MMP inhibitors inhibit angiogenic responses both in vitro and in vivo (Murphy et al. 1993; Anand-Apte et al. 1997; Benelli et al. 1994; Hiraoka et al. 1998). MMPs are involved in breast cancer initiation, invasion and metastasis (Duffy et al. 2000). At least two MMPs – MMP-2 and stromelysin-3 (MMP-11) – have been found to correlate with poor prognosis in patients with breast cancer (Duffy et al. 2000). Additionally, MMP-11-knockout-mice reacted to chemical mutagens with a reduced tumourgenesis (Masson et al. 1998). Therefore the inhibition of these proteinases may be helpful in preventing breast cancer and in blocking metastasis of established tumours (Duffy et al. 2000). Interstingly, when MCF-7 cells were grown as spheroids, they express significantly higher MMP-11mRNA levels than MCF-7 cells grown as monolayers (Kerjaschki et al. 2010). Three main ways to inhibit MMPs exist: the inhibition of signal transduction transmitted through the MAPKs – inhibition of the expression of MMPs; the use of TIMPs; and low molecular-weight synthetic inhibitors (Duffy and McCarthy 1998; Hiraoka et al. 1998). Whereby due to their

protein nature and their multiplicity of actions, TIMPs are unlikely to be used as anticancer molecules (Duffy et al 2000).

1.5.4 MMP-11

In contrary to other stromelysins, MMP-11 (stromelysin-3) has not yet been found to degrade any matrix protein, but to hydrolyse the serine proteinase inhibitor α1-proteinase inhibitor (Rei et al. 1994). MMP-11 is almost absent in normal adult organs (Peruzzi et al. 2009), but is highly expressed in tumour cell lines, and plays a major role in the control of cell proliferation – by liberating ECM-associated growth factors (Masson et al. 1998) – and tumour development, as it prevents cancer cell death through apoptosis and necrosis (Deng et al. 2005). The normal expression of MMP-11 during embryogenesis and tissue involution is associated with tissue remodelling and is located in stromal cells with contact to apoptotic epithelial cells, indicating that MMP-11 is a stromal-derived factor involved in epithelial cell survival (Fromigué et al. 2003). MMP-11 has been shown to be overexpressed in nearly all invasive primary carcinomas, which led to the suggestion that MMP-11 plays a major role in the initial development of epithelial malignancies (Fromigué et al. 2003). In these cases MMP-11 is synthetisised, as in vitro co-cultures models showed that its expression was induced in stromal cells after direct contact with tumour cells (Fromigué et al. 2003). MMP-11 suppresses cell apoptosis as it is able to activate cell survival pathways accompanied by activation of p42/p44 MAPK and Akt (Fromigué et al. 2003).

1.6 Nuclear factor kappa B (NF-κB)

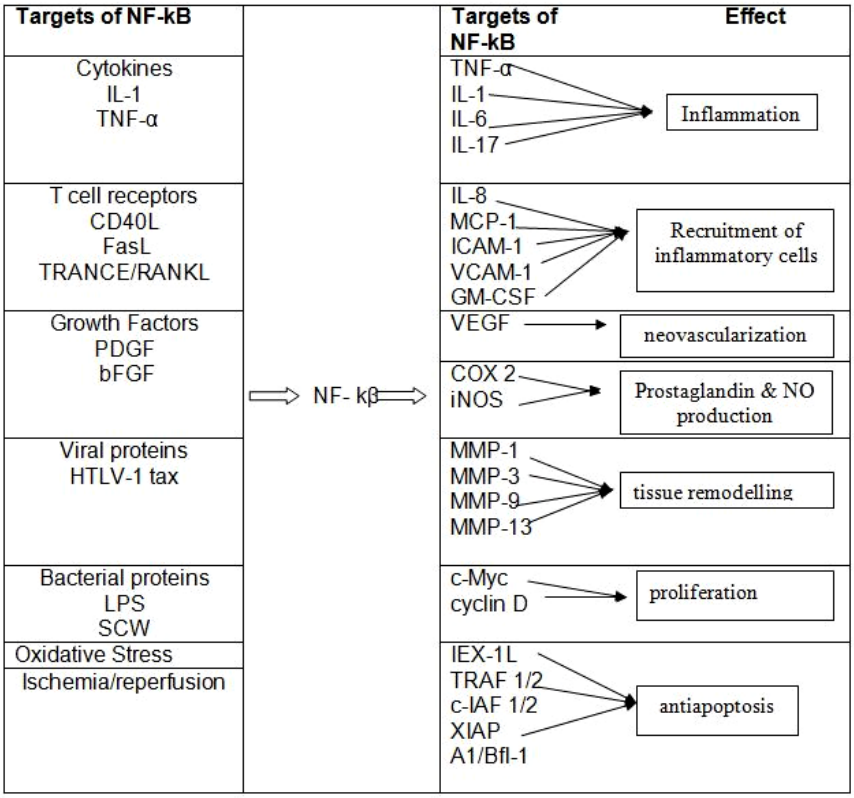
The protein complex NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is an important specific transcription factor, which is present in almost all mammalian cell types and tissues. NF-κB plays a major role in the regulation of the immune response to infection and inflammatory signalling via its ability to induce expression of genes encoding cytokines, cytokine receptors, and cell-adhesion molecules (Baldwin 1996; Ghosh et al. 1998). Incorrect regulation of NF-κB can be linked to inflammation, autoimmune diseases, and cancer. (Albensi and Mattson 2000) The role of NF-κB in cancer appearance involves the ability to control apoptosis (NF-κB activation can block cell-death pathways; Baldwin Jr. 2002) and cell-cycle progression, and possibly cell differentiation, angiogenesis and cell migration and -proliferation (Orlowski and Baldwin Jr. 2002).

Through attachment to certain regulatory sequences of the DNA, NF-κB is able to influence the transcription of certain genes. NF-κB subsumes (in mammals) five subunits: NF-κB1 (p50 and p105), NF-κB2 (p52 and p100), RelA (p65), RelB, and c-Rel (Ghosh and Karin 2002). These NF-κB- and Rel-proteins are able to build homo- (NF-κB-NF-κB protein attachment, or Rel-Rel-protein attachment) or heterodimers (NF-κB-Rel-protein attachment), and share a highly conserved 300 amino acid long N-terminal "Rel homology domain" (RHD), which is responsible for DNA binding, dimerisation, and I-κB binding (Gosh et al. 1998). Beside the RH-domain, Rel-proteins contain at least one transactivation domain in their C-termini, which leads to the activating function of Rel-proteins, whereas dimers without Rel-proteins do show an inhibitory function. (Sun and Andersson 2002)

NF-κB is able to bind at a specific DNA motive of about ten base pairs, the so-called κB-motive. In almost all cases, the attachment of NF-κB to this DNA-motive leads to an increased transcription of the dependent genes. About 200 different genes, like cytokines, chemokines, effector molecules of immunity, pro-survival factors, and adhesion molecules, are regulated by NF-κB (Table 3). (Sun and Andersson 2002) Genes that are regulated by NF-κB, as IL-1, ICAM-1, CINC, IL-8, and gro (Roebuck, 1999), COX-2 (Lee and Burckart et al. 1998; Kotake et al. 1998; Plummer et al. 1999), MMP-9 (Mun-Bryce & Rosenberg 1998; Lee and Burckart 1998), heme oxygenase-1, and iNOS (Kotake et al. 1998), can mediate cellular injury.

NF-κB plays a major role in responses to inflammatory signalling through Toll-like receptors, TNF receptors and the IL-1 receptor, as well as in response to signalling through antigen receptors of lymphocytes.

Table 3: Various targets and effects of NF-κB (Patel et al. 2009)



1.6.1 Activation and inhibition of NF-κB

In few cell types NF-κB is situated in the nucleus of the cell and is therefore constitutively active without external stimuli. This is true for B-lymphocytes and dendritic cells. In most other cell types NF-κB is inactive, because the nuclear localisation signals (NLS) of NF-κB proteins are masked by I-κB proteins (I-κBα, I-κBβ, I-κBγ or NEMO, I-κBε, and Bcl-3) and kept sequestered in an inactive state in the cytoplasm without any access to the nucleus situated DNA (Jacobs and Harrison 1998).

The activation of NF-κB is triggered by different stimuli, as the exposure to growth factors, cytokines (e.g. TNF-α and IL-1β), and mitogens, bacterial and viral infection (e.g. LPS, double-stranded RNA), stress inducing agents including proapoptotic and necrotic stimuli such as oxygen free radicals, ultraviolet light and γ-irradiation (Figure 18) (Karin and Ben-Neriah 2002; Tran et al. 1997), and by oncoproteins through signal transduction cascades (Baldwin 2001). The impact of such stimuli results in an activity change of cellular signal transduction pathways (e.g. MAP-kinase pathway), which are often regulated by phosphorylation. (Gilmore 2006)

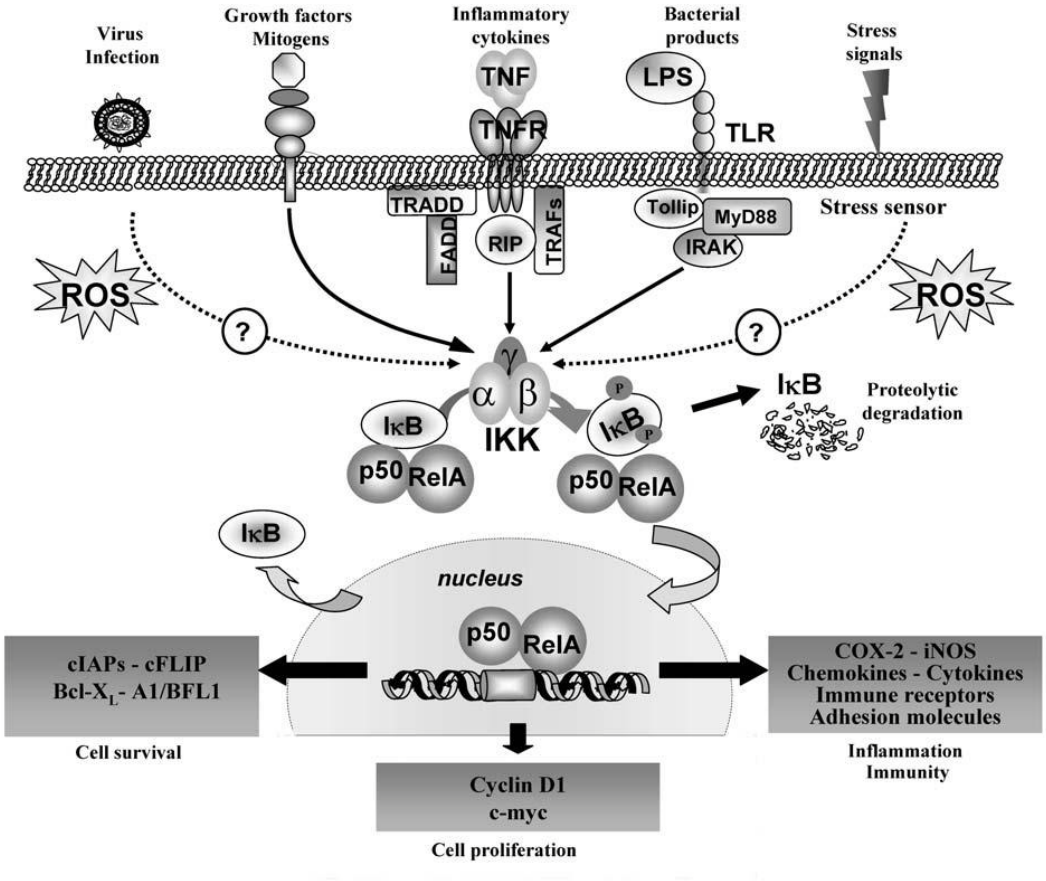


Figure 18: The NF-κB pathway. (Piva et al. 2006)
NF-κB heterodimers (e.g. p50/RelA) are held in the cytoplasm by I-κB inhibitory proteins (I-κB). Stimuli such as stress-inducing agents, exposure to inflammatory cytokines, mitogens, or to a diverse array of bacterial and viral pathogens lead to the activation of signalling cascades activating the IKK complex. Phosphorylation of I-κB by activated IKK is a signal for its ubiquitylation and proteasome-dependent degradation. Free NF-κB dimers translocate to the nucleus where they bind to the κB motive and activate the transcription of a variety of genes involved in the control of cell proliferation and survival, in the inflammatory and immune response, as well as autoregulatory genes, including I-κB itself. (Piva et al. 2006)

The last part of the activation of NF-κB is common in all signal transduction pathways: the activation of the I-κB-kinase complex (IKK: IKK-α-, and IKK-β-subunits) (Zandi et al. 1997). Stimuli such as bacterial lipopolysaccharide (LPS), negative-strand RNA viruses, double-stranded (ds)RNA, immunostimulatory DNA sequences (ISS-DNA), TNF-α (Figure 20) (May and Ghosh 1999; Li and Karin 1999), IL-1, and antigens, activate the IKK complex, which phosphorylates I-κB-proteins and induces their ubiquitination (Ghosh et al. 1998), that leads to the dissociation of I-κB from NF-κB, and the eventual degradation of I-κB by the 26S proteosome (Ghosh and Karin 2002). NF-κB molecules are therefore released from their inhibitors and can now enter the cell nucleus, where they can "turn on" the expression of specific genes (Sun and Andersson 2002). This activation then leads to the given physiological response (e.g. inflammatory or immune response, a cell survival response, or cellular proliferation) (Figure 19).

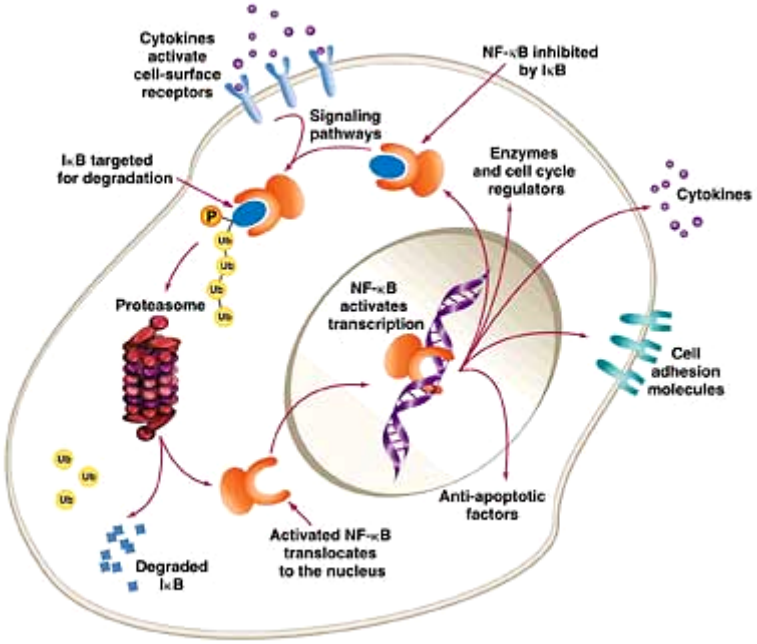


Figure 19: NF-κB mechanism of action (Albanell and Adams 2002).
In an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein I-κBα. The enzyme I-κB kinase (IKK) is activated by a variety of extracellular signals, released by integral membrane receptors. IKK phosphorylates the I-κBα protein, which results in ubiquitination, dissociation of I-κBα from NF-κB, and eventual degradation of I-κBα by the proteosome. The activated NF-κB is then translocated into the nucleus and binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function. (Gilmore 2006; Brasier 2006; Perkins 2007)

TNF may induce cell injury or cell protection, depending upon which genes are induced and in which cells the genes are induced in, and the circumstances of their induction (Figure 20) (Li and Karin 1999; Sharp et al. 2000). In contrast, IL-10 decreases the NF-κB activity by inhibiting IKKs, and by blocking NF-κB binding to target elements (Schottelius et al. 1999).

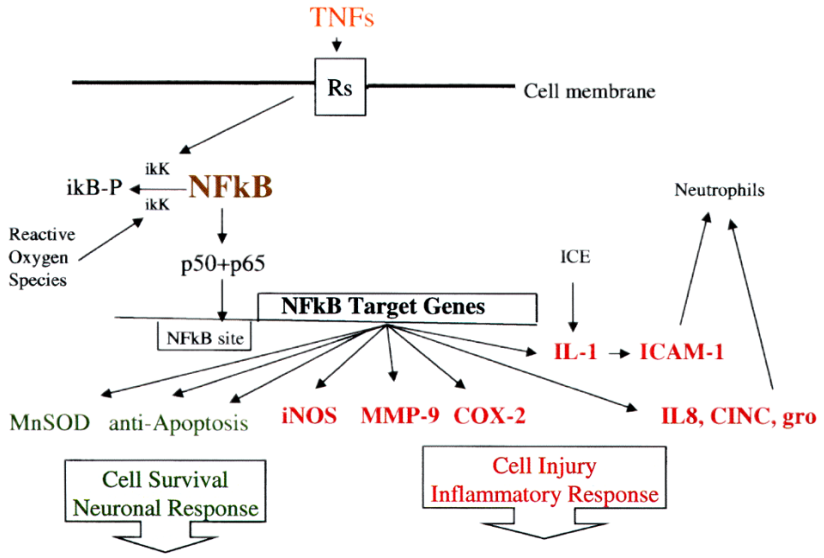


Figure 20: Mechanisms of TNF and NF-κB mediating cell survival or cell injury (Sharp et al. 2000)
The phosphorylation of I-κB and its release, results in NF-κB activation and binding of the p50-p65 complex to NF-κB sites in target genes. (Sharp et al. 2000)

The two main characteristics of NF-κB are its fast activation ("first responder" to harmful cellular stimuli; new protein synthesis not required) and its low specificity (stimulation of a wide variety of cell-surface receptors and numerous genes controlled), predestine NF-κB for the deployment in processes which need a rapid and broad change of the gene transcription. (Karin and Ben-Neriah 2000; Gilmore 2006)

Because of its essential role in the innate immunity, in inflammatory gene expression, B cell antigen receptor signalling, and the cell survival, mutations which inactivate NF-κB are generally lethal, and the partial loss of its function causes immunodeficiency.

1.6.2 NF-κB: cell survival and cancer

NF-κB is a critical factor for the regulation of cell proliferation and survival during immune, inflammatory, and stress responses (Karin and Lin 2002; Li and Verma 2002). For example NF-κB activates the expression of cyclin D1 and c-myc, which promotes the transition from the G1 into the S phase of the cell cycle (Guttridge et al. 1999; Karin et al. 2002; La Rosa et al. 1994). In inflammatory diseases NF-κB plays a major role, as an increased activation of NF-κB, and therefore an increased expression of many genes involved in immune and inflammatory responses is observed (Epstein et al. 1997). In inflammatory diseases adhesion molecules support the flow of inflammatory cells (e.g. neutrophils, eosinophils and T-lymphocytes) from the circulation to the inflammation site (Albelda et al. 1994). NF-κB regulates the expression genes that encode adhesion

molecules, and additionally it induces the expression of inflammatory cytokines (Epstein et al. 1997).

On the other hand, diverse studies show that NF-κB also plays a major role in carcinogenesis (Cogswell et al. 2000, Nakshatri et al. 1996, Sovak et al. 1997). Two mechanisms have been identified by which NF-κB acts to promote tumours. In tissue cells, that show DNA damages or mutations from toxins, radiation, or free radicals, activated NF-κB turns on genes that reduce apoptosis, and such immortalised cells reproduce in an unregulated manner (Karin and Greten 2005, Maeda et al. 2005, Vakkila and Lotze 2004). Diverse studies show that NF-κB activation can block cell-death pathways (Baldwin 2001). An increased production of cytokines and other growth factors such as IL-2, IL-6, GM-CSF, and CD-40 ligand (Karin et al. 2002), which support growth, replication, and invasion of cancerous cells, is the result of the NF-κB activation in inflammatory cells (Karin and Greten 2005, Becker et al. 2004, Greten et al. 2004). Therefore the NF-κB activation is required to prevent cells from apoptosis induced by TNF and other stimuli. The impact of NF-κB enables cancer cells to outlive non-cancerous cells and multiply, as NF-κB creates an environment that fosters cancerous tissue (Karin and Greten 2005). Additionally NF-κB stimulates the production of inflammatory signals, which support promoted cancer cells to spread to other tissues, locally (invasion) and at a distance (metastasis) (Karin and Greten 2005, Haffner et al. 2006). The suppression of NF-κB therefore could limit the proliferation of cancer cells. (Garg and Aggarwal 2002; Sethi et al. 2008) NF-κB is activated by most oncoproteins through signal transduction cascades (Baldwin 2001). Therefore drugs inhibiting NF-κB could be anti-cancer or cancer-preventive agents (Karin and Greten 2005). For example the inhibition of NF-κB in head and neck squamous cell carcinoma has already shown to inhibit cell survival and tumour growth (Duffey et al. 1999). The combination of NF-κB inhibition with drugs or cytokines, which induce cancer cell death, shows promise in cancer treatment (Li and Verma 2002). Nevertheless, as NF-κB is also involved in healthy immune responses, NF-κB inhibiting drugs should only be used for relatively short periods (during promotion and progression stages of cancer), in combination with chemotherapy or radiation treatments (Karin et al. 2002; Karin and Greten 2005).

"*The development of selective NF-κB inhibitors may represent a promising therapeutic tool to sensitise tumour cells to apoptosis and increase the efficacy of conventional anticancer drugs in a wide spectrum of malignancies*" (Piva et al. 2006).

1.6.3 Specific NF-κB inhibitor Bay-11

Variety of studies with tumour cell lines did reveal the proapoptotic acitivity of Bay 11-7082 and Bay 11-7085 (Nakanishi and Toi 2005). Bay-11 is a specific IKK inhibitor (Nakanishi and Toi 2005), and therefore represses the cytokine induced phosphorylation of I-κBα) and NF-κB activation. Due to the decreased nuclear translocation of NF-κB, the NF-κB activated expression of ICAM-1, VCAM-1, E-selectin, IL-6 and IL-8, decreased in TNFα treated HUVECS (IC_{50}: 5-10μM) (Pierce et al. 1997). The inhibition of I-κBα phosphorylation, celladhesion molecule expression and the stabilisation of I-κBα were found to be irreversible effects (Pierce et al. 1997).

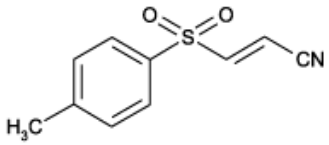


Figure 21: Structure of Bay-11 (Vonach 2010)
Molecular formula: $C_{10}H_9NO_2S$; molecular weight: 207.3 g/Mol

1.7 Inhibitors from natural resources

Marine organisms, bacteria, fungi and plants are screened successfully since many years for their anti-cancer-, anti-inflammatory-, and anti-bacterial-activities. Nowadays about 60% of all drugs used in the western medicine derive from natural compounds (Cragg et al. 2006). In our research we tested the anti-cancer-activities of diverse substances gathered from marine organisms and plants. For exapmle heteronemin is derived from marine sponges, xanthohumol can be isolated from hops, and berberine is a compound of a medicinal plant.

1.7.1 Heteronemin

Marine organisms have been reported to be sources of potential antitubercular agents (Wonganuchitmeta et al. 2004). Marine natural products have recently been recognised as a promising soruce of NF-κB inhibitors (Folmer et al. 2009). According to the study of Folmer et al. (2009) 18% of 263 extracts from marine organisms showed the potential to inhibit TNF-α induced NF-κB activation. Heteronemin is the dominant constituent of extracts of the marine sponge Hyrtios erecta (Crews and Bescansa 1986), and showed already antimycobacterial activity against M. tuberculosis (El Sayed et al. 2000).

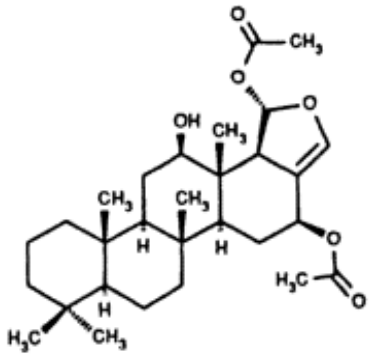


Figure 22: Structure of heteronemin (Crews and Bescansa 1986)
Chemical class: sesterterpene; molecular formula: $C_{29}H_{44}O_6$; molecular weight: 488.66 g/Mol

Additionally, heteronemin shows antitumour activity, which is based on its nature of being:

- antitubercular, cytotoxic, and an inhibitor of tyrosine kinases (Kazlauskas et al. 1976; Wonganuchitmeta et al. 2004; Rateb et al. 2009),
- a farnesyl transferase inhibitor (down-regulation of *ras* pathway) (Ledroit et al. 2004),
- an inhibitor of NF-κB activation (induced by TNFα); and
- an elicitor of apoptosis.

1.7.2 Xanthohumol

Xanthohumol is isolated from hops (*Humulus lupulus L.*), and from the Chinese medicinal plant *Sophoraflavesces Ait* (Naturalchemics 2009). Xanthohumol possesses anticancer and antiproliferative activities in several cancer cell lines such as human breast cancer (MCF-7), colon cancer (HT-29) and ovarian cancer (A-2780) cells (Miranda et al. 1999), down-regulates NF-κB activation by inhibiting its translocation into the nucleus (Colgate et al. 2007), and significantly reduces proliferation of the HCT 116-derived colon cancer cell line through the induction of apoptosis by downregulation of Bcl-2 and activation of the caspase cascade (Pan et al. 2005). Research has demonstrated xanthohumol's anti-inflammatory properties by inhibition of COX-1 and -2 activities (Gerhauser et al. 2002). Furthermore it is antiestrogenic without possessing intrinsic estrogenic potential (Gerhauser et al. 2002). Xanthohumol prevents carcinogenesis in the progression phase by inhibiting DNA synthesis and induction of cell cycle arrest in S phase, apoptosis, and cell differentiation (Gerhauser et al. 2002). Studies showed the ability of xanthohumol to repress both NF-κB and Akt pathways in endothelial cells and interferes with the angiogenic process, including inhibition of endothelial cell invasion and migration, growth, and formation of a tubular network (Albini et al. 2005). Furthermore xanthohumol did already cause cell cycle arrest in the S phase in estrogen receptor-negative mammary adenocarcinoma cell lines (e.g. MDA-MB-435) and therefore might inhibit the carcinogen-

induced and hormone-promoted proliferation at the level of DNA synthesis (Gerhauser et al. 2002).

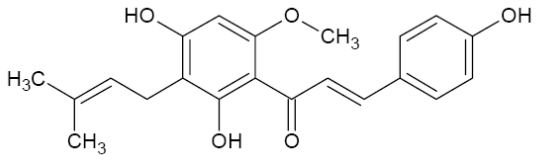


Figure 23: Structure of xanthohumol (Chemical Book 2009)
Chemical class: prenylated flavonoid; molecular formula: $C_{21}H_{22}O_5$; molecular weight: 354.396 g/Mol

1.7.3 Berberine

Berberine is a naturally occurring isoquinoline alkaloid, produced by a number of medicinal plants, such as *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric), *Berberis aquifolium* (Oregon grape), *Berberis lyceum* (Berberidacea) or *Tinospora cordifolia* (Issat et al. 2006), and is used (incl. its salts: berberine chloride, dihydrate, BCD) in the Indian Ayurvedic (Sathyavathi et al. 1987) and Chinese herbal medicine for treatment of e.g. inflammatory diseases (Tsang et al. 2009). Besides berberine is used as a natural yellow dye (colour index 75160), and shows beneath UV-light a strong fluorescence (Tsang et al. 2009).

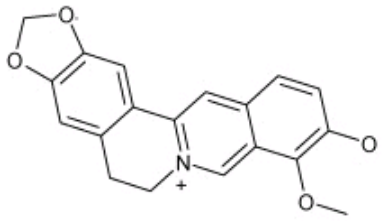


Figure 24: Structure of berberine (Khan et al. 2010)
Chem. class: isoquinoline alkaloid; molecular formula: $C_{20}H_{18}ClNO_4 2H_2O$; molecular weight: 407.85 g/Mol

Berberine exhibits anti-bacterial (Schiller 1995), anti-oxidative (Hwang et al. 2006), anti-inflammatory (Iizuka et al. 2000), and anti-carcinogenic activity (Nishino et al. 1986; Tsang et al. 2009), as it arrests cell growth (Khan et al. 2009; Tsang et al. 2009). Berberine inhibits cell migration and proliferation (cytostatic effect), and induces apoptosis (cytotoxic effect) in several human cancer cell lines (e.g. MCF-7 breast cancer cells, MDA-MB231) (Issat et al. 2006; Khan et al. 2009; Tsang et al. 2009). At high dose (300µM), berberine was localised in the nucleus, which may induces mitotic arrest (Tsang et al. 2009). It inhibits the activator protein 1 (AP-1), a key transcription factor in inflammation and carcinogenesis (Fukada et al. 1999); and COX-2 transcriptional activities (Fukuda et al. 1999a; Fukuda et al. 1999b; Issat et al. 2006). By the decreased production of urokinase-plasminogen activator (u-PA), and MMPs (MMP-2, and -9) through the FAK,

IKK and NF-κB mediated pathways (Ho et al. 2009), berberine shows inhibitory effects on invasion of cancer cells (Issat et al. 2006). As it is reported that MMP-2, -9, and u-PA require the NF-κB protein and AP-1, the inhibitory effect of berberine on NF-κB might be able to explain the suppression of MMP-2 and -9 gene expression (Ho et al. 2009).

Berberine acts as a therapeutic agent showing inhibitory effects on the invasive and metastasis capacity of malignant cells (e.g. tongue squamous cancer, lung cancer cells) (Issat et al. 2006), by preventing the degradation of I-κB, which blocks the translocation of NF-κB into the nucleus, it decreases the protein level of NF-κB (Ho et al. 2009). In prostate carcinoma cells, berberine induced antiproliferative effects, associated with G_1-phase arrest that correlates with the inhibition of expression of cyclin D1, -D2 and -E; Cdk2, Cdk4 and Cdk6; and increased expression of Cdk inhibitory proteins (Mantena et al. 2006). Furthermore, berberine suppressed the mevalonate pathway, which is the source of cellular cholesterol and lipid metabolites used for post-translational modification of cellular proteins, such as lamins, Ras and Rho proteins (Issat et al. 2006). Through this interference with the processing of Ras and Rho proteins, the cytostatic and cytotoxic effects of berberine may be explained (Issat et al. 2006).

1.7.4 GM6001

GM6001 (Galardin/Ilomastat) is a synthetic inhibitor of MMPs (MMP-1, -2, -3, -8, and -9) and prevents the release of TNF-α *in vivo* and *in vitro* (Solorzano et al. 1997). GM6001 is designed as a molecular mimic of MMP substrates, which allows it to enter the active site of MMPs, where it reversibly binds the critical zinc atom (Galardy 1993). GM6001 has shown to suppress the migration of human dermal microvascular endothelial cells (HDMVEC). This MMP-dependent inhibition of migration was associated with increased expression of adhesion proteins like VE-Cadherin. (Akahane et al. 2004) Experiments showed reduced collagen degradation in the presence of GM6001, which may limit the tumour expansion due to physical constraints (Almholt et al. 2008).

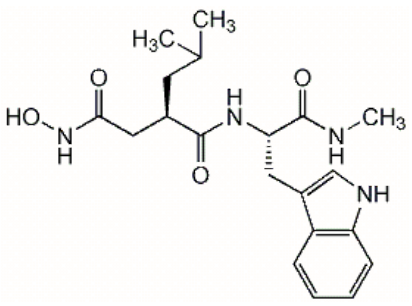


Figure 25: Structure of GM6001/Galardin/Ilomastat
Chemical class: hydroxamin acid; molecualr formula: $C_{20}H_{28}N_4O_4$; molecular weight: 388.47

2 Material and Methods

2.1 Material

2.1.1 Chemicals

Baicalein (EI-106) was purchased from Biomol (Hamburg, Germany). Bay-11-7082 was purchased from Biomol (EI-278) and from Calbiochem (Cat#196870) (Darmstadt, Germany). Xanthohumol was purchased from Naturalchemics (Homburg, Germany).
Heteronemin was supplied by Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (Hôpital Kirchberg, Luxembourg). GM6001 InSolution Cat#364206 was purchased from Calbiochem (Darmstadt, Germany). Berberine chloride dihydrate (purity 98.92 %) was purchased from Phytolab (Vestenbergsreuth, Germany). 12(s)-HETE Cat#34570 was supplied by Cayman Chemical (MI, USA).
For the production of MMP-11 down knock MCF-7^{mock} and MCF-7^{VEGFC} cells, Mission™ lentiviral transduction particles (Prod. Code SHVRS; five clones, IDs: TRCN0000050713, TRCN0000050714, TRCN0000050715, TRCN0000050716, TRCN0000050717), and polibrene (hexadimethrine bromide) Cat#H9268 from Sigma-Aldrich (Munich, Germany) were used. For the gap formation assays LECs were stained with cytotracker green Cat#C2925 purchased from Invitrogen (Karlsruhe, Germany).
Antibodies: Monoclonal antibody CD144 (VE-Cadherin) (PN IM1597) was supplied by Beckman Coulter (CA, USA). The polyclonal rabbit antibody paxillin (H-114) (SC-5574) and the monoclonal mouse α-Tubulin (DM1A) antibody Cat#sc-32293 were purchased from Santa Cruz Biotechnology (CA, USA). Monoclonal mouse antibody Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Cat#9106, monoclonal rabbit p44/42 MAPK (Erk1/2) (137F5) antibody Cat#4695, polyclonal rabbit antibody Phospho-Myosin Light Chain 2 (Ser19) Cat#3671, polyclonal rabbit Myosin Light Chain 2 antibody Cat#3672, monoclonal mouse antibody Phospho-Akt (Ser473) (587F11) Cat#4051, polyclonal rabbit Akt antibody Cat#9272, monoclonal rabbit antibody ROCK-1 (C8F7) Cat#4035, polyclonal rabbit ILK1 Antibody Cat#3862, and polyclonal rabbit MYPT1 antibody Cat#2634 were from Cell Signaling (MA, USA). Monoclonal mouse anti-β-Actin (clone AC-15) Cat#A5441 and monoclonal mouse Anti-Acetylated-Tubulin (clone 6-11B-1) Cat#T6793 were from Sigma-Aldrich (Munich, Germany). The polyclonal rabbit IgG Anti-phospho-MYPT1 (Thr696) Cat#07-251 was purchased from Upstate (NY, USA). The polyclonal rabbit phosphor-specific Actin (Tyr-53) antibody Cat#AP1671 was from ECM

Biosciences (KY, USA). Rabbit anti-S100A4 Cat#A08950 was purchased from Sigma (MO, USA). The polyclonal rabbit anti-mouse and anti-rabbit IgG were from Dako (Glostrup, Denmark). Polyclonal goat ARP2/3 subunit 1B antibody Cat#ab77084 was purchased from Abcam (MA, USA)

Western blotting: PVDF-membrane and the hyperfilms ECL – High performance chemiluminescence's film was purchased from GE-Healthcare (Amersham, Buckinghamshire, UK). Pierce-ECL Western Blotting Substrate Prod #32106 was from Thermo Scientific (Portsmouth, USA).

Real time PCR: The E-selectin Primer (TaqMan Gene Expression Assays Part No.4331182, Assay ID Hs00174057_m1), MMP-11 (stromelysin 3) (Assay ID Hs00171829_m1), and the TaqMan Universal PCR Master mix (No AmpErase UNG; PartNo.4324018) were purchased from Applied Biosystems (Vienna, Austria). RNeasy Mini Kit 50 and QIAshredder 50 (Cat#74104, Cat#79654) were purchased from QIAGEN (Hamburg, Germany). Superscript First Strand Synthesis System Cat#11904-018 was supplied by Invitrogen (Karlsruhe, Germany).

2.1.2 Cell culture

Different cell lines were used for the executed experiments. LECs (telomerase immortalized human lymph endothelial cells – T1S1) were grown in EGM2 MV medium (Clonetics #CC-4147) at 37°C in a humidified atmosphere containing 5% CO_2.

The breast cancer cell lines MCF-7^{mock} and MCF-7^{VEGFC} (VEGFC over expressing cell line) 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), 150µg hygromycin B (Roche #10843555, Vienna, Austria) at 37°C in a humidified atmosphere containing 5% CO_2. The MCF-7^{mock}-and MCF-7^{VEGFC}-MMP-11-knockdown 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), 150µg hygromycin B (Roche #10843555, Vienna, Austria) and 1µg/ml puromycin (Sigma Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 Methods

2.2.1 Gap formation Assay – MCF-7 spheroids-LECs monolayer co-culture

MCF-7 spheroids treated with chemicals: LECs were seeded in two ml EGM2 MV medium into 24 well plates (costar 3524). The MCF-7 cells (~3000 cells) were seeded in 150µl of a mixture of methylcellulose and MEM medium (supplemented with 10% FCS, 1% penicillin/streptomycin, 1% NEAA and 150µg hygromycin B), into a well of a round bottom microtiter plate (96 well) to allow spheroid formation (3000 cells/spheroid, with average diameter of ~ 300µm). After two days incubating the cells at 37°C in a humidified atmosphere containing 5% CO_2, the LECs were stained with cytotracker green (1:5000 dilution, final concentration 2µg/ml; Invitrogen, Molecular Probes #C2925) for one hour at 37°C. The MCF-7 cell spheroids (3000 cells/spheroid) were treated for 30 minutes at 37°C with different concentrations (5-100µM) of the chemical to be tested solved in DMSO. After the staining of the LECs and the 30 minutes treatment of the breast cancer cells, the MCF-7 spheroids were carefully transferred to the 24 well plate containing LECs. After four hours of incubation this MCF-7 spheroids-LEC monolayer co-culture, the gap sizes in the LEC monolayer underneath the MCF-7 spheroids were photographed and measured using an Axiovert (Zeiss) fluorescence microscope, using the FITC filter to visualise cytotracker(green)-stained LECs underneath the spheroids. Gap areas were determined with the Axiovision Re. 4.5 software (Carl Zeiss, Jena, Germany). As negative control MCF-7 spheroids were treated with DMSO. Each experiment was performed in triplicates and for each condition, the gap size under 12 and more spheroids was measured.

MCF-7 spheroids and/or LEC monolayer treated with chemicals: LECs and MCF-7 cells were grown as described above. After the staining of the LECs different settings were used:

a) negative control: MCF-7 spheroids were treated with DMSO for 30 minutes at 37°C, and were transferred onto the untreated LEC monolayer. After four hours of incubation at 37°C the co-culture with its gaps was photographed.
b) MCF-7 spheroid/LEC monolayer co-treatment: MCF-7 spheroids were treated for 30 minutes at 37°C with a certain concentrations of the chemical to be tested, and were transferred onto the untreated LEC monolayer. After four hours of incubation at 37°C the co-culture with its gaps was photographed.

c) MCF-7 spheroid treatment: MCF-7 spheroids were treated for 30 minutes at 37°C with a certain concentrations of the chemical to be tested. Afterwards the spheroids were washed with EGM2 MV medium and were transferred onto the untreated LEC monolayer. After four hours of incubation at 37°C the co-culture with its gaps was photographed.

d) LEC monolayer treatment: LECs were treated for 30 minutes at 37°C with a certain concentration of the chemical to be tested. Afterwards the LEC monolayer was washed with EGM2 MV medium, and untreated MCF-7 spheroids were transferred onto the pretreated LEC monolayer. After four hours of incubation at 37°C the co-culture with its gaps was photographed.

Each experiment was performed in triplicates, and for each condition the gap size under 12 and more spheroids was measured.

2.2.2 Proliferation Assay

To determine the proliferation rate of different cell lines, LECs and the different breast cancer cell lines were seeded into six well plates ($10x10^4$ cells/well), to enter a logarithmic growth phase. The cells were incubated in triplicates for 24, 48, and 72 hours. At each time point the cells were washed with PBS, trypsinised and counted manually with C-chips from Digital Bio (Seoul, Korea). Experiments were done in triplicates.

2.2.3 Apoptose Assay

The different cell lines were seeded into 24 well plates ($5x10^4$ cells/well). The cells were incubated for 24, 48, and 72 hours. At each time point the cells were washed with PBS, trypsinised, and gently suspended in 2ml medium. 100µl of the cell suspension and 5µl of the hoechst (33258) and propidium iodide mixture (200µl hoechst 5µg/ml, 100µl propidium iodide 2µg/ml, 700µl dest. water) were filled in a 96 well plate. After one hour of incubation, the double stained cells were examined under a fluorescence microscope with DAPI filter, photographed, analysed and counted manually. Experiments were done in triplicates.

2.2.4 Lentiviral transduction – MMP-11 knockdown

MCF-7^{mock} and MCF-7^{VEGFC} cells were grown in 24 well plates to 80% confluence in MEM medium supplemented with 10% FCS, 1% penicillin/streptomycin, 1% NEAA and 150µg hygromycin B. To increase the efficiency of the transduction and to prevent repulsion between cancer cells and virus particles (Mission® lentiviral transduction

particles) both, cancer cells and virus particles (five clones), were treated with polibrene® (hexadimethrine bromide) (recommended final concentration 8µg/ml), and the cell-viral particle mixture was put into the centrifuge at 2500 rpm for 90 minutes at 32°C (spin infection).

After 24 hours of incubation time at 37°C the cells were washed with PBS, trypsinised, and seeded in MEM medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% NEAA (without hygromycin) into a six well plate. The next day the medium was replaced by a fresh one containing the appropriate amount of puromycin (parting limit for MCF-7 cells according to previously performed kill curve experiments: 1µg/ml) for the selection of transduced cells. The next day and every 3-4 days the medium was replaced with fresh, puromycin containing medium, until resistant colonies could be identified. To ensure that only transduced cells survive this selection process, a control well with not transduced cells was parallel treated with puromycin containing medium. After the apoptosis of all control cells the selection was completed and the stable transduced cells could be used for further experiments.

2.2.5 RT-PCR

To measure the knockdown efficiency of the previously done lentiviral transduction (MMP-11 knockdown), a reverse transcription polymerase chain reaction (RT-PCR) was performed with the transduced MCF-7^{mock} and MCF-7^{VEGFC} cells (each cell line was transduced with five different lentiviral clones). Untreated MCF-7^{mock}scrambled and MCF-7^{VEGFC}scrambled cells (for control reasons) and the MMP-11 knockdown cells were seeded into 12 well plates (triplicates per cell line and per transduced lentiviral clone). RNA was isolated using the RNeasy Mini Kit 50 and QIAshredder 50 (QIAGEN). With superscript first strand synthesis system (Invitrogen 11904-018) 1µg of the RNA was reverse transcribed (cycler programme: 5 min. at 65°C). The resulting cDNA was amplified using TaqMan universal PCR master mix (Applied Biosystems) (cycler programme: 10 min. at 25°C, 50 min. at 42°C, 15 min. at 70°C, and 20 min. at 37°C) with the MMP-11 primer (Applied Biosystems). Duplicate samples amplified using GADPH primer (glyceraldehyde-3-phosphate dehydrogenasis) (Applied Biosystems) served as internal control. PCR products were analysed with the Abi Prism 7000 sequence detection system (Applied Biosystems), and the relative transcript expression was calculated using the $\Delta\Delta C_T$ method.

2.2.6 Western Blotting

LECs were seeded in 6cm dishes. To determine appropriate treatment time points with 12(S)-HETE, we first conducted time course experiments. Therefore the seeded LECs were incubated with 12(S)-HETE (1µM) for 10 minutes, 30 minutes, one-, two-, and four hours. The control cells stayed untreated. At each time point, the cells were washed twice with PBS and lysed with buffer containing 150mM NaCl, 0.1% triton-X100, 50mM tris pH 8.0, 1mM phenylmethyl-sulfonylfluorid (PMSF) and protease inhibitor cocktail (PIC). The lysates were centrifuged at 12,000rpm for 20 minutes at 4°C, and the supernatants were stored at -20°C.

The proteins of each lysate were separated by sodium dodecyl sulfate polyacrylamide Gel electrophoresis (SDS-PAGE) and electro-transferred onto Hybond PVDF-membranes (Amersham, Buckinghamshire, UK). The sample loading was controlled by staining the membranes with Ponceau S.

Afterwards, the membranes were washed with PBS (phosphate buffered saline; pH: 7.2) or TBS (tris buffered saline; pH: 7.6) and blocked in blocking solution (5% non-fat dry milk in TBS containing 0.1% tween-20 or in PBS containing 0.5% tween-20) for one hour at room temperature. After the blocking procedure the membranes were again washed with PBS/T or TBS/T (phosphate buffered saline/tween-20, or tris buffered saline/tween-20), and incubated with the first antibody (in blocking solution; dilution 1:500 – 1:1000) at 4°C overnight with gentle shaking. Subsequently the membranes were again washed with PBS/T or TBS/T, and incubated for one hour at room temperature with the second antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG; dilution 1:2000).

After the fourth washing procedure, the membranes were treated with ECL detection Kit (for ~1min.), the thus released chemiluminescence was subsequently captured when exposing the membranes to Amersham hyperfilms.

2.2.7 Statistical analysis

All performed experiments (RT-PCR, gap formation assay, apoptose- and proliferation assays) were analysed using Excel 2003 software, and Prism 5 software package (GraphPad, CA, USA), whereby significance was determined by student t-test (significance $p < 0.05$).

3 Results

3.1 Stimulation of LEC monolayer by 12(S)-HETE (Time course experiments)

To examine the impact of 12(S)-HETE on LECs, we treated LECs with 1µM synthetic 12(S)-HETE in time course experiments (10 minutes to 4 hours), which mimics the 3D co-culture system of MCF-7 spheroids and LECs, and further the invading of breast cancer cells to the lymphatic vasculature (Kerjaschki et al. 2010; Madlener et al. 2010). Figure 26-29 show the results of these time course experiments with 12(S)-HETE stimulated LECs.
VE-Cadherin is necessary for cell-cell adhesion and it is a marker for an epithelial and immotile phenotype. Stimulation of endothelial cells with 12(S)-HETE induces endothelial cell retraction (Honn et al. 1994a), thus VE-Cadherin expression showed in our experiments a transient downregulation (Figure 26). The phosphorylation of Erk1/2 decreased with time, showing a slight increase after four hours of incubation with 12(S)-HETE (Figure 26). Infact, we expected that Erk1/2 phosphorylation would increase upon 12(S)-HETE treatment. However, the dephosphorylation was consistent throughout the experiments, yet we can not interprete this effect.

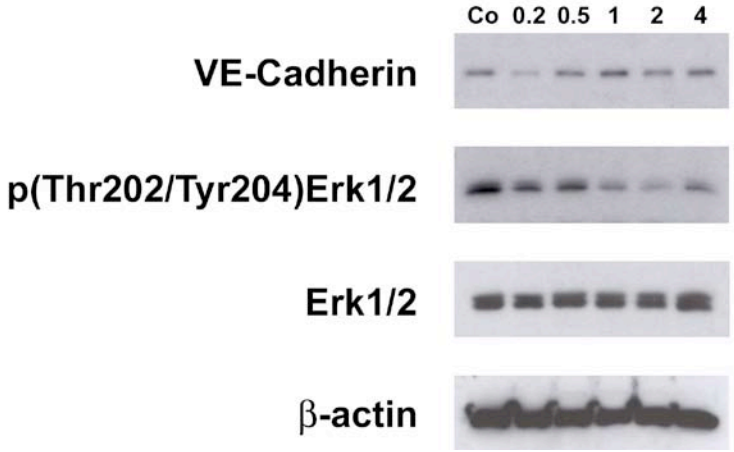


Figure 26: Time course with 12(S)-HETE treated LECs
LEC monolayers were incubated with 1µM synthetic 12(S)-HETE. After 10min, 30min, 1hour, 2hours, and 4hours cells were lysed and proteins analysed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by β-actin analysis.

Paxillin is a focal adhesion phosphoprotein, and is therefore necessary for the cell-ECM contact. In our experiment, the paxillin expression shows an increase after two hours of incubation LECs with 12(S)-HETE (Figure 27). Paxillin is associated with a mobile phenotype. Therefore our data indicate that cell motility was increased. S100A is a marker for the mesenchymal phenotype of LECs (Zeisberg and Neilson 2009). Since the treatment

of LECs with 12(S)-HETE induces the S100A expression after 30 minutes of incubation time (Figure 27) LECs acquired a more mesenchymal and mobile state. Tubulin is one major component of the cytoskeleton, and the acetylation of α-tubulin may play a major role in the differentiation of microtubule structure and function (Piperno and Fuller 1985; Weisenber 1981). Our results show that 12(S)-HETE has no impact on its expression and therefore on the structure of microtubules (Figure 27).

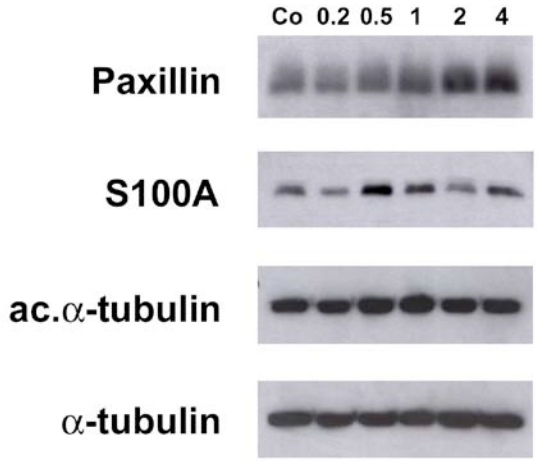


Figure 27: Time course with 12(S)-HETE treated LECs
LEC monolayers were incubated with 1µM synthetic 12(S)-HETE. After 10min, 30min, 1hour, 2hours, and 4hours cells were lysed and proteins analysed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by α-tubulin analyisis.

The Arp2/3 activity regulates the mesenchymal invasion, whereas ROCK-1 is crucial for amoeboid invasion (Paulitschke et al. 2009). The western blots of 12(S)-HETE stimulated LECs show a slight increase in expression of ROCK-1 and Arp2/3 (Figure 28), indicating the mobile phenotype of the retracted LECs.

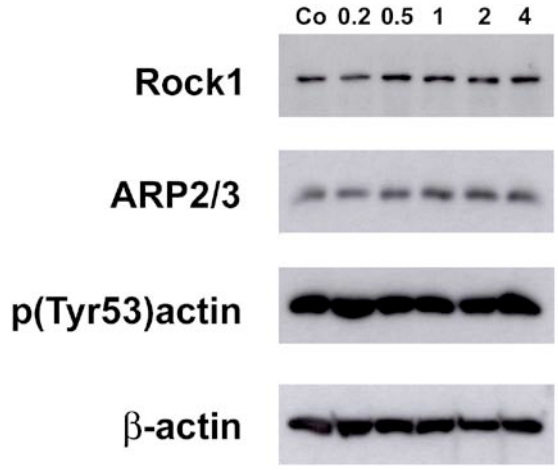


Figure 28: Time course with 12(S)-HETE treated LECs (Actinpathway)
LEC monolayers were incubated with 1µM synthetic 12(S)-HETE. After 10min, 30min, 1hour, 2hours, and 4hours cells were lysed and proteins analysed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by β-actin analysis.

Kerjaschki et al. (2010) could localise the phosphorylated light chain of myosin in LECs at the rim of the "gap", indicating the mobile phenotype in LECs. Our immunoblots show an increase in the expression of phosphorylated MLC2 and MYPT1 after incubation of LECs with 12(S)-HETE for one hour (Figure 29).

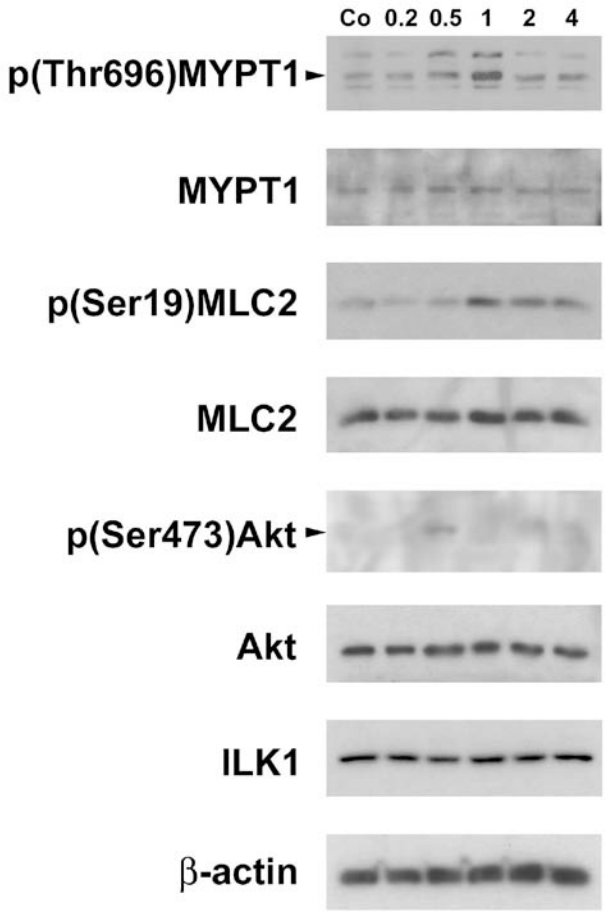


Figure 29: Time course with 12(S)-HETE treated LECs
LEC monolayers were incubated with 1µM synthetic 12(S)-HETE. After 10min, 30min, 1hour, 2hours, and 4hours cells were lysed and proteins analysed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by β-actin analysis.

The phosphorylation of Mypt1 at the rim of the "gap" (Kerjaschki et al. 2010) leads to its inhibition and the cytoskeletal reorganisation (Birukova et al. 2004a; Birukova et al. 2004b). Mypt shows a mitosis-specific phosphorylation and that the phosphorylation is reversed during cytokinesis (Totsukawa et al. 1999). In smooth muscle, myosin light chain kinase (MLCK) phosphorylates MLC2 at Thr18 and Ser19 (Ikebe and Hartshorne 1985), which is correlated with myosin ATPase activity and smooth muscle contraction (Tan et al. 1992). Akt is an important component in migratory and prosurvival signalling pathways (Franke et al. 1997; Burgering and Coffer 1995; Franke et al. 1995; Albini et al. 2005).

Whereas ILKs couple integrins and growth factors to activate pathways involved in cell survival, cell cycle control, cell-cell contact and cell motility (Wu and Dedhar 2001). Additionally ILK is a key component of cell–ECM adhesion structures (Wu and Dedhar 2000). Our results show barely detectable levels of phosphorylated Akt, with a slight increase after an incubation of LECs with 12(S)-HETE for 30 minutes (Figure 29). The expression of ILK does not seem to be effected by the treatment of LECs with synthetic 12(S)-HETE (Figure 29).

Our results indicate a 12(S)-HETE induced the mobile, mesenchymal, amoeboid phenotype in LECs (Table 4), correlating with the already observed formation of circular gaps in the LEC monolayer underneath MCF-7 spheroids (Figure 9; Kerjaschki et al. 2010; Madlener et al. 2010).

3.2 LOX inhibitor baicalein

To proof the LOX inhibitory function of baicalein (Li-Weber 2009), we performed control western blots by inhibiting LECs with baicalein (100µM) and then stimulating them with 12(S)-HETE. The results (Figure 30) show that baicalein does not influence the protein expression pattern in LECs. This clearly demonstrates that baicalein, which inhibits LOX, did not affect the response to 12(S)-HETE in LECs and therefore the effect of baicalein involves only the gap-forming mechanisms in MCF-7 spheroids, but not in LECs.

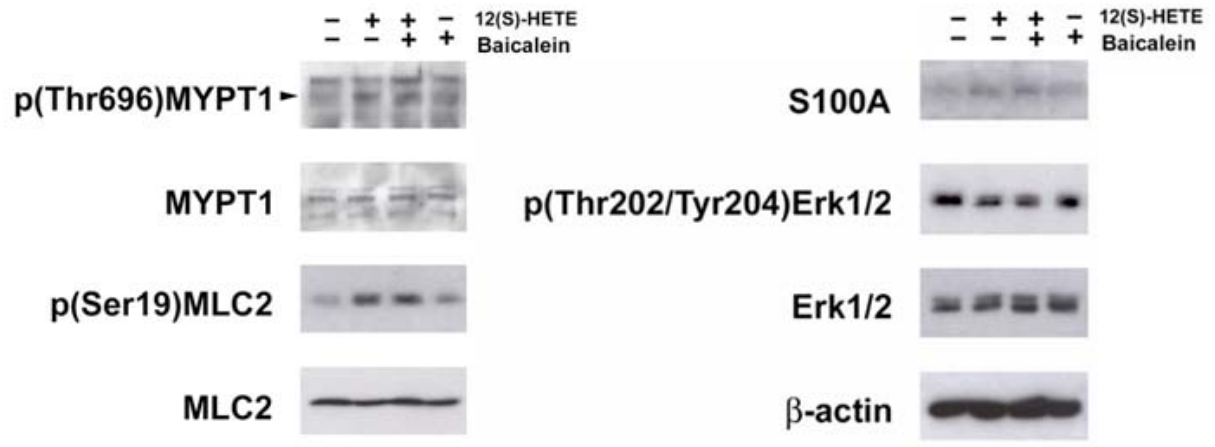


Figure 30: Analysis of baicalein inhibition
LECs were inhibited with 100µM baicalein and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by β-actin analysis.

3.3 NF-κB inhibition

The deregulation of NF-κB is associated with cancer development and inflammatory diseases (Folmer et al. 2009). NF-κB shows the ability to promote oncogenesis and cancer therapy resistance (as it is activated in cancer cells by several chemotherapies and by radiation; Baldwin 2001) through the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis and suppression of apoptosis (Orlowski and Baldwin 2002). NF-κB is activated in a variety of tumour cell lines grown in vitro (Baldwin 2001), e.g the NF-κB heterodimer p50-p65 has been shown to be activated in breast cancer cell lines and in some breast tumours (Sovak et al. 1997).

Targeting NF-κB in cancer can be realised through monotherapies that block different constituents in the NF-κB signal-transduction pathway. Thus the NF-κB nuclear translocation can be inhibited, through inhibition of I-κB degradation, by proteosome-, or IKK interaction inhibition (Orlowski and Baldwin 2002).

3.3.1 Bay-11

In our research, we performed gap formation assays, incubating LEC monolayer with bay-11-pretreated MCF-7 cell spheroids (Figure 31). According to the results an IC_{50} value of about 10µM bay-11 was observed. Additionally the experiments indicated the loss of attachment of cancer spheroids to the underlaying LEC monolayer at higher concentrations of bay-11 (>15µM), which let us assume that bay-11 inhibits a mechanism that is necessary for the adhesion of MCF-7 cells to LECs. The treatment with bay-11 provoked additionally a so called “dispersed” phenotype in the underlaying LECs (Figure 32 b, d; Figure 33 b), suggesting that LECs lost the contact to adjacent LECs but failed to move apart.

These results tempted us to investigate wether these effects were due to the inhibition of NF-κB in the MCF-7 cell spheroids or in the LEC monolayer. Therefore an additional gap formation assay was performed including four different settings: Serving as controls, untreated breast cancer spheroids were placed onto untreated LEC monolayers; 15µM bay-11-pretreated spheroids were placed onto untreated LEC monolayers; 15µM bay-11-pretreated spheroids were washed with PBS and then placed onto untreated LEC monolayers; and in the last setting untreated spheroids were placed onto 15µM bay-11-pretreated and PBS-washed LEC monolayers (Figure 31 and Figure 32).

As explored before bay-11 is an irreversible inhibitor and the PBS wash aimed to prevent a "wash over" of bay-11 from the spheroids to the LEC layer and vice versa. This way, the contribution of NF-κB to gap formation of MCF-7 spheroids or LECs could be discriminated. The results of this second bay-11 gap formation assay confirmed the irreversible inhibition of bay-11; because the PBS-unwashed bay-11-pretreated spheroids, and the bay-11-pretreated and PBS-washed spheroids provoked a similar decrease in gap sizes (Figure 31). Furthermore bay-11-pretreated and PBS-washed, versus bay-11-pretreated and PBS-unwashed spheroids showed a similar proportion of spheroids not attached to LEC monolayer. Nevertheless these two settings show an important difference, as the LECs in the first setting (in which bay-11-pretreated spheroids were placed PBS-unwashed onto LEC monolayer) showed the previously observed "dispersed" phenotype (Figure 32 b.), whereas the LECs in the setting with PBS-washed bay-11-pretreated spheroids looked normally (Figure 32 c.). Also in the last setting (bay-11-pretreated and PBS-washed LEC monolayer before untreated spheroids were placed ontop) the "dispersed" phenotype could be observed (Figure 32 d.), indicating that LECs lost their cell-cell contacts but did not migrate centrifugally as a result of direct contact with bay-11. Therefore, the lack of LECs migration provoked the dispersed pattern of LEC distribution. In contrast to the settings where spheroids were treated with bay-11, all spheroids were attached to the LEC monolayer in the last setting, in which LECs were treated with bay-11 and PBS-washed before bay-11-untreated spheroids were placed onto them.

Due to the "dispersed" phenotype observed when LECs were pretreated with bay-11 (Figure 32 d.), measuring the gap sizes underneath the spheroids was rather difficult. As LECs lost their cell-cell contacts "gaps" were observed, which occurred because of this lost cell-cell contact and therefore affected the gathered data (see Figure 31 right diagram: "LECs + Bay-11 15µM + PBS washed; and Figure 32 d); but as also the LEC's motility was reduced, the recognisable gaps were smaller than expected (Figure 31). Figure 33 shows that the size of LECs decreased dramatic when getting in contact with bay-11 (bay-11-untreated LECs' average length 53.25µm, and 15µM bay-11-pretreated LECs' average length: 36.25µm).

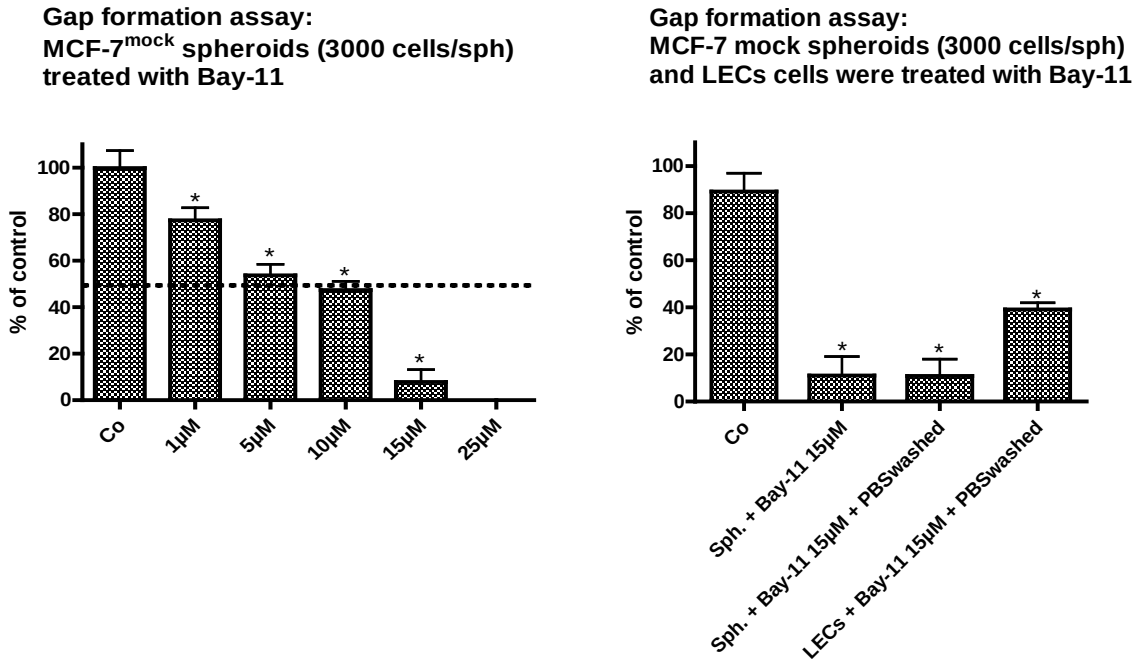


Figure 31: Impact of bay-11 on gap sizes in LECs induced by MCF-7 cell spheroids
Left figure: LEC monolayers were incubated for 4h at 37°C with bay-11 pretreated MCF-7^{mock} spheroids (3000 cells/sph.). MCF-7^{mock} spheroids were pretreated 30 min. with 1µM, 5µM, 10µM, 15µM, and 25µM bay-11. A share of spheroids treated with > 15µM bay-11 lost their attachment to the underlaying LEC monolayer. At a concentration of 25µM bay-11 all spheroids were not attached, causing the diminished gap size indicated in the figure. For control reasons gap sizes in LEC monolayer incubated with untreated MCF-7 spheroids were measured. ($p < 0.0346$) **Right figure**: To investigate the impact of bay-11 on cancer cells and on LECs a gap formation assay was performed including four settings. Untreated breast cancer spheroids were seeded onto LEC monolayer (controls); 15µM bay-11-pretreated spheroids were seeded onto LEC monolayer; 15µM bay-11-pretreated spheroids were PBS-washed and then seeded onto LEC monolayer; and untreated spheroids were seeded onto 15µM bay-11-pretreated and washed LEC monolayer. ($p < 0.0001$)

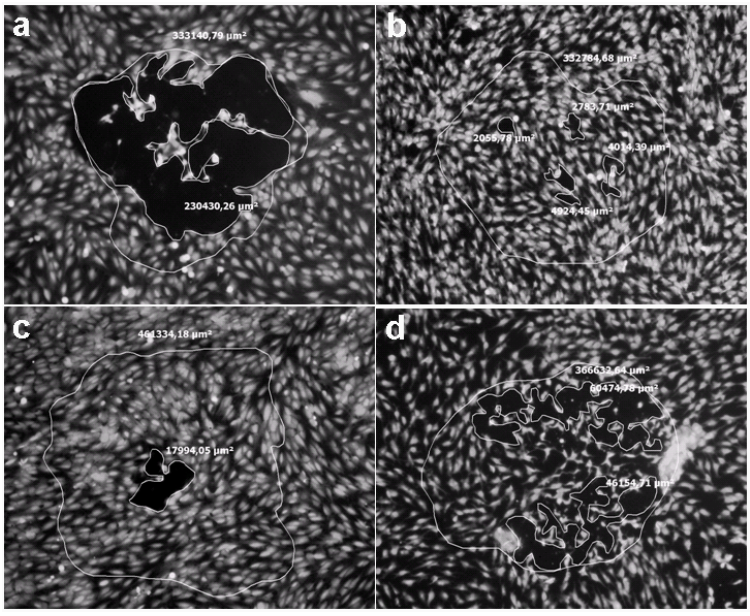


Figure 32: Gap formation assay with bay-11
a. Untreated MCF-7 spheroids were placed onto untreated LEC monolayer. **b**. MCF-7 spheroids were incubated for 30 minutes with bay-11 (15µM) and afterwards placed onto untreated LEC monolayer. **c**. MCF-7 spheroids were incubated for 30 minutes with bay-11 (15µM), afterwards washed with PBS, and placed onto untreated LEC monolayers. **d**. LEC monolayers were pretreated with bay-11 (15µM) for 30 minutes and afterwards washed with PBS. Untreated MCF-7 spheroids were placed onto the pretreated and PBS-washed LEC monolayers. Pictures were taken after 4 h of incubating the co-culture at 37°C.

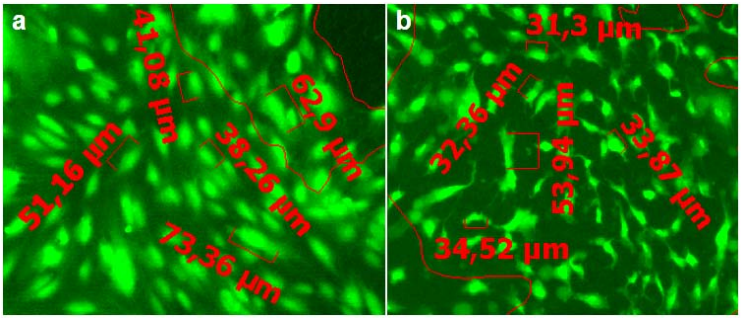


Figure 33: Lost cell-cell contact in LEC monolayer due to bay-11
a. Untreated LEC monolayer stained with celltracker green underneath an untreated MCF-7 spheroid. Average LEC length: 53.25 µm (n=15); **b**. LEC monolayer was pretreated for 30 minutes with bay-11 (15µM) and washed afterwards with PBS. Untreated MCF-7 spheroids were placed on the bay-11-pretreated LEC monolayers. Average LEC length: 36.25 µm (n=15); LECs were stained with cyto-tracker tagged in green. Pictures were taken after 4 h of incubating the co-culture at 37°C, and the length of LECs was measured with Axiovision software.

To investigate the previously observed impact of bay-11 on LECs ("dispersed" phenotype), we performed western blots, examining the levels of different proteins involved in cell mobility. To simplify the MCF-7 spheroids/LEC monolayer co-culture system, we treated LEC monolayer with 10µM bay-11, and afterwards with 1µM 12(S)-HETE to mimic the presence of MCF-7 spheroids (the factor provoking LEC retraction was identified as 12(S)-HETE which is secreted by MCF-7 spheroids). The results of the blots are shown underneath in Figure 34. Treating LEC monolayers with 12(S)-HETE results in an increase of the S100A protein level, whereas the addition of bay-11 leads to a decrease its expression (Figure 34).

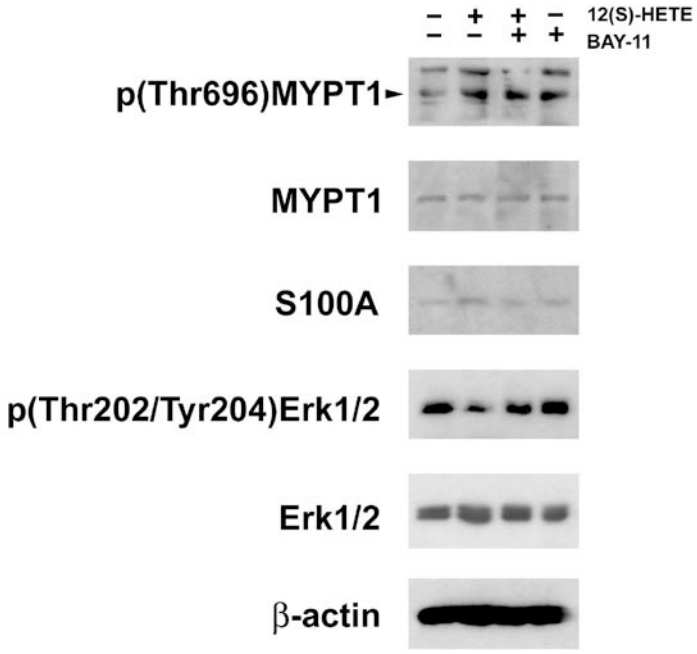


Figure 34: Analysis of bay-11 inhibition
LECs were inhibited with 10µM bay-11 and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. By β-actin analysis the sample loading was controlled.

The inhibition of bay-11 had two principally distinct effects: 1) The prevention of the adhesion of MCF-7 spheroids to LECs (at concentration >15µM), showing that MCF-7-LEC contact was mediated by NF-κB regulated adhesion molecules. 2) The inhibition of LEC migration induced by 12(S)-HETE which was secreted by MCF-7 spheroids and cause LEC gap formation underneath MCF-7 spheroids. Additionally the treatment with bay-11 prevented the 12(S)-HETE-induced increase of S100A, indicating the inhibition of the LECs mobility through bay-11, which correlated with NF-κB regulation of S100A.

Bay-11 and Baicalein – additive gap inhibition

The aim in drug development is to reach the best anti-cancer effect at the lowest possible concentration. This aim can either be reached through a highly effective drug that can be used at such a low concentration that only little sideeffects are observed, or through the co-treatment with different drugs at low level. In our research we examined the cooperative ability of bay-11 and baicalein. As each substance is believed to inhibit another pathway in cancer development, the treatment with both substances may increase the effectiveness. In order to investigate the cooperative effect of these substances we performed gap formation assays, whereby the results indicate an additive inhibition of these two substances. The treatment of MCF-7 spheroids with baicalein (100µM) or bay-11 (5µM, and 10µM) showed a decrease in gap sizes in LEC monolayer underneath the treated spheroids of about 30-40%. Whereas the simultaneous spheroid treatment with both substances (5µM bay-11 + 100µM baicalein; 10µM bay-11 + 100µM baicalein) resulted in a decrease of the LEC monolayer gaps induced by the treated spheroids by about 70-80% (Figure 35).

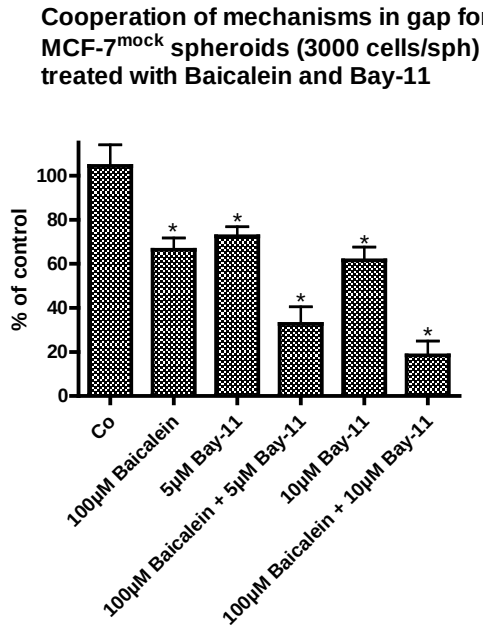


Figure 35: Cooperation of mechanism in gap formation (bay-11 and baicalein)
MCF-7 spheroids were treated for 30 minutes with bay-11 (10µM) and/or baicalein (100µM), and afterwards placed onto untreated LEC monolayers. This co-culture was incubated for 4 h at 37°C. ($p < 0.0042$)

Baicalein is a bonafide LOX inhibitor (Kerjaschki et al. 2010; Li-Weber 2009), whereas bay-11 is a NF-κB inhibitor. The results of the gap formation assays (Figure 35) are consistent with an additive inhibition as two independent mechanisms triggering gap formation are hit, and this indicates that bay-11 influences another pathway than baicalein.

3.3.2 Heteronemin

Since heteronemin was reported to inhibit NF-κB induction (Ledroit et al. 2004) this compound was tested in the gap formation assay. To investigate the dose dependent activity of heteronemin, concentrations from 1µM to 100µM were used. It was found that a 50% reduction (IC_{50}) in gap sizes was achieved at a concentration of 5µM heteronemin (Figure 36, left figure; Figure 37). In contrast to experiments with bay-11, the spheroid treatment with high heteronemin concentrations did not prevent the attachment of MCF-7 spheroids to LECs. Heteronemin concentrations of > 25µM provoked a similar "dispersed" LEC phenotype as observed with bay-11, indicating the lost cell-cell contact of LECs and inhibited migration (Figure 37 d). To examine wether the inhibition in gap formation by heteronemin-pretreated MCF-7 spheroids was reversible or not, we performed another gap formation assay with four different settings (analogans to the experiments using bay-11): Serving as controls, untreated breast cancer spheroids were placed onto untreated LEC monolayers; 5µM heteronemin-pretreated spheroids were placed onto untreated LEC monolayers; 5µM heteronemin-pretreated spheroids were washed with PBS and then

placed onto untreated LEC monolayers; and in the last setting untreated spheroids were placed onto 5µM heteronemin-pretreated and PBS-washed LEC monolayers (Figure 36, right diagram). The results of this second heteronemin gap formation experiment indicate an irreversible inhibition of heteronemin, as the induced gap sizes were similar in these two settings in which the spheroids were treated with heteronemin (+/- washed with PBS before placing them onto LEC monolayers; Figure 36, right diagram). When LECs were pretreated with 5µM heteronemin and washed with PBS before untreated spheroids were placed onto them, the induced gaps underneath the spheroids were about 50% bigger than the gaps induced by heteronemin-pretreated spheroids (Figure 36, right diagram; Figure 38).

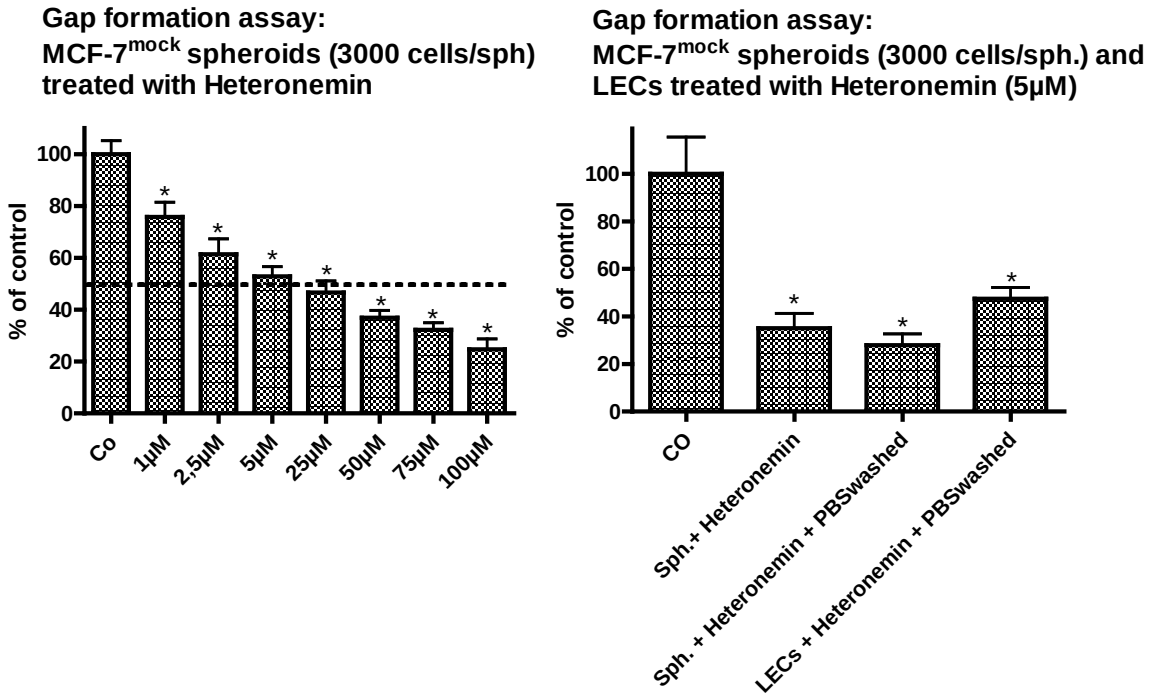


Figure 36: Impact of heteronemin on gap sizes in LECs induced by MCF-7 cell spheroids
Left figure: MCF-7^{mock} spheroids were pretreated 30 minutes with 1µM, 2.5µM, 5µM, 25µM, 50µM, 75µM, and 100µM heteronemin. LEC monolayers were incubated for 4h with heteronemin-pretreated MCF-7^{mock} cell spheroids (3000 cells/sph.). For control reasons gap sizes in LEC monolayer incubated with untreated MCF-7 spheroids were measured. ($p < 0.0055$) **Right figure**: To investigate the impact of heteronemin on cancer cells and on LECs, a gap formation assay was performed including four settings. Untreated breast cancer spheroids were placed onto LEC monolayer, serving as controls; 5µM heteronemin-pretreated spheroids were placed onto LEC monolayer; 5µM heteronemin-pretreated spheroids were washed with PBS and then placed onto LEC monolayer; and in the last setting untreated spheroids were placed onto 5µM heteronemin-pretreated and PBS-washed LEC monolayer. ($p < 0.0123$)

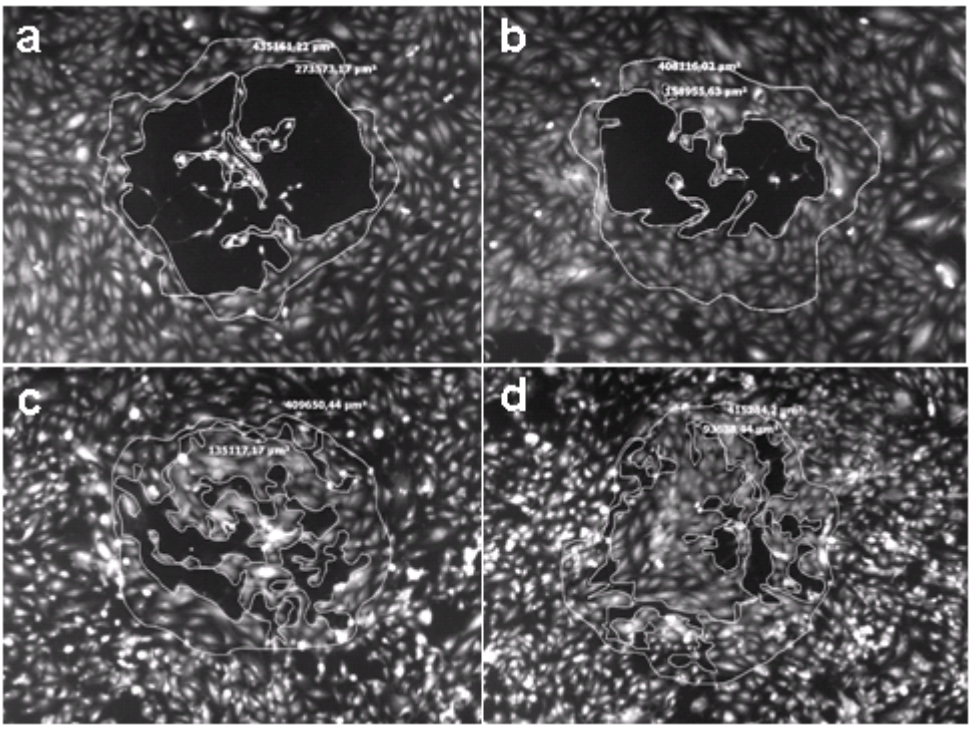


Figure 37: Gap formation assay with heteronemin
a. Untreated MCF-7 spheroids were placed onto untreated LEC monolayer. **b**. MCF-7 spheroids were incubated for 30 minutes with heteronemin (5µM) and afterwards placed onto untreated LEC monolayer. **c**. MCF-7 spheroids were incubated for 30 minutes with heteronemin (25µM) and afterwards placed onto untreated LEC monolayer. **d.** MCF-7 spheroids were incubated for 30 minutes with heteronemin (50µM) and afterwards placed onto untreated LEC monolayer. Pictures were taken after 4 h of incubating the co-culture at 37°C.

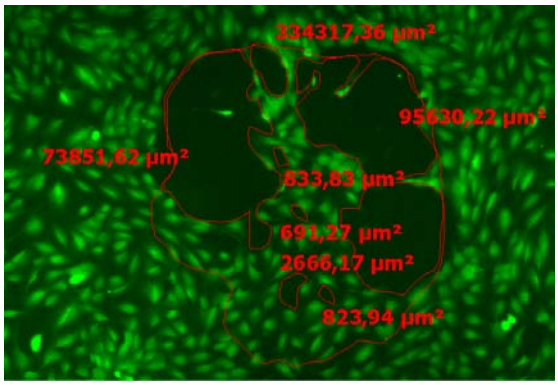


Figure 38: LECs pretreated with heteronemin (5µM)
LEC monolayers (stained with cyto-tracker tagged in green) were pretreated with heteronemin (5µM) for 30 minutes and washed with PBS, before untreated MCF-7 spheroids were placed onto the pretreated LEC monolayer. Picture was taken after 4 h of incubating the co-culture at 37°.

To further examine the impact of heteronemin on LECs, we performed western blots by treating LEC monolayers with heteronemin (5µM) and/or stimulating LECs with 12(S)-HETE (1µM). The level of phosphorylated MYPT1 shows a slight increase, whereas the expression levels of pErk1/2 and paxillin (marker of adhesion sites) show a drastic decrease (Figure 39) when LECs were treated with heteronemin.

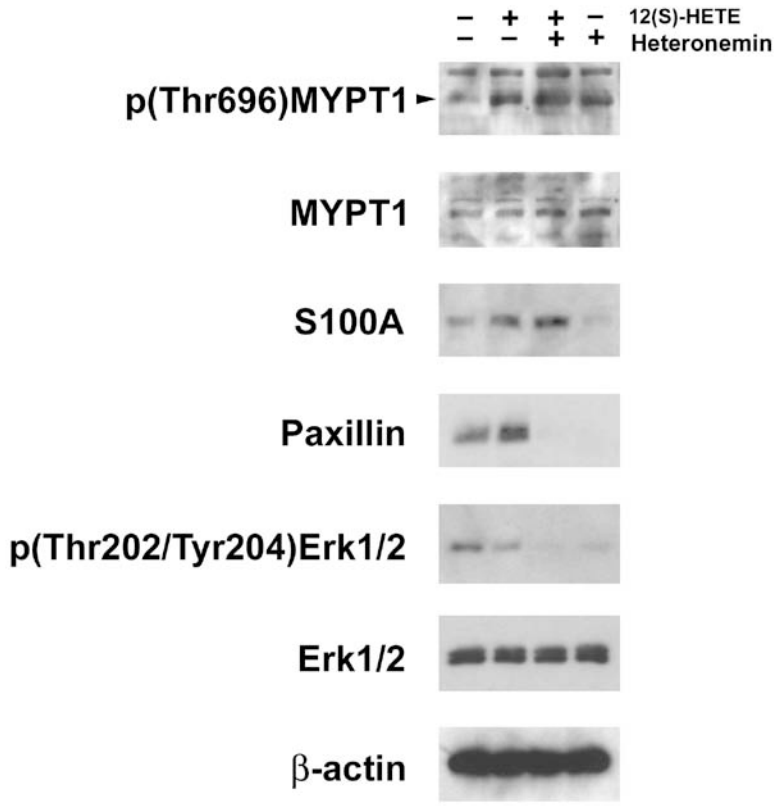


Figure 39: Analysis of heteronemin inhibition
LECs were inhibited with 5µM heteronemin and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. By β-actin analysis the sample loading was controlled.

Heteronemin showed at high concentrations an impact on the cell-cell contact between LECs, indicating that heteronemin inhibited NF-κB dependent mechanisms; whereas at low concentrations another mechanism(s) became inhibited which was responsible for the antimetastatic property. This could be related to the complete dephosphorylation of Erk1/2 and the complete inhibition of paxillin expression that is involved in cell migration. The levels of other mobility proteins (Figure 39) indicate a change in the actin-myosin interaction.

Heteronemin and Baicalein – no additive inhibition

To invetigate the cooperative impact of heteronemin, we performed gap formation assays with heteronemin and baicalein. MCF-7 cell spheroids were pretreated with 2.5µM and 5µM heteronemin and/or 100µM baicalein, before they were placed onto untreated LEC-monolayers (Figure 40). The treatment of spheroids with heteronemin (2.5µM and 5µM) alone decreased the induced gaps by 50-70%. The additional treatment of the MCF-7 spheroids with baicalein (100µM) did not further decrease the gap induction (Figure 40).

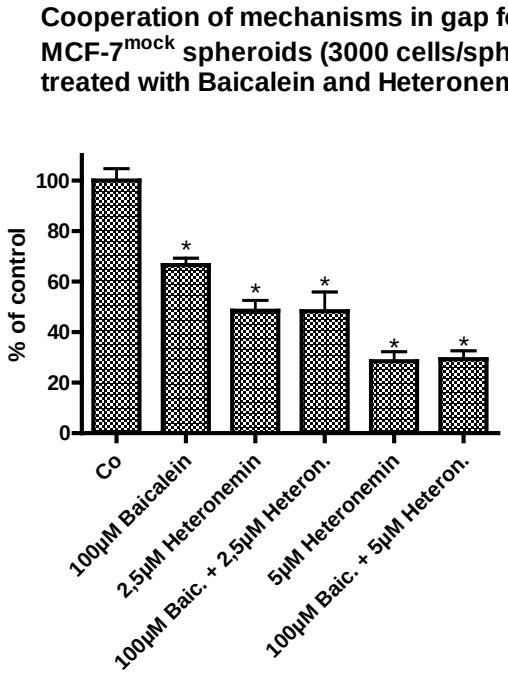


Figure 40: Cooperation of mechanism in gap formation (heteronemin and baicalein)
MCF-7 spheroids were treated for 30 minutes with heteronemin (2.5µM and 5µM) and/or baicalein (100µM), and afterwards placed onto untreated LEC monolayers. This co-culture was incubated for 4 h at 37°C. ($p < 0.0001$)

3.3.3 Xanthohumol

To examine the dose dependent effect of xanthohumol we performed again gap formation assays, by seeding MCF-7 spheroids, which were pretreated with 5-120µM xanthohumol for 30 minutes, onto untreated LEC monolayer. After four hours of incubating this co-culture the results indicated a reduction in gap sizes by about 50% at a concentration of 5µM xanthohumol (Figure 41, and Figure 42). At concentrations below 25µM of xanthohumol all spheroids were attached to the underlaying LEC monolayer, whereby at higher concentrations (50µM and 75µM) all spheroids lost their contact to LECs and therefore no gaps could be induced. In such high concentration also an abnormal LEC phenotype could be observed (similar to the "dispersed" phenotype), which increased with higher concentrations of xanthohumol (Figure 41 c).

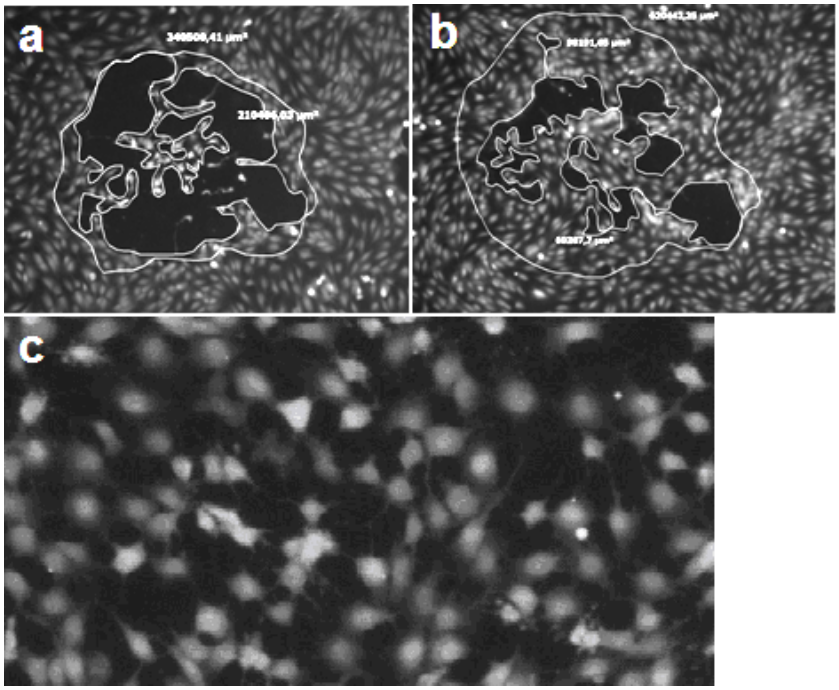


Figure 41: Gap induction by xanthohumol-pretreated MCF-7 spheroids
a. Untreated MCF-7 spheroids were placed onto untreated LEC monolayer. **b**. MCF-7 spheroids were treated with 5µM xanthohumol for 30 minutes, and then placed onto untreated LEC monolayer. **c**. Zoom of the LECs underneath MCF-7 spheroids treated with 120µM xanthohumol for 30 minutes, and then placed onto untreated LEC monolayer. As spheroids treated with >50µM xanthohumol did not attach to the underlaying LEC monolayer, no gaps could be observed underneath the spheroids. Pictures were taken after 4h incubating the co-cultures at 37°C.

To compare the impact of xanthohumol on MCF-7 spheroids to another cancer cell line, we performed another gap formation assay with HTB-26 (MDA-MB-231) breast adenocarcinoma cells (5000 cells/spheroid) instead of MCF-7 cells. HTB-26 cells were pretreated with xanthohumol (50µM, 75µM, and 100µM) or bay-11 (10µM) for 30 minutes before they were placed onto untreated LEC monolayers. The results of this second xanthohumol gap formation experiment show, that xanthohumol affects HTB-26 cell spheroids at much higher concentrations (50% gap inhibition with 100µM xanthohumol) compared to MCF-7 cell spheroids (50% gap inhibition with 5µM xanthohumol) (Figure 42), whereas the inhibitory effect of bay-11 seems to be similar in both cancer cell lines (IC_{50} ~ 10µM). This implicates, that either the primary activity of xanthohumol does not inhibit NF-κB in a way that is comparable to bay-11, or that the intracellular concentration of xanthohumol is lower in HTB-26 cells than in MCF-7 cells i.e. due to mechanisms that pump the drug out of the cell.

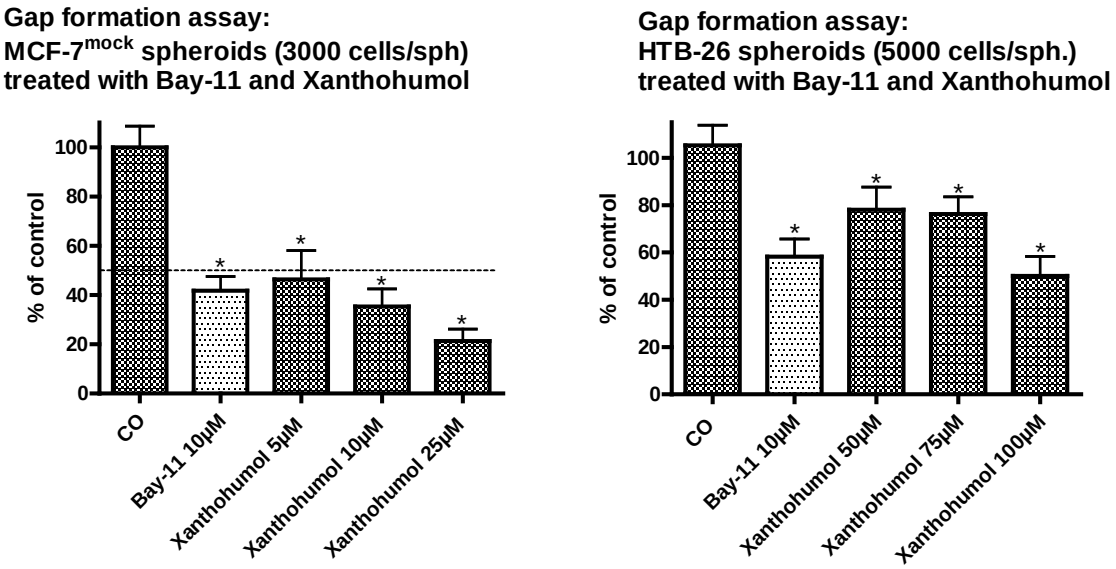


Figure 42: Impact of xanthohumol on gap sizes in LECs induced by MCF-7 cell (left figure) and HTB-26 (right figure) spheroids
Left figure: MCF-7 spheroids were treated with 5µM, 10µM, and 25µM xanthohumol for 30 minutes, before they were placed onto untreated LEC monolayers. For control reasons untreated spheroids were placed onto untreated LEC monolayer. To compare the gap size impact of bay-11 and xanthohumol, MCF-7 spheroids were treated with 10µM bay-11 for 30 minutes and then placed onto untreated LEC monolayers. After 4h of incubation the co-culture at 37°C gap sizes were measured. ($p < 0.0021$)
Right figure: HTB-26 cell spheroids were treated with 50µM, 75µM, and 100µM xanthohumol, and 10µM bay-11 for 30 minutes, before they were placed onto untreated LEC monolayers. For control reasons untreated HTB-26 spheroids were placed onto untreated LEC monolayer. After 4 h of incubation the co-culture at 37°C gap sizes were measured. ($p < 0.0475$)

In order to examine the effect of xanthohumol on LECs, we performed western blots, whereby LECs were treated with 12(S)-HETE in order to mimic the presence of breast cancer spheroids, and/or xanthohumol (25µM). The results are shown in Figure 43, indicating a loss in phosphorylated MYPT1 expression in LECs when treated with 12(S)-HETE and xanthohumol. Furthermore a drastic decrease of the phosphorylated MLC2 level after xanthohumol treatment could be observed. The expression levels of S100A and paxillin decrease as well. All these responses indicated that xanthohumol is a strong inhibitor of LEC motility. In contrast to bay-11 and heteronemin, xanthohumol increases the level of phosphorylated Erk1/2 in LECs (Figure 43).

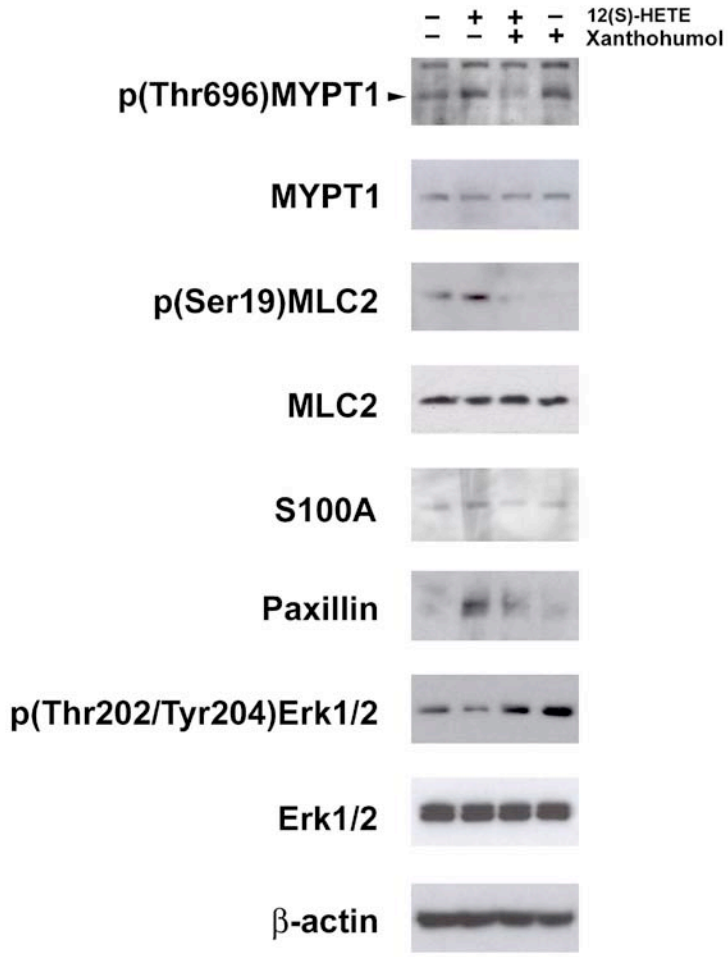


Figure 43: Analysis of xanthohumol inhibition
LECs were inhibited with 25µM xanthohumol and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. By β-actin analysis the sample loading was controlled.

3.3.4 Additional Rho/Rac inhibition

The familiy of Rho GTPases are small signalling G proteins that regulate many aspects of intracellular actin dynamics (Boureux et al. 2007; Bustelo et al. 2007). There are three main classes of Rho GTPases – Rac1, Cdc42, and RhoA – controlling cell protrusions during migration (Paulitschke et al. 2009). The GTPase Rho controls various cellular processes, such as cell adhesion and motility, enhancement of contractile responses, cytokinesis and transcriptional regulation (Nakagawa et al. 1996). Additionally RhoA encourages the retraction of actin, and Rac blocks the depolymerisation of actin (Sakamura et al. 2005). In multiple types of cancer an overexpression of Rho, Rac, and Cdc42 could be observed (Ellenbroek and Collard 2007).

3.3.4.1 Berberine

Berberine was shown to suppress the mevalonate pathway, which is the source of cellular cholesterol and lipid metabolites used for post-translational modification of cellular proteins, such as lamins, Ras and Rho proteins (Issat et al. 2006). Through this interference with the processing of Ras and Rho proteins, the cytostatic and cytotoxic effects of berberine may be explained (Issat et al. 2006).

To further examine the impact of berberine gap formation assays were performed. Therefore, MCF-7 spheroids were pretreated with 5-75µM berberine for 30 minutes and then placed onto untreated LEC monolayers. After four hours of incubating the LEC monolayer/MCF-7 spheroids co-culture at 37°C, the induced gaps were photographed and measured (Figure 44; and Figure 45). Berberine showed a dose dependent impact on MCF-7 spheroids' ability to induce gaps into the underlaying LEC monolayer, whereby a reduction in gap size by 50% could be observed at a treatment concentration of approximately 5µM berberine (Figure 44). In contrast to bay-11, or xanthohumol in high concentrations (> 50µM), berberine did not affect the attachment of MCF-7 spheroids to the underlaying LEC monolayer, even at high concentrations (75µM); and did not induce the "dispersed" phenotype of LECs.

As berberine shows strong auto fluorescence, the measurement of the induced gaps in the LEC monolayer was impaired at high berberine concentrations (Figure 45 d.).

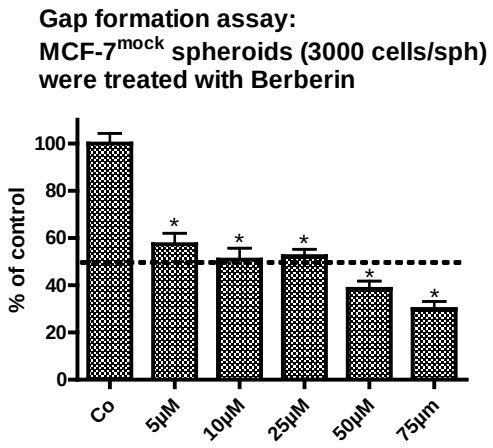


Figure 44: Impact of berberine on gap formation
MCF-7 spheroids were treated with 5µM, 10µM, 25µM, 50µM, and 75µM berberine for 30 minutes, before they were placed onto untreated LEC monolayers. For control reasons untreated spheroids were placed onto untreated LEC monolayer. Gap sizes were measured after 4 h of incubating the co-cultures at 37°C. ($p < 0.0001$)

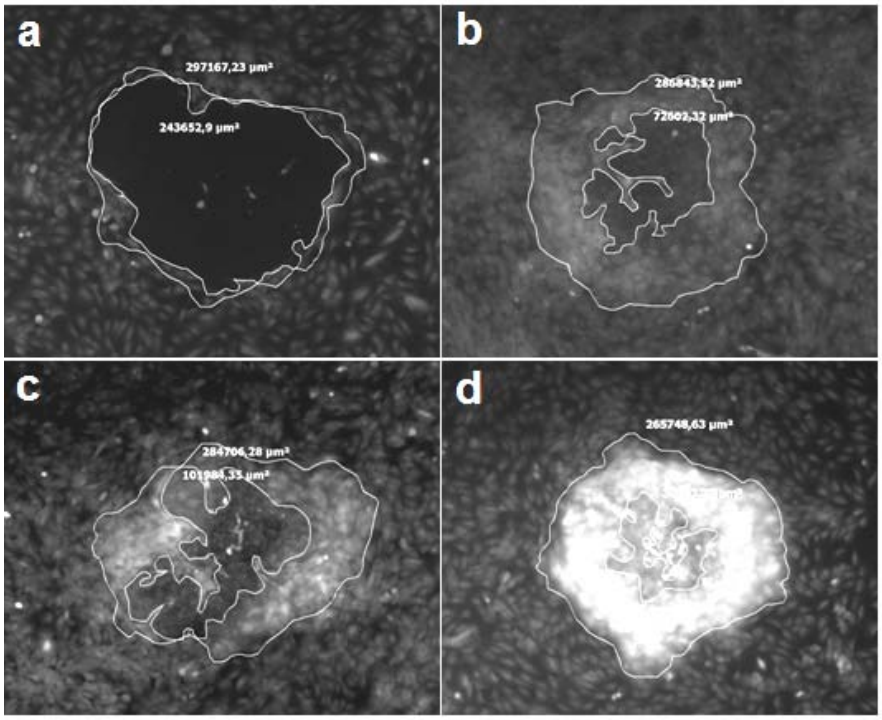


Figure 45: Gap induction by berberine treated MCF-7 spheroids
a. Untreated MCF-7 spheroids were placed onto untreated LEC monolayer. **b**. MCF-7 spheroids were treated with 5µM berberine for 30 minutes, and then placed onto untreated LEC monolayer. **c**. MCF-7 spheroids were treated with 25µM berberine for 30 minutes, and then placed onto untreated LEC monolayer. **d**. MCF-7 spheroids were treated with 75µM berberine for 30 minutes, and then placed onto untreated LEC monolayer. Pictures were taken after 4h incubating the co-cultures at 37°C. Berberine shows beneath UV-light a strong fluorescence (Tsang et al. 2009), which effects the measurement of gaps in the underlaying LEC monolayer.

To further examine the impact of berberine on LECs, we treated LEC monolayers with 12(S)-HETE (to mimic the presence of MCF-7 spheroids) and/or 5µM berberine. The resulting lysates were explored by western blotting (Figure 46). The immunoblots show that the expression levels of MYPT1 and phosphorylated MYPT1 increase with berberine treatment, whereas the levels of phospholylated MLC2 and paxillin decrease. The 12(S)-HETE induced decrease of Erk1/2 phosphorylation was prevented by simultaneous treatment with berberine (Figure 46).

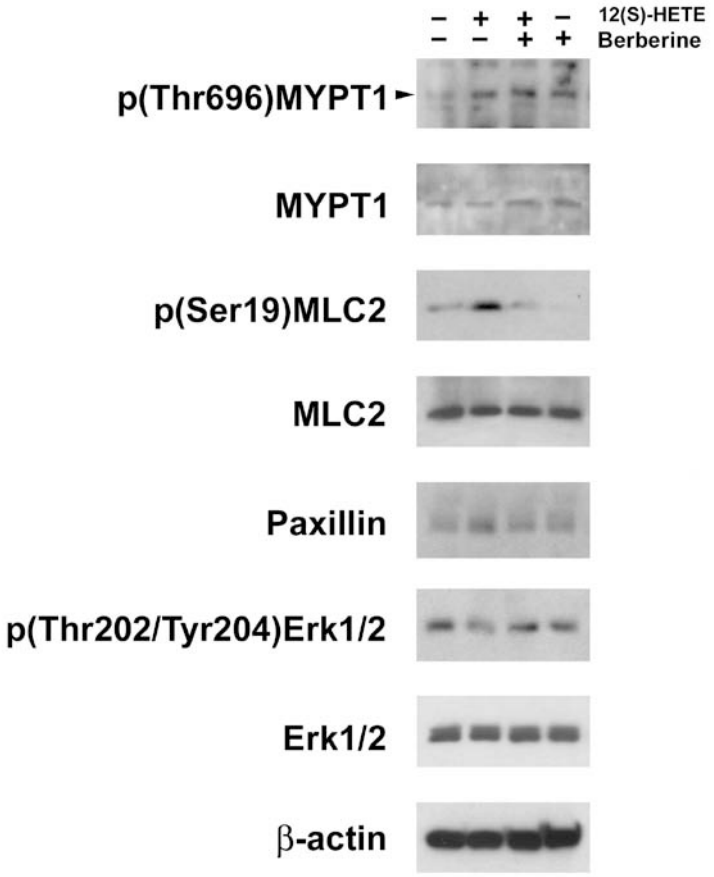


Figure 46: Analysis of berberine inhibition
LECs were inhibited with 5µM berberine and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. By β-actin analysis the sample loading was controlled.

Our data showed that berberine reorganised the cytoskeleton and decreased the mobile phenotype of LECs that was induced by the treatment with 12(S)-HETE,

3.4 MMP inhibition

The interaction of the tumour cells with the basement membrane is a critical event of tumour invasion that signals the initiation of the metastatic cascade. Therefore the inhibition of the degradation (by MMPs) of basement membrane components seems to be a major task. (Ray and Stetler-Stevenson 1994)

3.4.1 GM6001

It was recently shown that MMPs contribute to gap formation of LECs that were induced by MCF-7 spheroids (Madlener et al. 2010).
Treatment of MCF-7 spheroids with the broadsprectrum MMP inhibitor GM6001, or more specific treatment with TIMP2, MMP-2 inhibitor and MMP-9 inhibitor resulted in a LEC

gap size reduction by ~ 20-25% (Kerjaschki et al. 2010; see Table 6), which is consistent with the prominent role of these MMPs in metastasing breast cancer.

To complete these data, the impact of GM6001 on the mobility of LECs was examined by western blotting. Treatment with GM6001 resulted in a slight decrease in pMYPT1 and pErk1/2, a slight increase of pMLC2, and a strong increase of the cell-ECM adhesion protein paxillin when LECs where treated with GM6001 (Figure 47).

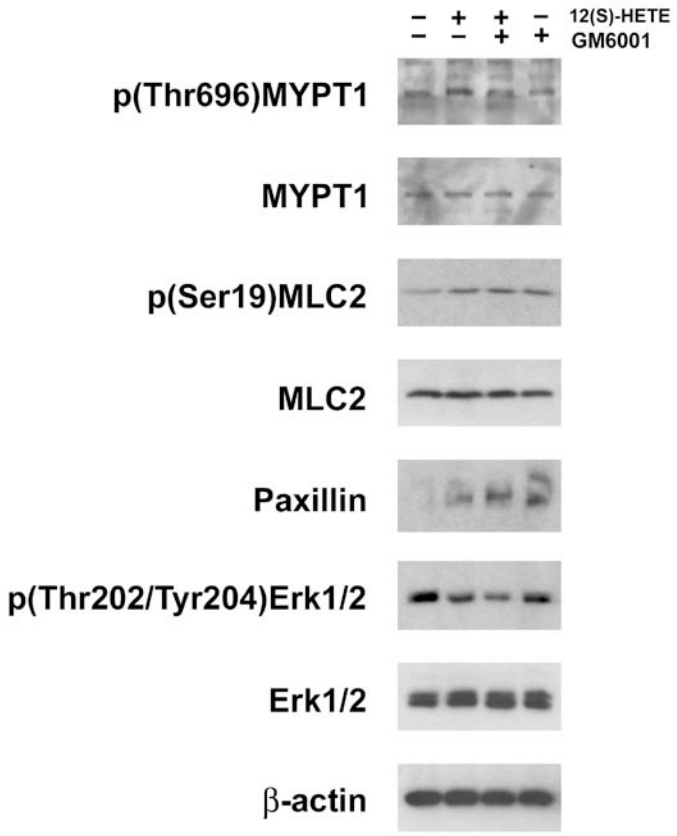


Figure 47: Analysis of GM6001 inhibition
LECs were inhibited with 20µM GM6001 and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. By β-actin analysis the sample loading was controlled.

In order to reveal the impact of the MMP inhibitor GM6001 on the gap induction of HTB-26 cell spheroids into LEC monolayer, we performed gap formation assays with GM6001-pretreated HTB-26 cell spheroids (Figure 48). The results show that neither baicalein nor GM6001 had an impact on the gap induction in LEC monolayer. Therefore, the major mechanism of HTB-26, which is responsible for the formation of gaps in LEC monolayer, is NF-κB, because 10µM bay-11 inhibited gap formation by 50% (Figure 42).

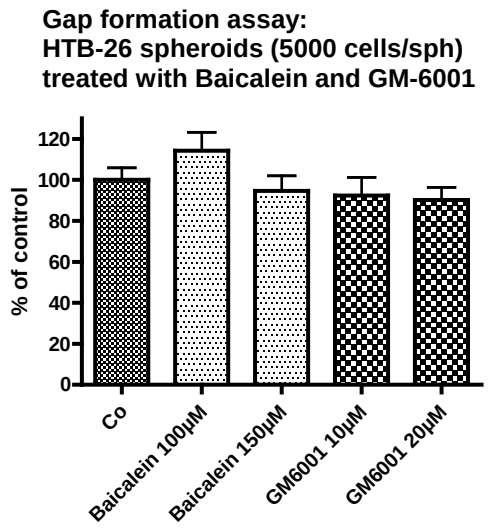


Figure 48: Impact of baicalein and GM6001 on gap induction through HTB-26 cell spheroids
HTB-26 cell spheroids (5000 cells/spheroid) were pretreated with 100µM and 150µM Baicalein, and 10µM and 20µM GM6001 for 30 minutes before they were seede onto untreated LEC monolayer. The co-culture was incubated for 4 h at 37°C before gap sizes were measured. ($p > 0.05$)

3.4.2 MMP-11 knockdown

There exists no specific small molecule inhibitor of MMP-11. In order to show the correlation between expression of MMP-11 and gap induction in LEC monolayers (MMP-11 expression correlates with poor progrognis of breast cancer patients), we used the lentiviral transduction of siRNA targeting MMP-11 mRNA. Five clones ("clone 13-17") of lentiviral transduction particles were used, whereby at least one construct from each gene set is expected to show a knockdown efficiency of over 70%. RT-PCR demonstrated the depletion of the MMP-11 mRNA. As the clone "16" showed the most efficient knockdown results (Figure 49), the MCF-7^{VEGFC}-MMP-11-knockdown cells produced with this clone were used for further experiments.

Although the expression of MMP-11 protein was decreased in MCF-7^{VEGFC}-MMP-11-knockdown cells by about 70% (Figure 49 left diagram), the LEC gap formation induced by these spheroids showed a reduction of only ~ 20% which is consistent with previous experiments using GM6001 (Kerjaschki et al. 2010; Madlener 2010) (Figure 49 right diagram; Figure 50).

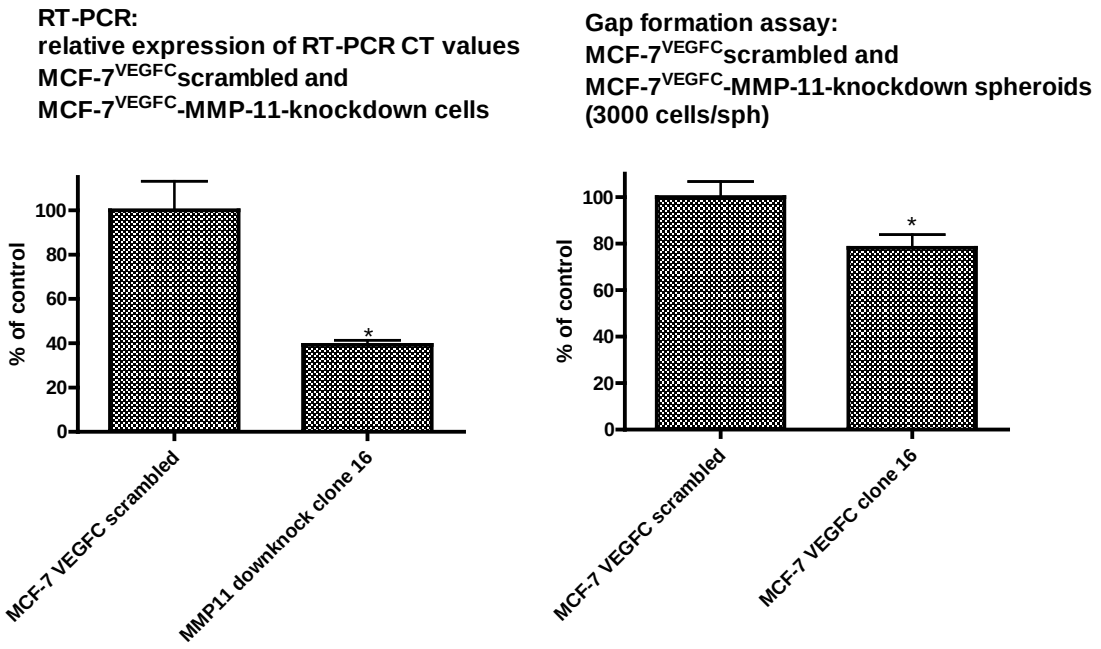


Figure 49: Relative expression of MMP-11 (RT-PCR); gap formation assay with MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids
Left diagram: Clone 16's efficiency in MMP-11 knock down (p=0.0445). As negative control empty vector transfected (scrambled) MCF-7^{VEGFC} cells were measured (100%) by RT-PCR. **Right diagram**: Gaps induced by MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids in LEC monolayers (p = 0.0182). MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids were placed untreated onto untreated LEC monolayers. After 4 h of incubating the co-cultures at 37°C gap sizes induced by the spheroids were measured.

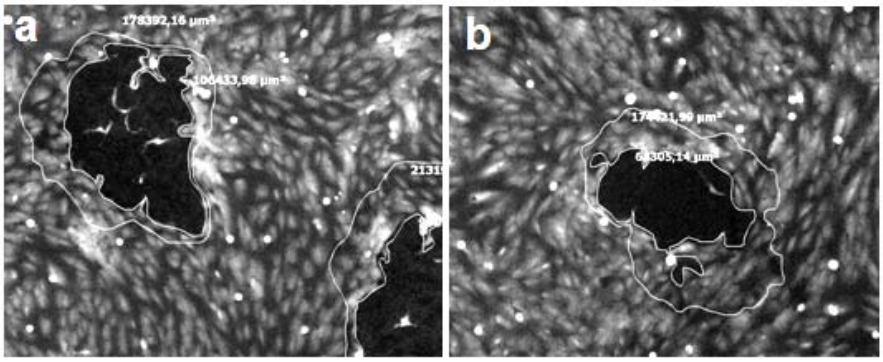


Figure 50: Decrease in gap sizes induced by MMP-11 knockdown spheroids
a. Untreated MCF-7^{VEGFC} scrambled cell spheroids were placed onto untreated LEC monolayers. **b**. Untreated MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids (clone 16) were placed onto untreated LEC monolayers. After 4 h of incubating the co-cultures at 37°C gap sizes were measured.

In order to examine the cooperation of MMP-11 depletion in breast cancer cell spheroids and the spheroid treatment with bay-11 and/or baicalein, we performed gap formation assays. Therefore MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids were treated with 10µM bay-11 and/or 100µM baicalein. As negative control scramble RNA transfected MCF-7 cell spheroids were treated with 10µM bay-11 and 100µM baicalein or solvent (DMSO). Figure 51 shows the results of the assay, indicating the loss of the additive inhibition of bay-11 and baicalein when treating MMP-11 downknocked cells, which was

due to the impact of DMSO on the gap induction (Figure 51 right diagram). According to the assay's results gaps induced by MCF-7^{VEGFC} scramble spheroids were getting smaller by about 20% when spheroids were treated with DMSO (0.1%), whereas gaps induced by MCF-7^{VEGFC}-MMP-11-knockdown spheroids are getting larger by about 15% when spheroids were treated with DMSO (0.1%). This effect of DMSO counteracts the impact of the MMP-11 depletion (Figure 51 right diagram).

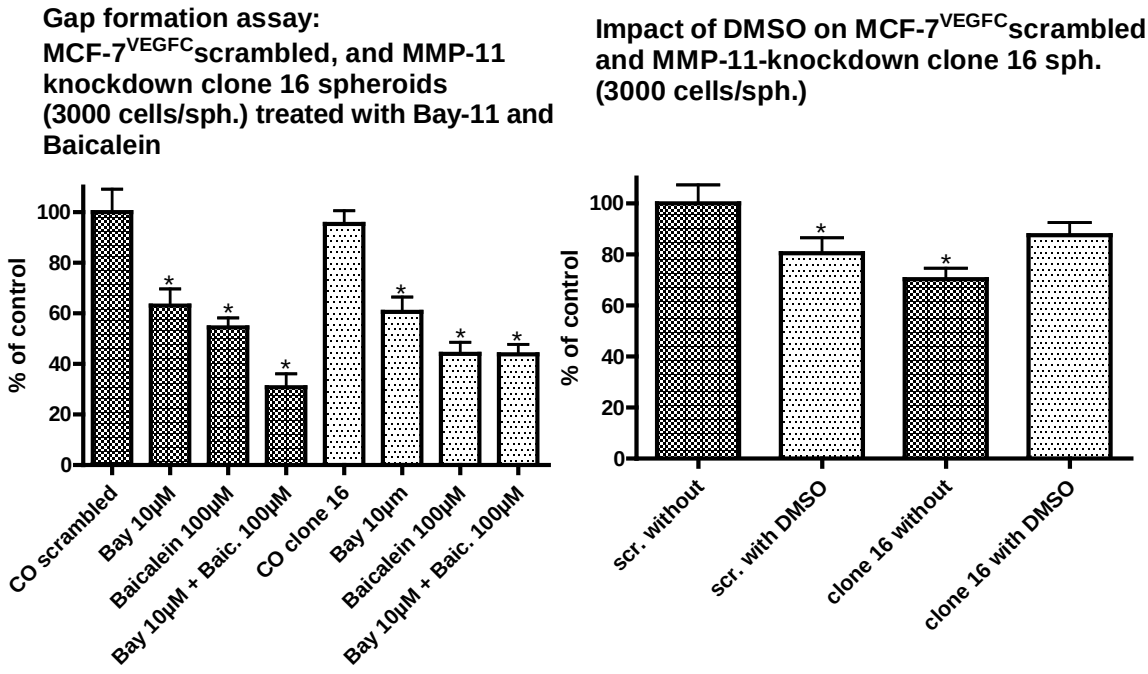


Figure 51: MMP-11 knockdown in cooperation with bay-11 and baicalein (left figure); the impact of DMSO on the gap formation induction in LEC monolayers (right figure)
Left diagram: MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids treated with 10µM bay-11 and/or 100µM baicalein. As positive control scramble MCF-7^{VEGFC} spheroids were treated with 10µM bay-11 and/or 100µM baicalein. As negative control both cell types were used untreated (untreated MCF-7^{VEGFC} scramble as 10%). Spheroids were placed onto untreated LEC monolayers. After 4 h of incubating the co-cultures at 37°C induced gap sizes were measured. ($p < 0.0023$); **Right diagram**: Scramble MCF-7^{VEGFC} cell spheroids and MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids were treated with 0.1% DMSO. As negative control untreated cell spheroids were used. DMSO treated and untreated spheroids were placed onto LEC monolayers. After 4 h of incubating the co-cultures at 37°C gap sizes were measured. ($p < 0.0416$)

In order to examine the cooperative effect of MMP-11 depletion and the treatment with heteronemin, we performed again gap formation assays with MCF-7^{VEGFC}-MMP-11-knockdown spheroids treated with 5µM heteronemin and/or 100µM baicalein. As negative control MCF-7^{VEGFC} scramble cell spheroids were used. Also in these experiments no additive inhibition of baicalein and heteronemin was observed (Figure 52), whereby the triple-inhibitory effect of MMP-11 depletion, LOX inhibition (baicalein), and NF-κB inhibition (heteronemin) showed a suprising result, as the gap sizes induced by MMP-11-knockdown spheroids co-treated with heteronemin AND baicalein were bigger than treated

with baicalein alone (Figure 52). The inhibitory impact of DMSO on the MMP depletion effect could also be seen in this gap formation assay (Figure 51 right diagram).

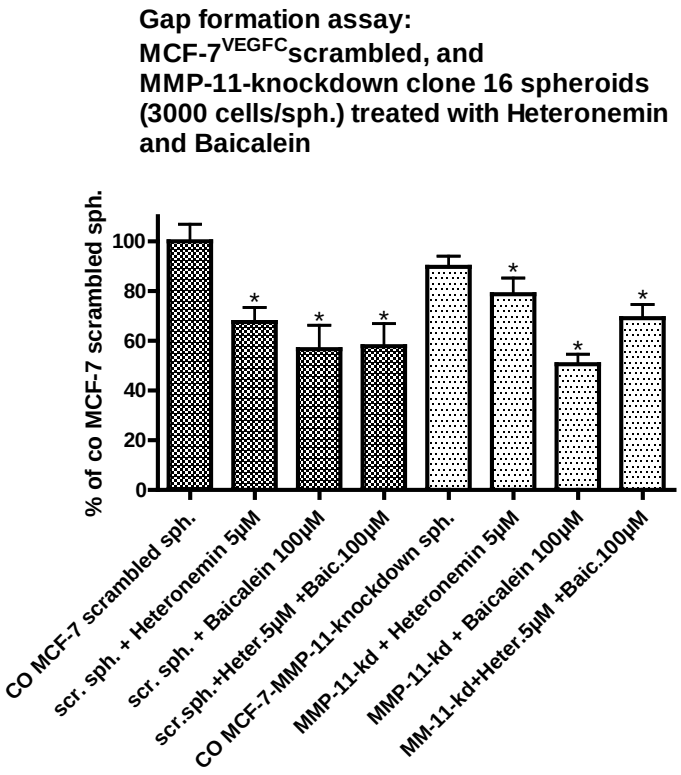


Figure 52: MMP-11 knockdown in cooperation with heteronemin and baicalein
MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids treated with 5µM heteronemin and/or 100µM baicalein. As positive control not transduced MCF-7^{VEGFC} spheroids were treated with 5µM heteronemin and/or 100µM baicalein. As negative control both cell types were used untreated (untreated MCF-7^{VEGFC} scramblee as 10%). Spheroids were placed onto untreated LEC monolayers. After 4h of incubating the co-cultures at 37°C induced gap sizes were measured. ($p < 0.0359$)
(MMP-11-kd sph. ….. MCF-7-MMP-11-knockdown spheroids)

4 Discussion

Metastasis is a multi-step process including the cellular transformation and growth of the primary tumour, the dissociation of tumour cells, the invasion of the stroma, the entering of the circulation by detached and embolised tumour cells, extravasation into distant organs, and the growth and development of secondary tumours (Fidler 2003).

Already 1979 Kramer and Nicolson described the interactions of tumour cells with vascular endothelial cell monolayers, showing that various tumour cells are able to "*attach to endothelial monolayers and cause morphologic changes, such as rupture of endothelial-endothelial cell interactions leading to retraction of endothelial cells and exposure of extracellular matrix, as well as their propensities to invade and underlap retracted endothelial monolayers and continue migration*" (Kramer and Nicolson 1979)

We tried to mimic the interactive process of breast cancer cells invasion to the lymphatic vasculature, by using a 3D co-culture system of MCF-7 cancer cells and telomerase-immortalised human lymphendothelial cell layers. Thereby three mechanisms of metastasis were investigated. Lipoxygenases metabolise arachidonic acid to biologically active eicosanoids that may play a major role in tumour promotion, progression and metastasis (Nie et al. 2003; Nie and Honn 2004; Yoshimura et al. 2004). Together with metalloproteases that loosen inter-endothelial junctions and matrix attachment (Deryugina and Quigley 2006), lipoxygenases released by MCF-7 tumour spheroids support the centrifugal migration of lymphatic endothelial cells away from the sites of contact with tumour cells (Kerjaschki et al. 2010; Madlener et al. 2010). The third mechanism investigated for its involvement in cancer metastasis was NF-κB, as its deregulation is associated with cancer development and inflammatory diseases (Folmer et al. 2009). NF-κB shows the ability to promote oncogenesis and cancer therapy resistance (Baldwin 2001) through the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis and suppression of apoptosis (Orlowski and Baldwin 2002).

In our study different natural and synthetic substances were tested for their metastasis inhibiting ability. Therefore our investigations include gap formation assays using 3D co-cultures, consisting of MCF-7 breast cancer cell spheroids (Madlener et al. 2010) and human lymphendothelial cell monolayers (Schoppmann et al. 2004), whereby the cancer spheroids were treated with the different substances (bay-11, baicalein, heteronemin, xanthohumol, berberine, GM6001). To further investigate the impact of these substances and their inhibited mechanisms, we performed western blotting showing the correlative

alterations in the motility of lymphendothelial cells indicating an immobile phenotype and consequently an inhibition of metastasis.

Induction of LECs with 12(S)-HETE shows mobile phenotype induction in LECs

12(S)-HETE (mainly produced by platelets, leukocytes, smooth muscle, epithelium, neuron, and fibroblast cells) (Spector et al. 1988) induces a protein kinase C-dependent cytoskeletal rearrangement and retraction of microvascular endothelial cells, and an increased tumour cell adhesion to exposed ECM (Tang et al. 1993; Honn et al. 1994a; Kerjaschki et al. 2010). In previous experiments 12(S)-HETE showed little or no effects on actin-binding proteins (filamin and tropomyosin) or on microtubules (Tang et al. 1993), as 12(S)-HETE induces a protein kinase C-dependent cytoskeletal rearrangement of microvascular endothelial cells, which seems to be mediated through enhancing phosphorylation of proteins that comigrated with myosin light chain, actin, and vimentin (Tang et al. 1993). Studies showed that 12(S)-HETE treatment resulted in an increase in the filamentous polymeric (F) actin content in the cytoskeleton, and enhances phosphorylation of myosin light chain, which may contribute to increased stress fibre formation (Rice et al. 1998).

Our results indicate a 12(S)-HETE induced the mobile, mesenchymal, amoeboid phenotype in LECs (Table 4), correlating with the already observed formation of circular gaps in the LEC monolayer underneath MCF-7 spheroids (Figure 9; Kerjaschki et al. 2010; Madlener et al. 2010).

Table 4: Induction of LECs with 12(S)-HETE: change of motility proteins' expression

	VE-Cad-herin	**pErk1/2**	**Erk1/2**	**Paxillin**	**S100A**	**Rock-1**	**ARP2/3**
+ 12(S)-HETE	decrease (min. at 0,2h), then increase (max. at 1h)	decrease (min. at 2h)	no change	increase (max. at 4h)	increase (max. at 0,5h)	increase (max. at 0,5h)	increase (max. at 1h)
	pMypt1	**Mypt1**	**pMLC2**	**MLC2**	**pAkt**	**ILK**	**ac. α-tubulin**
+ 12(S)-HETE	increase (max. at 1h)	increase (max. at 0,5h and 1h)	increase (max. at 1h)	increase (max. at 1h)	weak expression, increase (max. at 0,5h)	no change	no change

LEC monolayers were incubated with 1µM synthetic 12(S)-HETE. Lysates were taken after 10min, 30min, 1 h, 2 h, and 4 h. Analyses were performed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by β-actin analysis.

Stimulation with 12(S)-HETE induces microvascular endothelial cell retraction (Honn et al. 1994a). Upon 12(S)-HETE treatment cell-cell adhesion is reduced as confirmed by a transient downregulation of the VE-Cadherin expression (Figure 26), leading to a reduced epithelial and increased mobile phenotype of LECs. This triggering effect of 12(S)-HETE on VE-cadherin downregulation in LECs was already observed in previous experiments (Vonach 2010). Paxillin expression was increased upon 12(S)-HETE treatment of LECs (Figure 27), and also this is associated with an increased mobile phenotype of LECs, because paxillin (focal adhesion phosphoprotein) is necessary for cell-ECM contact, and its increased expression could already be associated *in vivo* and *in vitro* with enhanced endothelial cell motility (Roll et al. 2000). Furthermore S100A, a marker for a mesenchymal phenotype (mesenchymal transition encompasses cell mobility) (Zeisberg and Neilson 2009), could be activated through the 12(S)-HETE treatment of LECs (Figure 27).

ROCKs cause the formation of actin stress fibres and focal adhesions generating an amoeboid movement (Sahai and Marshall 2003); whereas the Arp2/3 activity regulates the mesenchymal invasion (Paulitschke et al. 2009). The western blots of 12(S)-HETE stimulated LECs show a slight increase in expression of both proteins (Figure 28), and therefore also these marker proteins indicate the mobile phenotype of LECs treated with 12(S)-HETE, which causes retraction and formation of gaps in the confluent cell layer that may serve as entry ports for metastasing cancer cells. In smooth muscle, myosin light chain kinase (MLCK) phosphorylates MLC2 (Ikebe and Hartshorne 1985), which is correlated with myosin ATPase activity and smooth muscle contraction (Tan et al. 1992). ROCK phosphorylates Ser19 of smooth muscle MLC2, which regulates the assembly of stress fibres (Totsukawa et al. 2000). MYPT1 (myosin-binding subunit of myosin phosphatase) is the regulatory/targeting subunit of the myosin phosphatase, which regulates the interaction of actin and myosin in response to signalling through the GTPase Rho (Feng et al. 1999). Previous experiments could associate phosphorylated MYPT with the mobile phenotype in LECs (Kerjaschki et al. 2010). Our results show an increase in the expression of both proteins – phosphorylated MLC2 and MYPT1 – after one hour of incubating LECs with 12(S)-HETE (Figure 29), which is another biochemical indicator for cell mobility. ILK is a key component of cell–ECM adhesion structures (Wu and Dedhar 2000). Additionally ILK is required for the activation of Akt, and is thus essential in Rac mediated actin cytoskeleton reorganisation (Paulitschke et al. 2009), as Akt blocks the depolymerisation of actin (Sakamura et al. 2005). Therefore ILK regulates cancer motility and invasiveness

(Filipenko et al. 2005). Furthermore ILK is involved in regulating Erk, and it can directly phosphorylate Akt, and MLC, regulating smooth muscle contraction and possibly cell motility in non-muscle cells (Wu and Dedhar 2001; Persad et al. 2001). When ILK is over expressed in normal epithelial cells cell-cell adhesion is lost, due to the down regulation of E-cadherin expression (Winder and Ayscough 2005). Our results show a low degree of phosphorylated Akt, with a slight increase after 30 minutes of incubating LECs with 12(S)-HETE (Figure 29), but the expression of ILK seems not to be effected by the treatment of LECs with synthetic 12(S)-HETE (Figure 29). Furthermore 12(S)-HETE seems to have no impact on the expression of acetylated α-tubulin and therefore on the structure of microtubules (Figure 27), which is in correlation with previous experiments that showed little or no effects of 12(S)-HETE on actin-binding proteins (filamin and tropomyosin) or on microtubules (Tang et al. 1993).

The LOX inhibitor baicalein shows little effect on LECs

To proof the LOX inhibitory function of baicalein (Li-Weber 2009), we performed control western blots by inhibiting LECs with baicalein (100µM) and then stimulating them with 12(S)-HETE. The results (Figure 30) show clearly that baicalein has no impact on 12(S)-HETE treated LECs (Figure 30; Table 5), which is in contrast with previous studies showing an increase of pErk by 12(S)-HETE treatment and its decrease due to baicalein treatment (Chen et al. 2008). Thus the phosphorylation status of Erk altered by 12(S)-HETE is dependent of the cell type.

Table 5: Impact of inhbitors on LEC protein expression correlated with 12(S)-HETE-induced motility

12(S)-HETE induction of:	**pMypt1**	**Mypt1**	**pMLC2**	**MLC2**	**Paxillin**	**S100A**	**pErk1/2**	**Erk1/2**
Inhibition with:								
Baicalein								
Bay-11						**inh.**	**inh.**	
Heteronemin	↑	↑			↓		↓	
Xanthohumol	↓		↓		**inh.**	**inh.**	**inh.**	
Berberin	↑		**inh.**		**inh.**		↑	
GM6001	**inh.**		↑		↑		↓	

LECs were pretreated with 10µM bay-11, 100µM baicalein, 5µM heteronemin, 25µM xanthohumol, 5µM berberine, or 20µM GM6001 and/or stimulated afterwards with 1µM 12(S)-HETE. After cells were harvested, the protein lysates were analysed by western blotting. Untreated LECs were used as negative control. The sample loading was controlled by β-actin analysis.

↑ ….. increase in expression level independent of 12(S)-HETE treatment

↓ ….. decrease in expression level independent of 12(S)-HETE treatment

inh. …. specifically inhibits 12(S)-HETE effects

Bay-11 inhibits phosphorylation of I-κB*α*, and leads to reduced cell motility

As above mentioned, 12(S)-HETE was shown to decrease e.g. the level of VE-cadherin expression (Vonach 2010), and therefore increases the possibility for cancer metastasis (Kerjaschki et al. 2010). The loss of E-cadherin is a key event in EMT (Thiery 2002), as it enables local invasion and dissemination of cancer cells. The treatement of MCF-7 breast cancer speroids with 10µM bay-11 caused an induction of VE-cadherin expression (Vonach 2010), leading to a loss of MCF-7 invasiveness and LEC-migration. Our gap formation experiments are in accordance with these findings as the gap sizes in the LEC monolayer underneath bay-11-pretreated (10µM) (30 minutes) MCF-7 spheroids also showed a reduction of gap formation by 50% underneath MCF-7 spheroids (Figure 31), indicating the reduced ability for LEC motility. Furthermore a cloudy cell distribution ("dispersed" phenotype) in the LEC monolayer could be observed when LECs got in contact with bay-11 (Figure 31), indicating a loss in cell-cell contact by 12(S)-HETE secretion and a loss of the ability of centrifugal migration. The inhibition of adhesion molecule expression leads to reduced MCF-/ spheroid-LEC contact and therefore to decreased gap sizes which was accomplished by treatment with bay-11 (Figure 33), and a high proportion of bay-11-pretreated spheroids did not attach to the underlaying LEC monolayer, even when bay-11-pretreated spheroids were washed with PBS before placed onto LEC monolayer. Furthermore bay-11-treatment of LECs caused inhibition of cell migration and therefore the formation of only small gaps (Figure 31).

Therefore, inhibition of bay-11 had two principally distinct effects: 1) The prevention of the adhesion of MCF-7 spheroids to LECs (at concentration >15µM), showing that MCF-7-LEC contact was mediated by NF-κB regulated adhesion molecules. 2) The inhibition of LEC migration induced by 12(S)-HETE which was secreted by MCF-7 spheroids and cause LEC gap formation underneath MCF-7 spheroids.

The examination of the levels of different marker proteins involved in cell mobility, EMT (epithelial-mesenchymal transition), and metastasis showed a decrease of S100A by bay-11 in LECs (Figure 34; Table 5). S100A acts as an angiogenic factor, stimulating the mesenchymal motility and invasiveness of endothelial cells (Takenaga et al. 1994, Jenkinson et al. 2004, Ambartsumian et al. 2001, Schmidt-Hansen et al. 2004). Treating LEC monolayers with 12(S)-HETE results in an increase of the S100A protein level, whereas co-treatment with bay-11 prevents this increase (Figure 34), indicating the inhibition of the LECs mobility through bay-11, which correlated with NF-κB regulation of S100A.

Bay-11 is an inhibitor of NF-κB activation, as it inhibits the cytokine (TNFα) induced phosphorylation of I-κBα. Whereby, this inhibition, and the inhibition of cell adhesion molecule expression and the stabilisation of I-κBα are found to be irreversible effects (Pierce et al. 1997). This enabled us to study the distinct effect of bay-11 on MCF-7 spheroids and on LEC monolayers (Figure 31).

Bay-11 and baicalein – additive inhibition in gap formation

When treating MCF-7 spheroids and LECs with bay-11 AND baicalein NF-κB- and LOX-inhibition, an additive inhibitory effect was observed on gap formation (Figure 35). These results underscore the potential of cooperating drugs for the management of metastasising cancer.

Heteronemin inhibitor of TNFα induced NF-κB activation

Heteronemin has previously been shown to be cytotoxic, an inhibitor of tyrosine kinases (Kazlauskas et al. 1976; Wonganuchitmeta et al. 2004; Rateb et al. 2009), and as bay-11, an inhibitor of TNFα induced NF-κB activation.

In the gap formation assay, 5µM heteronemin reduced the gap size to 50% (Figure 36, left figure). In contrast to experiments with bay-11, the spheroid treatment with heteronemin did not result in a decreased ability of the MCF-7 spheroids for the attachment to LECs. Our experiments show an irreversible inhibition of heteronemin (Figure 36, right figure) and the results indicate that heteronemin at even low concentrations (~ 5µM) reduced MCF-7 triggered gaps but did not affect the attachment of cancer spheroids to LEC monolayers. At higher concentrations (> 25µM) heteronemin shows also an impact on the cell-cell contact between LECs, as it provoked a “dispersed” like phenotype in the LEC monolayer (Figure 37 c. and d.). This indicates, that heteronemin inhibited NF-κB dependent mechanisms at considerably higher concentrations (25µM) compared to bay-11. Thus another mechanism(s), which became inhibited by already low heteronemin concentration (5µM) was responsible for the antimetastatic property. This could be related to the complete inhibition of paxillin expression that is involved in cell migration. It was shown that the absence of Erk1/2 decreased paxillin levels *in vitro* and *in vivo* what further reduced endothelial cell motility (Figure 39; Table 5) (Roll et al. 2000). This is in full accordance with our findings because 5µM heteronemin caused the complete dephospholylation of Erk1/2. Erk1/2 – activated by mitogens, growth factors, and cytokines (Roux and Blenis 2004) – phosphorylates and regulates the activities of a variety

of proteins (e.g. paxillin), and its signalling pathways acitivate the *ras* small GTPase, which is a frequently mutated oncogene in human cancers and is a downstream effector of EGFR and induces the cell cycle by increasing cyclin D1 expression (Roberts and Der 2007). The levels of other mobility proteins (Figure 39; Table 5) indicate a change in the actin-myosin interaction, as the pMYPT1 expression slightly increases in heteronemin- AND 12(S)-HETE-treated LECs. Previous experiments did already show that the phosphorylation of MYPT1 leads to the phosphatase inhibition and the cytoskeletal reorganisation (Birukova et al. 2004b).

Heteronemin and Baicalein – no additive inhibition

In contrast to bay-11, the combinatorial treatment with heteronemin and baicalein did not show an additive inhibitory effect on the MCF-7 spheroid induced gap formation in LEC monolayers (Figure 40). As previously described, baicalein was shown to inhibit the LOX pathway (Tong et al. 2002a, and b), and chemokines (fibroblastic eotaxin) (Nakajima et al. 2001). Our data indicate that heteronemin inhibited the MAPK (Erk1/2) pathway. The reason why inhibition of MAPK and LOX do not cause additive effects remains however unclear.

Xanthohumol inhibits I-κB phosphorylation, leading to reduced cell mobility

The hops constituent xanthohumol was shown to down-regulate the NF-κB activation (Colgate et al. 2007). In the LEC gap formation assay low concentrations of xanthohumol (IC_{50} ~ 5µM) reduced MCF-7 spheroid triggered gaps (Figure 42). At high concentrations (> 50µM) all xanthohumol-pretreated spheroids lost the contact to the underlaying LEC monolayer. At such high concentrations also an abnormal LEC phenotype could be observed (similar to “dispersed” phenotype), which increased with higher concentrations of xanthohumol (Figure 41 c.), and indicated that high xanthohumol concentrations inhibited NF-κB, whereas at low concentrations another additional mechanism prevented gap formation as it was observed with heteronemin.

When pretreating HTB-26 (MDA-MB-231) breast adenocarcinoma cell spheroids with xanthohumol the results indicate that xanthohumol affects this cell line only at much higher concentrations (IC_{50} ~ 100µM) (Figure 42). Compared to bay-11, which inhibits the gap formation induced by MCF-7 spheroids and HTB-26 spheroids at similar concentrations (~ 10µM), xanthohumol seems to affect different pathways in the two breast cancer cell lines. This reduced inhibitory effect of xanthohumol on the HTB-26

cells' ability for gap formation indicates differences between the MCF-7 and the HTB-26 cell line, but furthermore supports the notion that xanthohumol affects an additional mechanism in MCF-7 cells, but not in LECs and this most likely involves 15 lipoxygenases function (Kerjaschki et al. 2010; Madlener et al. 2010), because this enzyme provides the major effect on LEC gap formation and is expressed in MCF-7 cells, but not in HTB-26 cells (Kerjaschki et al. 2010).

The MCF-7 cancer cell line is in contrast to the HTB-26 cell line estrogen-receptor positive (Roomi et al. 2005). Previous studies have already shown that xanthohumol blocks the effect of estrogen, as xanthohumol binds its receptor, which may lead to the prevention of breast cancer (Gerhauser et al. 2002). It is however highly unlikely that this is the reasons why xanthohumol inhibits gap formation induced by MCF-7 cell spheroids, because colon cancer spheroids induce gaps by the same mechanisms as MCF-7 cells, yet colon cancer cells do not express ER receptors (Kerjaschki et al. 2010).

The analyses of expression levels of different motility marker proteins indicate a loss in phosphorylated MYPT1 in LECs when treated with 12(S)-HETE AND xanthohumol, which decreases the ability for cytoskeletal reorganisation. This lost ability and the drastic decrease of the phosphorylated MLC2 level after xanthohumol treatment will clearly lead to inhibited cell mobility and therefore reduced gap formation. The 12(S)-HETE-induced expression levels of S100A and paxillin are suppressed, indicating the loss of a mesenchymal mobile phenotype of 12(S)-HETE stimulated LECs when pretreated with xanthohumol. In contrast the level of phosphorylated Erk1/2 increased when LECs were treated with xanthohumol (Figure 43; Table 5) and counteracted 12(S)-HETE mediated dephosphorylation.

Previous research has already revealed that xanthohumol represses the NF-κB translocation into the nucleus by inhibiting the protein kinase IKK, which phosphorylates I-κBα (Albini et al. 2005). Moreover, xanthohumol inhibits the phosphorylation/activation of Akt in endothelial cells, indicating effects of xanthohumol on endothelial cell mobility (Albini et al. 2005). In summary, xanthohumol seems to interfere with angiogenic processes, such as the inhibition of endothelial cell invasion and migration, growth, and network formation of tubular-like structures (Albini et al. 2005), and this observation is supported by the here presented data.

Berberine inhibits I-κB degradation and LEC mobility

Berberine has already been proved to exhibit anti-carcinogenic activity (Nishino et al. 1986; Tsang et al. 2009), as it arrests cell growth (Khan et al. 2009; Tsang et al. 2009), cell migration and proliferation (cytostatic effect), and induces apoptosis (cytotoxic effect) in several human cancer cell lines (e.g. MCF-7 breast cancer cells, MDA-MB231) (Issat et al. 2006; Khan et al. 2009; Tsang et al. 2009). Berberine acts as a therapeutic agent, showing inhibitory effects on the invasive and metastasis capacity of malignant cells (e.g. tongue squamous cancer, lung cancer cells) (Issat et al. 2006). By preventing the degradation of I-κB, which blocks the translocation of NF-κB into the nucleus, it decreases the protein level of NF-κB (Ho et al. 2009).

Our data indicate that berberine has a dose dependent impact on MCF-7 spheroids' ability to induce gaps into the underlaying LEC monolayer, whereby a reduction in gap size by 50% could be observed at a treatment concentration of approximately 5µM berberine (Figure 44). In contrast to bay-11, or xanthohumol, high concentrations (> 50µM) of berberine did not impact the attachment of MCF-7 spheroids to the underlaying LEC monolayer, and did not induce a "dispersed" phenotype of LECs.

Further analysis with western blotting indicated that 5µM berberine reorganised the cytoskeleton and decreased the mobile phenotype of the LECs that was induced by the treatment with 12(S)-HETE, because the expression levels of pMYPT1 increased, and the 12(S)-HETE induced levels of pMLC2 and paxillin were repressed (Figure 46; Table 5). Furthermore berberine counteracted the 12(S)-HETE mediated decrease of Erk1/2 phosphorylation (Figure 46; Table 5).

Many previous studies have shown that anti-tumour activities of berberine may be mediated in part through the suppression of NF-κB activation (Pandey et al. 2008; Yi et al. 2008; Hu et al. 2008; Enk et al. 2007). Here we provide evidence that the anti-cancer activity of berberine involves also the regulation of Erk1/2 MAPK and consequently of paxillin, as well as a severe interference with the activation of MLC2 and therefore berberine inhibited two major mobility mechanisms.

Ras/Rho inhibition by berberine

Furthermore, berberine was reported to suppress the mevalonate pathway, which is the source of cellular cholesterol and lipid metabolites used for post-translational modification of cellular proteins, such as lamins, Ras and Rho proteins (Issat et al. 2006). Through this interference with the processing of Ras and Rho proteins, the cytostatic and cytotoxic

effects of berberine may be explained (Issat et al. 2006). As Rho activity can suppress apoptosis, contribute to the loss of cell polarity, and alter adhesion proteins, which contributes to EMT of cancerous cells (Ellenbroek and Collard 2007), its inhibition by berberine may repress EMT, which is in accordance with our results of reduced LEC mobility due to berberine treatment.

GM6001 a MMP inhibitor and prevents TNF-α release: increasing the mobile phenotype

GM6001 (Galardin/Ilomastat) is a synthetic inhibitor of MMPs (MMP-1, -2, -3, -8, and -9), as it mimics the MMP substrates and enters/binds the active site of MMPs (Galardy 1993); and prevents the release of TNF-α *in vivo* and *in vitro* (Solorzano et al. 1997). Former work from our lab showed that GM6001, TIMP2, MMP-2-, andMMP-9-inhibitors inhibited MCF-7-induced gap formation of LECs by ~ 20% each (Table 6; Kerjaschki et al. 2010; Madlener et al. 2010). The inhibitory effect of simultaneous treatment with GM6001 and baicalein hat an additive effect (Kerjaschki et al. 2010). Therefore, GM6001 and baicalein were also tested with HTB-26 spheroids (Figure 48).

Neither GM6001 nor baicalein decreased the gap size in LEC monolayers triggered by HTB-26 spheroids (Figure 48). Since HTB-26 cells do not express any LOX enzymes (Kerjaschki et al. 2010), baicalein was ineffective and thus HTB-26 induced LEC gaps by another mechanism. As GM6001 did not reduce the gap size induced by HTB-26 spheroids it seems that also MMPs did not contribute to this process. Hence, the studies on the alteration of biochemical mobility markers upon treatment of LECs with 12(S)-HETE applies only to a limited type of cancers. Hence the common mechanism, by which MCF-7 cells and HTB-26 cells induce LEC gap formation, is by the NF-κB pathway (Figure 42), whereas in MCF-7 cells two additional mechanisms contribute to LEC gap formation, namely MMPs and LOXs. This is in agreement with our observation that the gaps induced by MCF-7 spheroids are much larger than those induced by HTB-26 spheroids (Figure 13).

Table 6: Inhibitors used in the MCF-7 spheroid/LEC gap assay (Kerjaschki et al. 2010)

	Target of Inhibitor	**n**	**Reduction in gap area** (+/- SEM)	**p value**
Arachidonic acid metabolism				
Aspirin 200 µM	COX1/2	12	0	-
NS-398 20 µM	COX2	9	0	-
NDGA 75 µM	Pan-LOX	16	88.3% (+/- 11.0)	<0.0001
Caffeic acid 200 µM	5-LOX, 15-LOX	12	52.4% (+/- 33.9)	0.0024
Metalloproteases				
GM6001 20 µM	Pan –MMP	10	**25.1%** (+/- 15.5)	0.0481
TIMP1 200 ng/ml	Soluble MMPs	9	0	-
TIMP2 200 ng/ml	Membrane-MMPs	10	26.7% (+/- 11.9)	0.0193
MMP2-Inhib II 20 µM	MMP2	18	**21.8%** (+/- 19.7)	0.0258
MMP9-Inhib I 20 µM	MMP9	15	**26.2%** (+/- 19.4)	0.0358
MMP13 Inh 20 µM	MMP13	20	0	-
MT1MMP-IgG 10µg/ml	MMP14	9	0	-
Aprotinin 1000U/ml	Non-MMP protease inhibitor	11	0	-
Radical scavengers				
Probucol 200 µM	Lipid peroxidation, ROS	9	0	-
Mannitol 100 µM	OH-radical	10	0	-
Catalase 600 U/ml	H_2O_2 metabolism	11	0	-
Carboxy-PTIO 200 µM	NO-radical	9	0	-

TIMP1 (CC1062), TIMP2 (CC3327), Polyclonal rabbit anti-MT1MMP antibody (AB8102; inhibitory antibody) were from Chemicon. GM6001, MMP2 inhibitor II, MMP9 inhibitor I, MMP13 (collagenase 3) inhibitor, and Carboxy-PTIO were from Calbiochem. Nordihydroguaiaretic acid (NDGA) was from Cayman Chemical. Aspirin, NS398, D-Mannitol, Catalase, Probucol, and Aprotinin were from Sigma. Caffeic acid was from Biomol. Statistical analysis and SD refer to the highest tested concentration. The paired two tailed t-test was used. (Kerjaschki et al. 2010)

The examination of expression levels of different mobility proteins in GM6001-treated (20µM) LECs indicated a prevention of actin-myosin changes upon 12(S)-HETE stimulation, as the level of pMYPT1 decreased, whereas the level of pMLC2 increased upon exposure to 12(S)-HETE as well as to GM6001, or to both (Figure 47; Table 5). This induction of the mobile phenotype could also be seen through the strong increase (Figure 47; Table 5) in the expression of paxillin, which is a cell-ECM adhesion phosphoprotein and a marker for the mobile phenotype (Deakin and Turner 2008; Ross et al. 2000). The GM6001 treatment of LECs furthermore decreased the expression levels of pErk1/2 (Figure 47; Table 5). Therefore, Erk1/2 activity and paxillin expression seemed to be disconnected in presence of GM6001. In contrast to our data, GM6001-treated human dermal microvascular endothelial cells (HDMVEC) showed a time-dependent decrease in paxillin expression (Akahane et al. 2004), whereas suppression of HDMVEC migration by GM6001, which correlated with VE-cadherin accumulation at cell-cell junctions increase,

was also observed in our experiments.

MMP-11 knockdown reduced gap sizes by about 20%

Kerjaschki et al. (2010) found that MCF-7 cells express significantly higher levels of MMP-11 when grown as spheroids compared to monolayers.

MMP-11 is over expressed in the majority of human carcinomas, including breast carcinoma, and leads to increased cell numbers and tumour volumes and it mediates cell-matrix adhesion, which is a prerequisite for cell migration. (Kasper et al. 2007). Hence we studied the role of MMP-11 in the formation of LEC gaps.

The knock down of MMP-11 expression (MMP-11 depletion) was shown to suppress cells growth *in vitro* and *in vivo*, and to inhibit cell proliferation and tumourigenicity (Deng et al. 2005). In order to show the correlation between expression of MMP-11 and gap formation induction in LEC monolayers, we used the lentiviral transduction of siRNA to target MMP-11 mRNA. Although the expression of MMP-11 protein was decreased by about 70% in MCF-7^{VEGFC}-MMP-11-knockdown cells, compared to control MCF-7^{VEGFC} cells (Figure 49 left diagram), the gap formation of MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids showed a reduction of ~ 20% (Figure 49 right diagram; Figure 50). This is a minor contribution of MMPs to gap formation, and this is in accordance with previous experiments showing that the specific inhibition of MMP-9, and MMP-2 reduced gap formation also by 20-25% (Table 6) (Kerjaschki et al. 2010).

Through the co-treatment of MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids with bay-11 (10µM) AND baicalein (100µM), we wanted to examine the triple inhibitory effect – MMP-11 depletion, NF-κB inhibition, and LOX inhibition – on the spheroids' ability for gap induction in LEC monolayer. Results of the assay indicate the loss of the additive inhibition of bay-11 and baicalein when treating MCF-7^{VEGFC}-MMP-11-knockdown cells. We found that the solvent DMSO obscured specific and additive effects on gap size reduction (see "Impact of DMSO on gap induction by MCF-7 spheroids"). Hence, the triple-inhibitory effect of MMPs, LOX, and NF-κB could not be determined in this type of assay. Also the co-treatment of MCF-7^{VEGFC}-MMP-11-knockdown cell spheroids with heteronemin (5µM) AND baicalein (100µM) did not result in an additive inhibition (Figure 52), and DMSO showed an detrimental impact on gap induction by MCF-7 spheroids.

Impact of DMSO on gap induction by MCF-7 spheroids

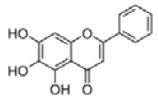
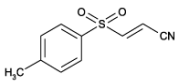
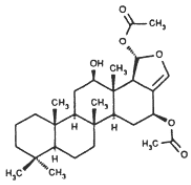
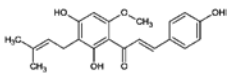
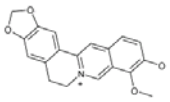
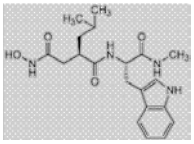
Our results gained from the gap formation assays using MCF-7^{VEGFC}-MMP-11-knockdown and scramble MCF-7^{VEGFC} cell spheroids indicate an impact of DMSO on the spheroids' ability to induce gap sizes in LEC monolayers. The gap sizes induced by control MCF-7^{VEGFC} spheroids are getting smaller by about 20% when spheroids were treated with DMSO (0.1%), whereas gaps induced by MCF-7^{VEGFC}-MMP-11-knockdown spheroids are getting larger by about 15% when spheroids were treated with DMSO (0.1%). This fact compensates the impact of the MMP-11 depletion (Figure 51 right diagram). Therefore the triple-inhibitory effect of MMPs, LOX, and NF-κB can not be determined in this type of assay, as DMSO is required as solvent for baicalein. The impact of DMSO indicates a possible transcription inhibitory effect. Some literature did already reveale the suppressive activity of DMSO onto the transcription of the c-*myc* and myeloperoxidase (MPO) genes (Antoun et al. 1991). This could have also affected the transcription of shMMP-11 RNA.

Summary: Impact of inhibitory substances

We used in our research substances different in origin and different in impacts. All tested substances had the capacity to inhibit LEC gap formation triggered by MCF-7 breast cancer cell spheroids in the underlaying LEC monolayer. Even at low concentrations (5µM) they inhibited either the activation of NF-κB, or the expression of MMPs (Table 7). Additionally, the tested substances showed a significant impact on the motility of LECs, by cytoskeletal reorganisation and the inhibition of mobile/mesenchymal phenotypes, leading to a decrease in the ability of cancer cell spheroids to induce gaps into lymphendothelial layers (Table 7).

There is a basic understanding to develop a rationale support to regular cancer therapies consisting of surgical resection, radiation, and chemotherapy; by the usage of anticancer drugs derived from natural compounds. Amongst others our study investigated natural compounds derived from hops, medical plants, and marine organisms to combat lymph node metastasis of ductal breast cancer emboli; which all posses a significant ability to inhibit lymphendothelial gap induction by cancer cells, at even low concentrations. Nowadays about 60% of all drugs used in the western medicine derive from natural compounds (Cragg et al. 2006). However there are still unexplored resources in herbal medicines which need to be explored (Cragg et al. 2006).

Table 7: Summary of our findings

	Origin	**Structure**	**Target of inhibition**	**IC_{50} (MCF-7 cells)***	**Impact on LEC****
Inhibitor					
Baicalein	Roots of *Scutellaria bai-caleinsis*		inhibition of 12/15-LOX and their metabolite 12(S)-HETE	100µM	no effect on LECs
Bay-11	n.s.		inhibits cytokine induced phosphorylation of I-κBα and activation of NF-κB	10µM	inhibition of mesenchymal motility, and lost cell-cell contact
Hetero-nemin	Extracts of marine sponge *Hyrtios erecta*		inhibits TNF-α induced NF-κB activation; possible inhibition of MAPK pathway (>25µM)	5µM	change in actin-myosin interaction, cytoskeletal reorganisation, and reduced cell motility; >25µM lost cel-cell contact
Xantho-humol	Hops (*Humulus lupulus L.*)		inhibits IKK, which phosphorylates I-κBα; possible impact on 15-LOX function	5µM	decreased ability for cytoskeletal reorganisation, and loss of mesenchymal mobile; >50µM lost cell-cell contact phenotype
Berberine	Medicinal plants e.g. *Berberis vulgaris*		prevents degradation of I-κB, blocking translocation of NF-κB; mevalonate pathway suppression (Ras/Rho proteins); possible impact on the MAPK pathway	5µM	cytoskeletal reorganisation, decreased mobile phenotype
GM6001	synthetic		inhibitor of MMPs (binds to active site of MMPs); prevents release of TNF-α	20µM (reduced gap size by 25%)	change in actin-myosin interaction, increase of mobile phenotype

* Gap formation assays were performed, treating MCF-7 cell spheroids with different concentrations of each substance to be tested. After incubating the pretreated spheroids for 30 minutes at 37°C, they were seeded onto untreated LEC monolayer. After 4h of incubating the co-cultures at 37°C gap sizes were measured and pictures were taken.

** Western blots were performed, measuring the protein expression levels of lysates of inhibitor and 12(S)-HETE treated LEC monolayers.

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Veröffentlichungen

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Curriculum Vitae

Personal information

Name	Katharina Viola
Date of birth	23rd November 1981
Place of birth	Mexico City
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Family status	Unmarried, no children

Education

Presumably Sept. 2010	**Master's degree in Biology** Diploma thesis: "*The inhibition of 12(S)-HETE stimulated mobility and gap formation of lymphendothelial cells"* (Grade: 1)
Since Oct. 2002	**Studies of Biology, Specialisation in Anthropology** University of Vienna, Faculty of Life Sciences
March 2009	**Master's degree in International Business Administration** Diploma thesis: "*The implementation of economic oriented topics in the education of artists"* (Grade: 1)
Oct. 2001 – March 2009	**Studies of International Business Administration, Specialisation in Logistics and Organisation** University of Vienna; Faculty of Business, Economics and Statistics (BWZ)
June 2001	**High school degree** High school for financial occupations (HBLA Wien 19), Specialisation in Environmental Economics and Hotel Business

Work experience

March 2009 – Apr. 2010	**Diploma project at the Medical University of Vienna,** Institute of Cancer Research (ICR) Supervising tutor: Univ. Prof. Dr. Georg Krupitza, Clinical Institute for Pathology
June 2007 – Dec. 2007	Employment at **Kotànyi GmbH** (Wolkersdorf), Department for Supply Chain Management
Feb. 2002 – Jan. 2003	Employment on project basis at **Finance Trainer International Ges.m.b.H** (Vienna)
July 1999 – Sept. 1999	Internship at the **Hotel Schrannenhof** (Klosterneuburg)

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