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**MECHANISMS OF CANCER CELL INTERACTION WITH THE STROMA, IN
PARTICULAR THE INTRAVASATION OF MDA-MB231 BREAST CANCER
SPHEROIDS THROUGH THE LYMPHENDOTHELIAL BARRIER**

verfasst von / submitted by

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**THIS DISSERTATION IS DEDICATED
TO MY BELOVED PARENTS
LUBIS HUSAINY AND ANDI FRISTIATI PANGERANG
FOR THEIR ENDLESS LOVE, PRAYER,
SUPPORT AND ENCOURAGEMENT**

Verily, with the hardship, there is relief.

(Q.S. Al-Insyirah)

Dear my Lovely Family, I am going back home

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ABBREVIATIONS

12(S)-HETE	hydroxyeicosatetraenoic acid, "S" stereoisomer	EGF	Epidermal growth factor
12-HETER	12(S)-HETE receptor (syn. GPR31)	ERBB4	Erb-B2 Receptor Tyrosine Kinase 4
3D	3-dimensional	ERKs	Extracellular-signal-regulated kinases
ALOX	arachidonate lipoxygenase	FAK	Focal adhesion kinase
ALOX12/15	lipoxygenase 12/15	FCS	fetal calf serum
BEC	blood endothelial cell	HUVEC	Human umbilical vein endothelial cell
BLT2	Leukotriene B4 receptor 2 (syn. BLT2 receptor, BLT2R, low affinity LTB4 receptor)	ICAM-1	intercellular adhesion molecule 1
CAF	cancer associated fibroblast	I κ B α	Inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells
CAT	collective to amoeboid transition	IL	Interleukin
CCID	Circular chemo repellent induced defect	IP3	inositol trisphosphate
COX2	Cyclooxygenase-2	LEC	lymph-endothelial cell
CRC	colorectal cancer	LPS	Lipopolysaccharide
CXCL1	C-X-C motif ligand 1	MAT	Mesenchymal to amoeboid transition
CXCR4	Chemokine (C-X-C Motif) Receptor 4	MDSCs	Myeloid-derived suppressor cells
CYP1A1	Cytochrome P450 1A1	MET	MET Proto-Oncogene, Receptor Tyrosine Kinase
DAG	diacyl-glycerol	miR	MicroRNA (micro ribonucleic acid)
EC	endothelial cell	MLC2	myosin light chain 2
ECM	Extracellular matrix components	MMPs	matrix metalloproteinases
EETs	Epoxyeicosatrienoic acids	MYLK	myosin light chain kinase

NEMO	NF-kappa-B modulator	essential	RELB	V-Rel	avian reticuloendotheliosis viral oncogene homolog B
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells		RHOA	Ras homolog gene family, member A	
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-Cells 1		RHOF	Ras homolog family member F	
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in BCells 2		ROCK	Rho-associated protein kinase	
PAR1	Protease-activated receptor 1		Ser19	serine 19	
PKC	protein kinase C		Si-RNA	Small interfering ribonucleic acid	
PLC, PROX1	phospholipase C Prospero homeobox protein 1		SOX18 TEM	SRY-related HMG-box 18 Transendothelial migration	
PS	penicillin/streptomycin		TGFβ	transforming growth factor-β	
REL	V-Rel	avian	TNFα	Tumour necrosis factor α	
RELA	V-Rel	avian	VEGF	vascular endothelial growth factor	
	reticuloendotheliosis oncogene homolog	viral			
	reticuloendotheliosis oncogene homolog A	viral			

1.SUMMARY

The most common cause of death from cancer is not the primary tumor itself, but the secondary tumour, namely metastasis. Metastasis is end progress of cancer development where tumour cells have to go through a process by the switch on/off a variety of molecular/biochemical mechanisms that are communicated through distinct intra-or intercellular pathways that are referred to metastatic cascade. Breast cancer cells disseminate mostly through the lymphatic vasculature and the colonisation of the lymph nodes is a prognostic marker, which determines the fate of the patient.

In order to identifying molecular mechanism of active compounds suspected of having potential anti-metastatic activity, in particular, the intravasation of cancer cells into the lymphatic vessel, a three dimensional (3D) in vitro assay is used. SOX18 and PROX1 are endothelium-specific transcription factors. Therefore, the particular contribution of SOX18, as well as mechanistic insight into its mode of action requires further investigation. Therefore, elucidating the precise mechanisms which are triggered by 12(S)-HETE, SOX18, PROX1 and NF-kB (which is regulated by SOX18), to produce large gaps within the endothelial cell wall, so-called "circular chemically induced defects" (CCIDs) was among the major aims of this doctoral thesis.

A CCID or known as circular chemorepellent-induced defects assay was used to investigate the potentiality of intravasation through the 3D co-culture model. These model are consist of spheroids from triple negative breast cancer MDA-MB231 and estrogen dependent MCF-7, or colon cancer SW620 which are placed on the top of a confluent lymph endothelial cell (LEC). Molecular (siRNAs) and pharmacological inhibition, Western blotting analysis, qRT-PCR were utilized to elucidate the molecular mechanisms of breast cancer cell intravasation through lymphatic barriers. Furthermore, anti-intravasative mechanisms of flavonoids and FDA-approved drugs were studied.

In the MDA-MB231 model, CCID formation was induced by 12(S)HETE dependent on SOX18 and its transcriptional target PROX1. The upregulation of RELA, NFKB1, and ICAM-1 were also dependent on SOX18. 12(S)-HETE secreted by breast cancer cells triggered a signal that was transduced towards SOX18 by GPR31 and BLT2. Furthermore,

The release of intracellular Ca^{2+} is triggered by activation of MLC2 by 12(S)HETE, which in turn activates MYLK which was independent of GPR31/12-HETER.

Focusing on the regulation of NF- κ B, NF- κ B indicated a positive (auxiliary) feed-back loop between SOX18-NF- κ B-SOX18 was induced by SOX18 in LECs. This mechanism might be specific to LEC since 12(S)-HETE did not induce SOX18 expression in HUVECs (human umbilical vein endothelial cells – blood endothelial cells). siRNA-mediated inhibition of SOX18 and PROX1 attenuated CCID formation to a similar extent as siRNA-mediated inhibition of RELA, NF- κ B1 or NF- κ B essential modulator.

FDA-approved clinical drugs have additional activities that they have not originally been developed for. The screening of approximately 600 FDA approved drugs, which was performed earlier by this group, revealed ~ 15 drugs which inhibited cancer spheroid-induced CCID formation in LEC or BEC monolayers. They include Fenofibrate, Vinpocetine, Carbamazepine, Ketotifen, Niflumic acid, dyclonine and bepridil hydrochloride. Some of the drug drugs inhibited CYP1A1 activity, intracellular Ca^{2+} release and the expression of motility proteins as putative intravasation mechanisms. The flavonoids: luteolin and apigenin, suppressed pro-intravasative trigger factors in MDA-MB231 specifically MMP1 expression and CYP1A1 activity. These flavonoids which are present in medicinal plants, spices, herbs and fruits, attenuated CCID formation.

Taken together, as breast cancer cells predominantly spread through the lymphatic vasculature, inhibiting cancer cell intravasation may reduce the ability of breast cancer cells to metastasize. Therefore, inhibiting intravasation could be a strategy to develop medicines for anti-metastatic intervention.

2. ZUSAMMENFASSUNG

Die häufigste Todesursache bei Krebs ist nicht der Primärtumor selbst, sondern der Sekundärtumor, nämlich die Metastasierung. Die Metastasierung ist der Endpunkt der Krebsentstehung, bei der Tumorzellen einen Prozess durchlaufen müssen, indem sie eine Reihe von molekularen / biochemischen Mechanismen ein-/aus-schalten, die durch intra- oder inter-zelluläre Signalwege weitergeleitet werden und so die metastatische Kaskade bilden. Brustkrebszellen streuen zumeist über die lymphatische Vaskulatur und die Besiedlung der Lymphknoten ist ein prognostischer Marker, der das Schicksal der Patientin mitbestimmt.

Um den molekularen Mechanismus von Wirkstoffen zu identifizieren, von denen angenommen wird, dass sie eine potentielle antimetastatische Aktivität haben, insbesondere die Intravasation von Krebszellen in das Lymphgefäß, wird ein dreidimensionaler (3D) In-vitro-Assay verwendet. SOX18 und PROX1 sind endothelium-spezifische Transkriptionsfaktoren. Daher ist es nötig, den besonderen Beitrag von SOX18, sowie dessen Mechanismus betreff seiner Wirkungsweise weiterhin zu untersuchen. Deshalb war das wesentlichste Ziel dieser Dissertation, die genauen Mechanismen, die durch 12(S)-HETE, SOX18, PROX1 und NF-kB (welches von SOX18 reguliert wird) „circular chemo-repellent induced defects (CCIDs - das sind Öffnungen in der lymph-endothelialen Barriere)“ hervorrufen, zu untersuchen.

Ein CCID oder bekannt als zirkulärer Chemorepellent-induzierter Defekt-Assay wurde verwendet, um die Möglichkeit von Intravasation durch das 3D-Co-Kulturmodell zu untersuchen. Diese Modelle bestehen aus Sphäroiden von dreifach negativem Brustkrebs MDA-MB231 und Östrogen-abhängigem MCF-7 oder Dickdarmkrebs SW620, die auf der Spitze einer konfluenten Lymphendothelzellen (LEC) angeordnet sind. Molekulare Hemmung (durch siRNAs) und pharmakologische Hemmung, Western blot Analyse und qRT-PCR beleuchteten die molekularen Mechanismen der Brustkrebs-Intravasation durch lymphatische Barrieren. Weiters wurden anti-intravasative Mechanismen von Flavoniden sowie von Arzneimitteln, die durch die FDA zugelassen sind, studiert.

Im MDA-MB231 Modell, wurde CIDD-Bildung durch 12(S)-HETE in Abhängigkeit von SOX18 und seinem transkriptionellen target PROX1 induziert. Die Hochregulation von RELA, NFKB1 und ICAM-1 war ebenfalls abhängig von SOX18. 12(S)-HETE, welches von

Brustkrebszellen sezerniert wird, löste ein Signal aus, welches durch 12-HETER und BLT2 an SOX18 weitergeleitet wurde. Darüber hinaus wird die Freisetzung von intrazellulärem Ca^{2+} durch Aktivierung von MLC2 durch 12 (S) HETE ausgelöst, was wiederum MYLK aktiviert, das unabhängig von GPR31 / 12-HETER ist

Mit Augenmerk auf NF- κ B zeigte sich, dass SOX18 und NF- κ B durch direktes (positives) feed-back miteinander verbunden waren. Dieser Mechanismus scheint spezifisch für LECs zu sein, da 12(S)-HETE in HUVECs (Blutendothel Zellen; BECs) SOX18 nicht induzierte. Hemmung von SOX18 und PROX1 durch siRNAs hemmte CCID-Bildung ähnlich stark wie Hemmung von RELA, NFKB1 oder NEMO durch siRNAs.

Arzneimittel, die durch die FDA (Food and Drug Administration, USA) zugelassen sind, besitzen zusätzlichen Aktivitäten für die sie ursprünglich gar nicht entwickelt wurden. Ein von dieser Gruppe zuvor durchgeführtes screening von ca. 600 FDA-zugelassenen Drogen ergab, dass etwa 15 davon Krebs-Spheroinduzierte CCID-Bildung in LEC und BEC monolayern hemmten. Einige davon sind z.B. Fenofibrate, Vinpocetine, Carbamazepine, Ketotifen, Nifumid, Dyclonine und Bepridil. Manche dieser Drogen hemmten CYP1A1 Aktivität, intrazelluläre Ca^{2+} -Freisetzung und die Expression von motility-Proteinen als mutmaßliche Intravasations-Mechanismen. Die Flavonoide Luteolin und Apigenin hemmten pro-intravasative Auslöser in MDA-MB231 Zellen wie etwa MMP1 Expression und CYP1A1 Aktivität. Diese Flavonoide, die in Heilpflanzen, Gewürzen, Kräutern und Früchten enthalten sind, hemmten CCID-Bildung.

Zusammenfassend sei festgehalten, dass Brustkrebszellen hauptsächlich über lymphatische Gefäße streuen und Hemmung der Krebszell-Intravasation könnte die Fähigkeit von Brustkrebszellen verringern, zu metastasieren. Daher könnte das Hemmen der Intravasation eine Strategie sein, um Medikamente für anti-metastatische Intervention zu entwickeln.

3. INTRODUCTION

3.1 Breast Cancer

There are several types of cancer that often cause mortality in women and men, including lung cancer, bronchus and colorectal. In addition, there are also special cancers predominated occurring in women, namely breast cancer and prostate cancer is mainly occurring in men (Siegel et al.2015). Lung cancer is leading with around 27% from 50% fatalities of all cancer deaths among these four types of cancers in women (Siegel et al. 2015). The main threat and the reason for most cancer deaths are not the primary neoplasias, but secondary tumours, the metastases (Leber and Efferth. 2008), which destroy the function of colonized organs (Fig.1). Breast cancer is the number one leading cause of cancerous deaths among women worldwide with poor prognosis and tangled cellular coding that requires in-depth understandings of signaling cascades involved in cell proliferation and cancer progression. In general breast cancer has 3 major types invasive, non-invasive and other include Paget's disease of the nipple that accounts 1-4% of breast cancer (Bodai and Tusio, 2015). In accordance to data from the GLOBOCAN 2012, more than one million cases of breast cancer have been documented globally, produced by the International Agency for Research on Cancer (IARC), estimate that breast cancer incidence was as high as 1,676,600 new cases in that year (Ferlay, et al, 2015). Approximately 25% to 30% of women with invasive breast cancer will die of their disease (Harris et al, 1992). Thus, the deaths experienced by about 75% of cancer patients can be caused by something outside cancer itself. (Richie and Swanson, 2003). In some cases, mortality occurs at a young age or less than 35 years with a more aggressive cancer diagnosis, while other cases occur in very old patients with age above 75 years which have the comorbid disease that accelerates breast cancer death.(Smith H, 1996). (Costanza, 2001).

Breast cancer is the leading cause of cancer death in productive women aged 20 to 59 years, (Siegel et al.2015). Nowadays, with the advance of modern research in the field of technology in diagnosing and curing breast cancer, however, there are still some clinical problems that need to be resolved urgently. Some of these problems include: (1) who can be prevented and when to prevent cancer? (2) Specific and sensitive methods required for diagnosing, (3) causes of tumor development (4) how can breast cancer be treated? (5) how to predict the resistance which caused by therapy, along with prevention and overcomes it ?. Overcoming these problems are increasingly difficult due to some facts in molecular and clinical level

about the heterogeneity of this disease (Perou, et al, 2000, Sorlie et al, 2001). It should be a priority to further improve the better technology in the diagnosing of a breast in order to find new specific targets for therapy of breast cancer.

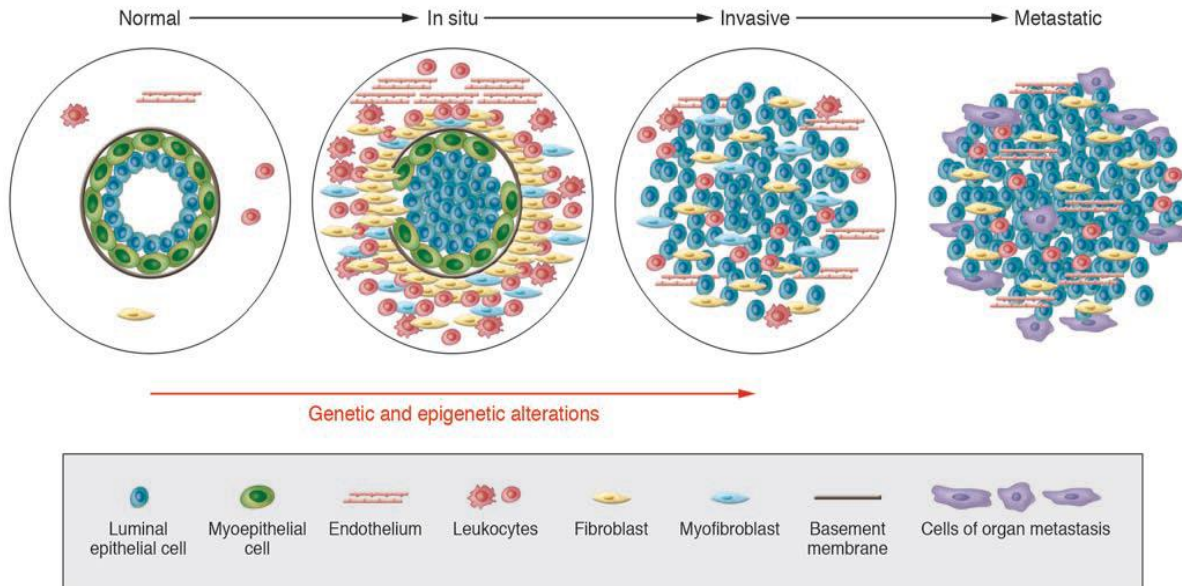


Figure 1 **Breast tumor model expansion (Polyak, J Clin Invest 2007)**

Progression of carcinoma in the schematic view from normal, in situ, invasive, and metastatic. Normal breast ducts are composed of the basement membrane and a layer of luminal epithelial and myoepithelial cells. Cells composing the stroma include various leukocytes, fibroblasts, myofibroblasts, and endothelial cells. In in situ carcinomas, the myoepithelial cells are epigenetically and phenotypically altered and their number decreases, potentially due to degradation of the basement membrane. At the same time, the number of stromal fibroblasts, myofibroblasts, lymphocytes, and endothelial cells increases. Loss of myoepithelial cells and basement membrane results in invasive carcinomas, in which tumor cells can invade surrounding tissues and can migrate to distant organs, eventually leading to metastases.

3.2 Metastasis

Breast cancer–related deaths are predominantly due to the "untreatable" nature of metastatic breast cancer (MBC) at the moment. By the time the patient was first diagnosed with primary breast cancer, less than 50% eventually developed into metastasis, interestingly, approximately 6% of patients have metastases in the early of diagnosis. (Jing Lu, et al, 2009). In most cases, metastasis has already occurred by the time cancer is observed (Schroeder et al. 2012). The classical simplification of metastasis which includes multiple steps (Fig.2). Metastasis is a multi-step phenomenon and an early step in this process is called tumour intravasation, which is when cells (particularly breast and colon cancer cells) enter the lymphatic vasculature. The number of lymph nodes that are colonised by breast cancer cells

(the "lymph node status") is a prognostic marker (Sobin et al. 2009) and a rate-limiting step determining distant organ metastasis (Kerjaschki et al. 2011).

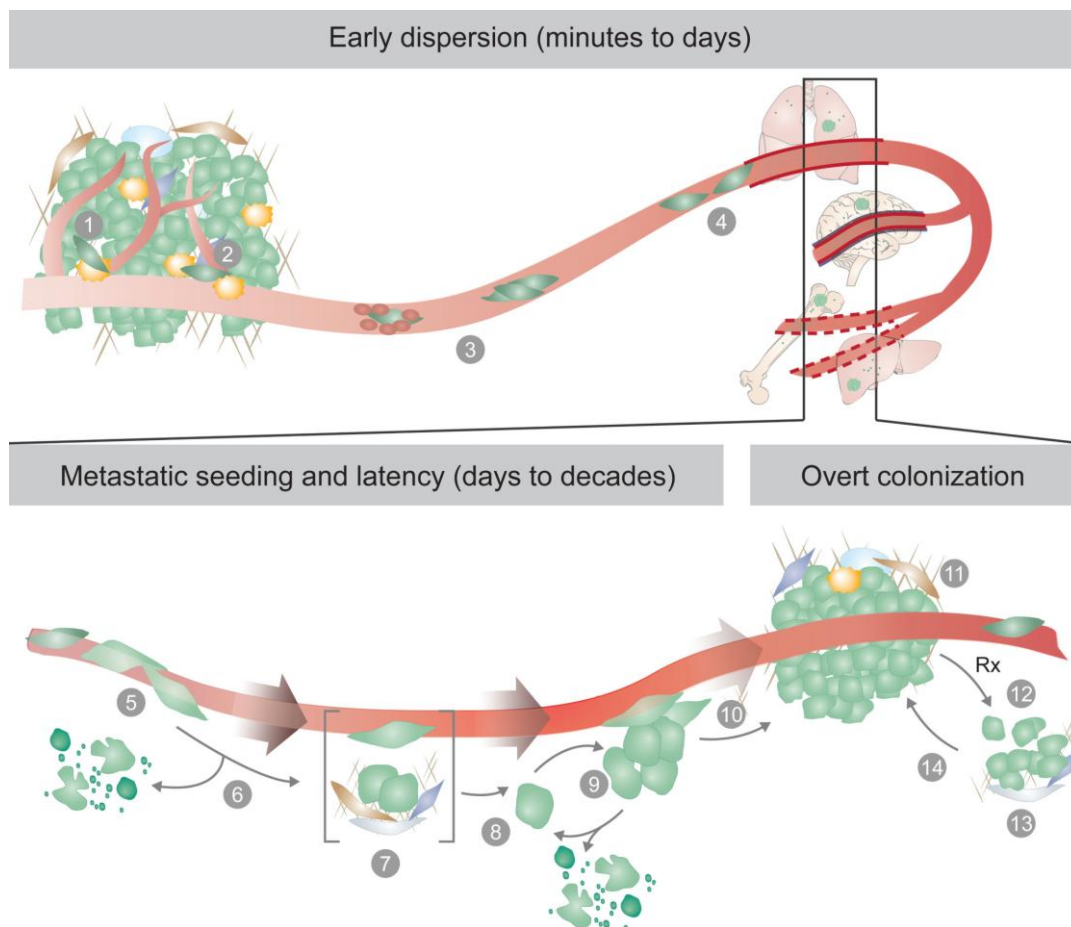


Figure 2. **The multiple steps of metastasis** (Massague and Obenauf, Nature, 2016)

Metastasis occurs through multiple steps: (1) local invasion of primary cancer cells in the primary tumor, (2) Cancer cells enter the lymphatic or blood vessels in a process called intravasation, (3) circulation of cancer cells (4) Cancer cells survive and travel through circulation or lymphatic system and, (5) Cancer cells leave lymphatic or blood vessels in a process called extravasation. After extravasation, metastatic colonization comprises, Cancer cells adapt, grow and colonize secondary tissues.

Along with the improvement of cancer therapies in the last decades, the therapeutic strategies that specifically target metastasis are still far from being efficient. Surgical intervention is the major strategy to treat few patients with metastatic cancer, 80% of patients who have metastatic cancer have a survival time of fewer than 5 years, in spite of surgery and other adjuvant treatments have been done (Steeg 2006).

3.2.1 Local Invasion

In order to initiate the metastatic process, cancer cells need to break away from the primary tumor. The ability of cells to invade and migrate must be possessed in order to enable the cell to move and degrade. Prior to end in the lymphatic vessels, these cells pass through the extracellular matrix of the surrounding tissue and into the blood vessels. Cancer cells then reach the secondary site via a highway provided by the blood vessels and lymphatics. Clinical indications of metastasis can be characterized by cancer cells in the lymph nodes. This may be derived from lymph nodes due to a direct relationship between the location of the formation of the primary tumor and lymphatic vessel which is the pathway out of tumor cells (Joyce and Pollard, 2009).

There are several factors during tumour development including MMP1 and MMP9 which belongs to extracellular matrix metalloproteinases, chemokines as pro-migratory factor, EGF and FGF-1 as growth factors and cathepsins (Kassenbrock et al, 2010, Quail DF, Joyce , 2013) secreted by Cytoskeletal rearrangements within the cancer cells (Hall A, 2009), combined with the action of adhesive interactions) which will drive cancer cell invasion and migration through the stroma. Cancer cells may migrate as single cells boring a path through the extracellular matrix (Giampieri S, et al., 2009), move along collagen fibers (Roh-Johnson M, et al,2014) or migrate collectively as ensembles that forge ahead from the tumour invasion front (. Friedl and Gilmour, 2009). Cancer cells undergo the morphological and phenotypical changes such as "epithelial to mesenchymal transition'(EMT) which will be induced by tumour microenvironment by producing specific growth factors (e.g. TGF- β and) and cytokine which facilitated inflammation (Reddy et al. 2012). Amoeboid migration (rounded; CAT, MAT) is dissociation process of individual cells from cell clusters to achieve the features of mobile amoeboid cells (Pankova et al. 2010; Brabek et al. 2010; Thiery et al. 2009; Wong and Hynes 2006).

3.2.2 Intravasation

Intravasation is the process when cancer cells successfully enter the lymphatics-or blood. In order to intravasate, the surrounding tissue into the blood vessels must first be invaded by tumor cells. Thus, Local angiogenesis will be triggered by the tumor, the invaded blood vessels have weak cell-cell junctions so that cancer cells can easily enter the blood vessels (Weis and Cheresch, 2011). Transendothelial migration (TEM) is a condition where in the

3.2.3 Survival in the circulation

Cancer cells need to survive in the vessel as well as at the disseminating site to develop metastatic after circulating tumour cells arrest in microvessels in distant tissues. In order to protect the cells from immune attack and stress of a hostile environment, cancer cells need Platelets, Regulatory T cells (Treg cells) and macrophage to survive (Fig.4) (Kitamura et al, 2015). Platelets play an important role after intravasation (Aisha, E. C. et al.2004). Due to protecting cancer cells from NK cells, platelet and the resultant fibrin clot formation should be activated and promote early survival cancer cells in circulation and metastatic sites (Kitamura et al, 2015). When clot formation initiating the tumour, vascular cell adhesion molecule 1 (VCAM1) and vascular adhesion protein 1 (VAP1; also known as AOC3 will be expressed by activated endothelial cells. VCAM1 and VAP1 recruits macrophage (Ferjancic, Š. et al). Cancer cell bind to CD49d expressed by the macrophages, which increases metastasis (Chen, Q et al, 2011). To increase survival of circulating metastasis-initiating needs activation of RANKL which is secreted by Treg cells in a primary mammary tumour (Tan, W. et al.2011).

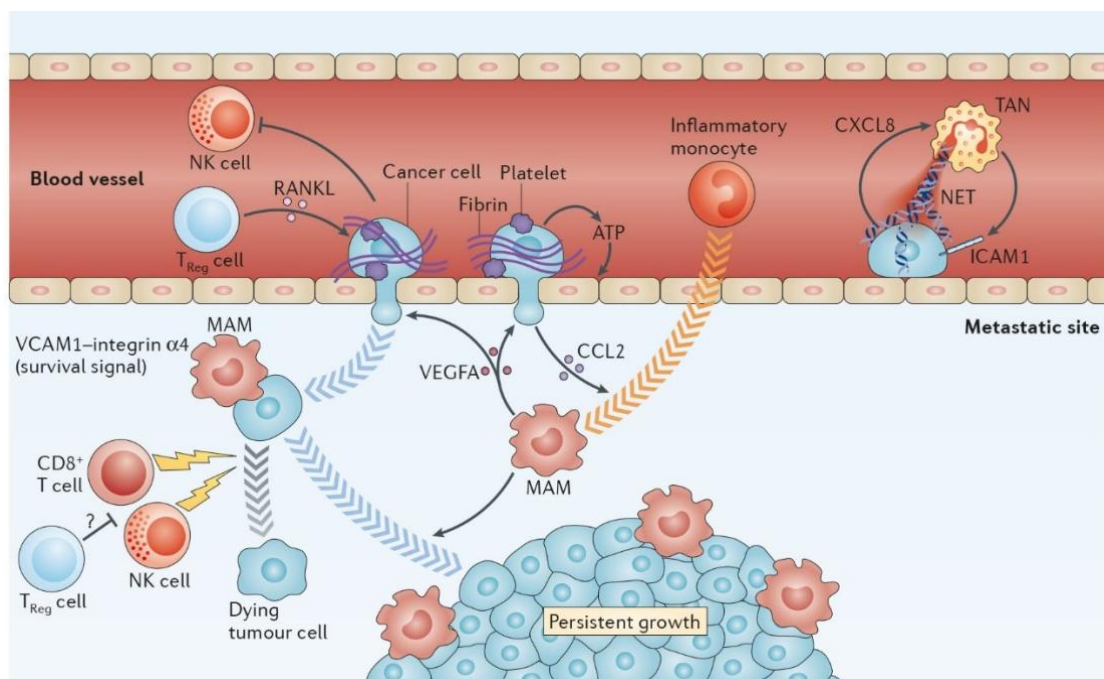


Figure 4. Survival in the circulation (Kitamura et al, Nature Reviews, 2015)

In order to survive after leaving primary sites, cancer cells need to exist in the circulation. This survival process is helped by platelets, regulatory T cells (Treg cells) and macrophage. Fibrin clot will be formed by platelets and others survival signal and by tumour-associated macrophages (TAMs). TAMs then activate AKT signaling via vascular cell adhesion molecule 1 (VCAM1) on tumour cells, as well as regulatory T (Treg) cells, through the production of receptor activator of nuclear factor- κ B ligand (RANKL).

3.2.4 Extravasation

In cancer metastasis especially on extravasation regulation, chemokines, and their seven transmembrane-spanning G protein-coupled receptors have important roles (DeNardo, D. G. et al. 2009, Casazza, A. et al. 2013). The adhesion of cancer cell to endothelial cells marked as the first step of extravasation, tumour cells then cross the endothelium known as transendothelial migration (TEM) and final step is when cancer cells invasion into surrounding matrix (Chen et al. 2013). It is noteworthy that different tumour types preferentially metastasize to target different organs. Breast cancer cells preferentially metastasize to the bone, brain, lung, and liver; prostate cancers metastasize to the bone; and lung cancers metastasize to the bone, liver, and brain (Casazza, A. et al. 2013).

Extravasation of cancer cells is promoted by metastasis-associated macrophages (MAMs). MAMs are the result of the differentiation of inflammatory monocytes due to the secretion of the chemokine ligand CC 2 (CCL2) of the trapped cancer cell in emboli. Vascular endothelial growth factor A (VEGFA) is secreted by MAMs, then affect the growth of emigrated cancer cells and their survival. At metastatic sites, cell tumors play a role in secreting the chemokine ligand 8 (CXCL8) resulting in interactions between tumor-associated neutrophils (TANs) that can enhance the entrapment of circulating cancer cells with intercellular adhesion molecule 1 (ICAM1). This mechanism occurs due to the ability of TANS to producing neutrophil extracellular traps (NETs) (Shibue et al. 2012).

There are several molecules that play a role in the proliferation of cancer cells in the surrounding tissues, including focal and adhesion kinase (FAK) and Ras homolog family member F (RHOF) races, these two molecules together with the DIAPH3 protein and integrin $\beta 1$ are driven the interaction between the cancer cells which is extravasating and the basement membrane around the blood vessels. Prior to interacting, the formation of filopodium-like protrusions is triggered by a tumor cell that has crossed the endothelial wall and invaded the matrix surrounding the blood vasculature (Shibue et al. 2012).

3.2.5. Colonization

Colonization of metastasis is a biological state formed by cancer cells on the secondary site, to achieve this level, the cancer cells must survive and grow then break free from their primary site and survive the circulatory system (Szmulewitz, et al. 2015). The successful colonization outcome is characterized when the tumor rapidly expanding macrometastasis. Interestingly, the composition of the microenvironment of each organ sites where is the cancer cell colonized in secondary tissue are unique (Fig. 5). (Nguyen, et al, 2009)

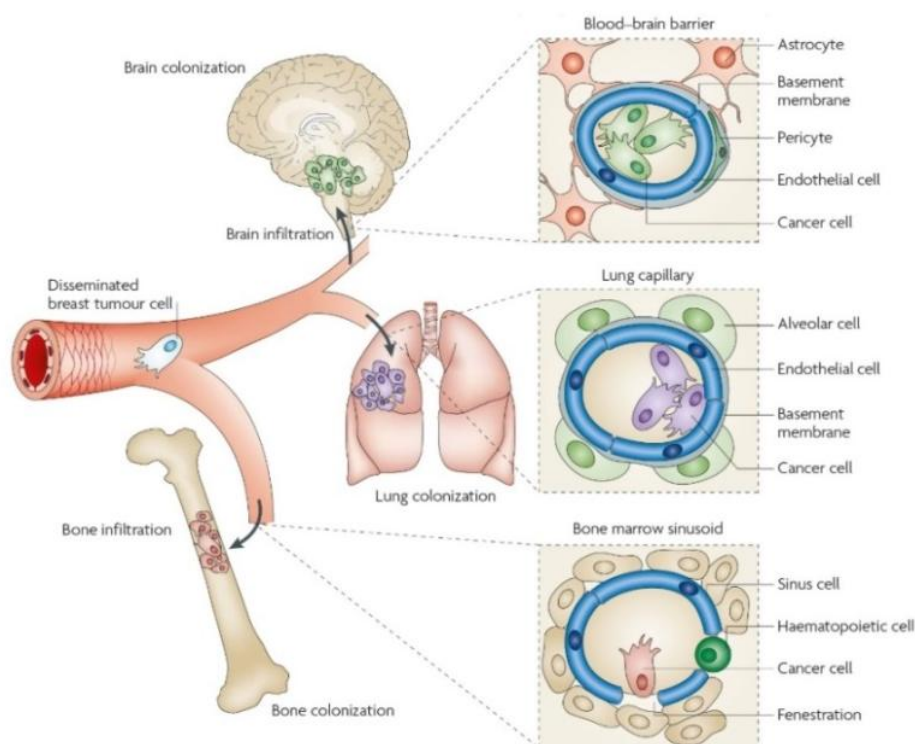


Figure 5. **Metastasis of breast tumour cell.** (Nguyen et al, Nature 2009)

The potential barriers to metastasis in different sites are exemplified by the case of breast cancer and the anatomy of capillary walls in different target tissues. Breast cancer cells entering the circulation can infiltrate a distant organ if they carry the necessary functions for extravasation. The fenestrated structure of bone marrow sinusoid capillaries is more permissive to cancer cell infiltration than the contiguous structure of lung capillary walls. Brain capillaries are more difficult to penetrate, owing to the unique nature of the haematoencephalic barrier. Infiltration through these barriers selects for tumour cells that express the necessary extravasation functions.

There are several mechanisms of extravasated cancer cells after their reach new environmental as successful colonization markers, including (i) the ability oncogenic signaling together with gene induction to modify the distant tissue, (ii) the influence of inhibitor molecules and immune system function that can interfere with the life cycle of

cancer cells , (iii) survival signals in new environments influenced by stimulation due to the interaction between resident cells and stem cell niches, and (iv) in order to facilitate the proliferation of the cancer cells and their growth microenvironment, cell populations have to be able to modify or transform new host microenvironment (Jin and Mu 2015; Obenauf and Massagué 2015).

3.3 Three-dimensional *in vitro* assay (CCID assay) resembling intravasation

As we know, about 90% of cancer deaths are caused by metastasis. Metastasis is a major life-threatening factor of patients with cancer.. Metastasis is multiple processes which includes multiple steps and involves not only tumour cells but also stromal and vascular interactions and environmental stimuli. In general, the first step of metastasis is the process of intravasation where the invasion by primary tumor cells into blood vessels and lymphatics vasculature. In case of breast cancer, cancer cells are more frequent to the lymphatic routes, consequently, prognostic markers for outcomes in patients could be shown by tumor cells colonization in lymph nodes (Carlson et al. 2009).

Due to conveniently and easy to set up for high throughput screening, two dimensional (2D) monolayer cell culture models were the most of previous *in vitro* models used in cancer research. However, these 2D monolayer models had a limitation, lack of stromal environment as this is important for the metastatic process (Kim 2005). To overcome some of these limitations, Kim had been developed a technique called three dimensional (3D) aggregates. The advantages of this technique are caused by various components of cell phenotypes, such as necrotic cells and proliferating and nonproliferating cells. This system also provides a very similar situation within the intact tumor, in the presence of non-vascularised tumor regions (Yamada and Cukierman 2007). Thus, this 3D cell culture model is suitable to identify the interaction between the tumor and the stroma in relation to the drug discovery. In some types of cancer, the first step of metastasis is the intravasation of primary tumour cells into the blood vasculature and lymphatics (Lovitt et al. 2014).

It was recently demonstrated that MCF-7 ductal breast cancer cells express 15LOX and secrete its metabolite 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), which is also produced by 12LOX. 12(S)-HETE forces junction retraction (Honn et al. 1994) between lymph endothelial cells (LECs) of the lymphatic vessel barrier adjacent to a tumour. This

results in large gaps within the endothelial cell wall, so-called "circular chemically induced defects" (CCIDs), which are entry gates for breast cancer emboli into lymphatics thereby causing lymph node metastases (Kerjaschki et al. 2011). An assay, which correlates with the situation in human patients and was validated in SCID mice, measures the size of CCID formation (Fig. 6; Kerjaschki et al. 2011) and can be modified for other cancer cell lines.

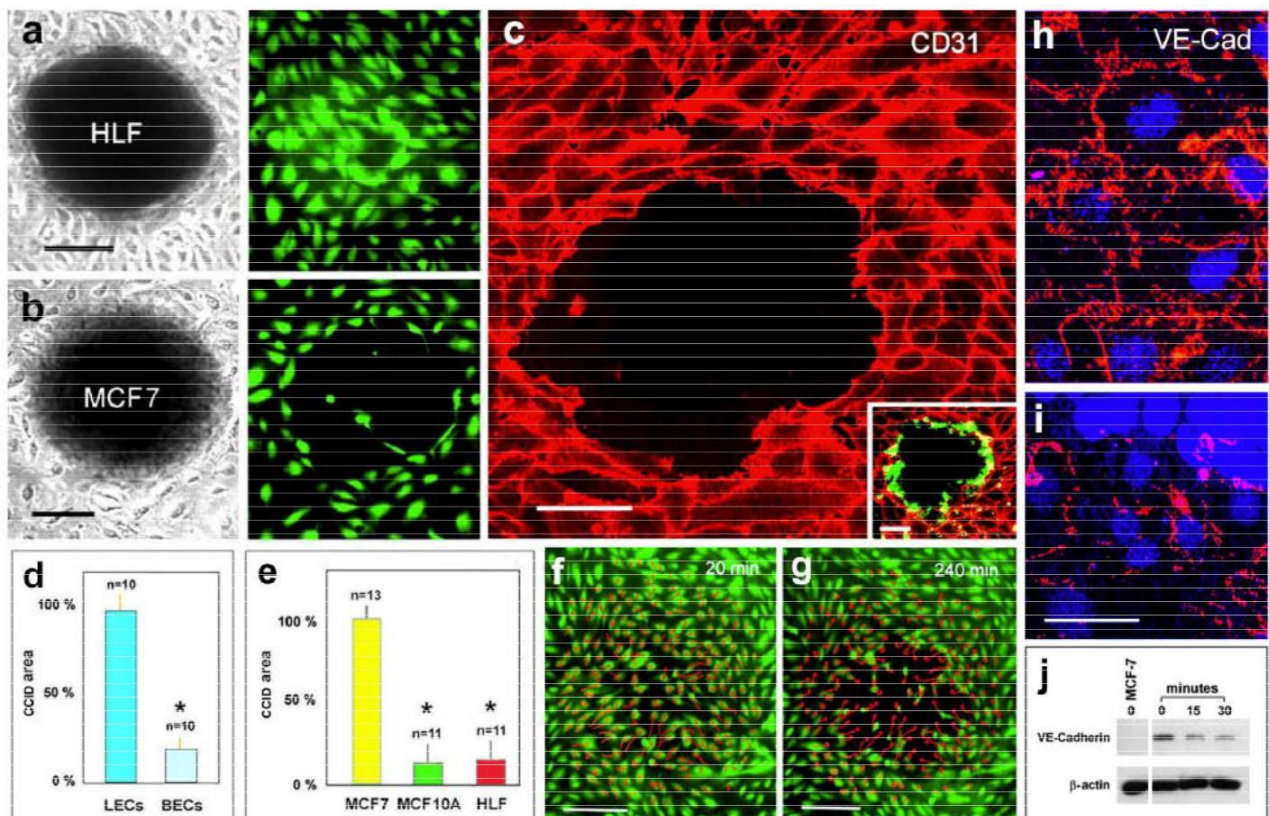


Figure 6. MCF-7 Breast cancer spheroid induces CCID in LECs monolayers and interfere VE-cadherin at the CCID's. (Kerjaschki et al. 2011)

a, After 4 h of co-incubation, human lung fibroblasts (HLF) spheroid of fails to induce the gap in a monolayer of human lymphatic endothelial cells (LECs, cyto-tracker tagged in green). **b**, MCF7 spheroids induce gap formation on CCIDs. **c**, A MCF7 spheroid induced CCID is outlined when the LEC borders are stained for CD31 (red, confocal image). **Insert**, LECs (demarcated with CD31 in red) at the margin of the CCIDs show expression of MYPT1 (green) indicating cell mobility (confocal image). **d**, MCF7 spheroids induce CCIDs preferentially in LEC monolayers (left bar), but significantly less (14.3% of lymphatics, $p=0.0047$) in monolayers of microvascular blood endothelial cells (BECs, right bar). **e**, When compared to spheroids of MCF7 cells (left bar, 100%), CCID 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%, $p < 0.0001$). Bars indicate SEM. **f** and **g**, Tracings of LEC migration (red lines, starting positions are marked by circles) beneath a spheroid during a 4 h co-incubation. **h**, Confocal image show continuous LEC junctions of VE-cadherin at distance from a spheroid. **i**, At the spheroid's margin the VE-cadherin pattern is disrupted. **j**, Confluent LECs monolayer were incubated with 1 μ M 12(S)-HETE for 2 different times 15 and 30 min, or with solvent (0) and cell lysates were immunoblotted with antibodies to VE-cadherin or β -actin. As controls VE-cadherin⁺ MCF7 cells were used. Scale bars: **a**, **b**, **f** and **g** 100 μ m, in 25 μ m, **h** and **i** 50 μ m. (Kerjaschki et al. 2011).

3.4 Mechanism of the arachidonic acid metabolite 12(S)-HETE contributing to CCID formation

Arachidonic acid is a molecule that plays a role in cell signaling in humans. Lipoxygenase enzymes include ALOX12 and ALOX15 using arachidonic acid either as a substrate or the metabolites such as 12 (S) HETE which is involved in several types of cancer (Kerjaschki et al. 2011). In addition, enzyme epoxygenase and cyclooxygenase also reported plays role in cell signaling. 12(S)-HETE induces CCIDs in lymphatic endothelial cell monolayers (Fig.7) (Vonach et al. 2011; Kerjaschki et al. 2011).

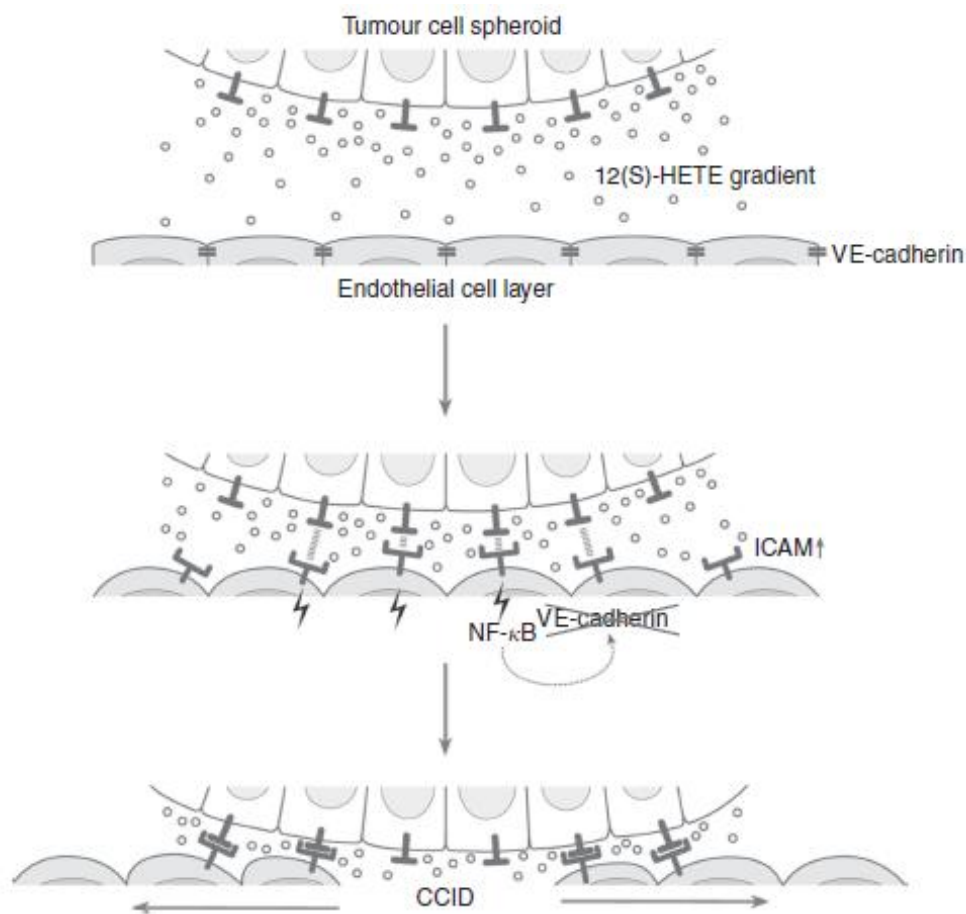


Figure 7: Interaction between tumour cell spheroid and endothelial cells. (Vonach et al. 2011)

MCF-7 tumour cell spheroid is secreted 12(S)-HETE and causes NF-κB activation associated with ICAM-1 expression. Tumour spheroid attaches to endothelial cells and additionally NF-κB inhibits the adhesion protein VE-cadherin. Finally, a migration of endothelial cells occurs and CCID is formed

Besides ALOX12/15 cytochrome P450 isoenzyme CYP1A1 is also able to catalyse 12(S)-HETE formation (Capdevila et al. 2000, Powell et al, 2015) and triple negative MDA-MB231 breast cancer cells secrete 12(S)-HETE through CYP1A1 activity, thereby causing CCID formation in LEC barriers (Nguyen et al. 2016). There are several factors that influence the transcription of CYP1A1, including (i) aryl hydrocarbon receptors (AHR), (ii) environmental factors which can be resisted by different the cells in various ways and (iii) mediators , such as a cytosolic ligand-activated transcription factor that work to bind polyaromatic hydrocarbons in the process of transcription (Feng et al, 2013). In MDA-MB231 cells the expression of CYP1A1 induces 12(S)-HETE synthesis which then activates 12(S)-HETE receptor (12-HETER) in LECs, triggers Ca^{2+} -release and MLC2-activation, which contributes to CCID formation in LEC monolayers (Nguyen et al. 2016).

3.5 Mechanism of NF- κ B activity contributing to CCID formation

In order to regulate several gene expressions of proteins and cytokines is required a protein complex called Nuclear factor- κ B or known as NF- κ B (Simone and Vucic, 2012). The inactive form of NF- κ B is located in the cytoplasm, this form binding several molecules such as NF- κ B inhibitors (IkB and IkB α) and several related ankyrin repeat-containing proteins (Fig.8). On the activation of NF- κ B, IkB and IkB α as NF- κ B inhibitors will be phosphorylated and degrade through the proteasome. Thus, on regulation some gene expression such as cytokines and growth factors, NF- κ B must translate into the nucleus and eventually bind to the promoter region (Fabre et al. 2012; Körber et al., 2013).

NF- κ B including two types of pathways: classical (or canonical) and alternative (or non-canonical) pathways. The canonical pathway is affected and activated by several signals such as IL-1 (interleukin -1), , lipopolysaccharide (LPS) and tumour necrotic factor α (TNF α) and has a crucial role in the regulation of inflammation and immunity. This pathway including NEMO, p105/NF- κ B1, RELA, IKK α and IKK β . On the other hand, The non-canonical pathway including NIK, p100/NF- κ B2 and RELB are triggered by CD40 ligand, lymphotoxin- β 2 which belongs to tumor necrosis factor cytokine and B cell activating factor (Fig.8) (Oeckinghaus et al. 2011).

By a pathway, which has not been elucidated yet, 12(S)-HETE activates NF- κ B in LECs causing ICAM-1 expression and adhesion to the tumour emboli, which is rate limiting for CCID formation. Activation of NF- κ B plays a role in several mechanisms of tumour cells

such as proliferation and angiogenesis (Brown et al. 2008; Flister et al. 2010). NF- κ B is a transcription factor usually responsible for the cytokine-inducible expression of a plethora of pro-inflammatory genes. It has been shown that NF- κ B activity of tumour cells, as well as, LECs contribute to CCID formation, as separate treatment of either tumour spheroids or LECs with Bay 11-7082, a specific irreversible I κ B α inhibitor attenuated CCIDs (Vonach et al. 2011). The inhibition of NF- κ B translocation with Bay11-7082 or siRNA-mediated suppression of RELA and NF- κ B1 significantly attenuated CCID formation by downregulating the expression of NF- κ B and its target ICAM-1 in LEC monolayers (Nguyen et al. 2015). SOX18 reportedly regulates NF- κ B during inflammation and angiogenesis upon cytokine treatment (Basilio J et al. 2013)

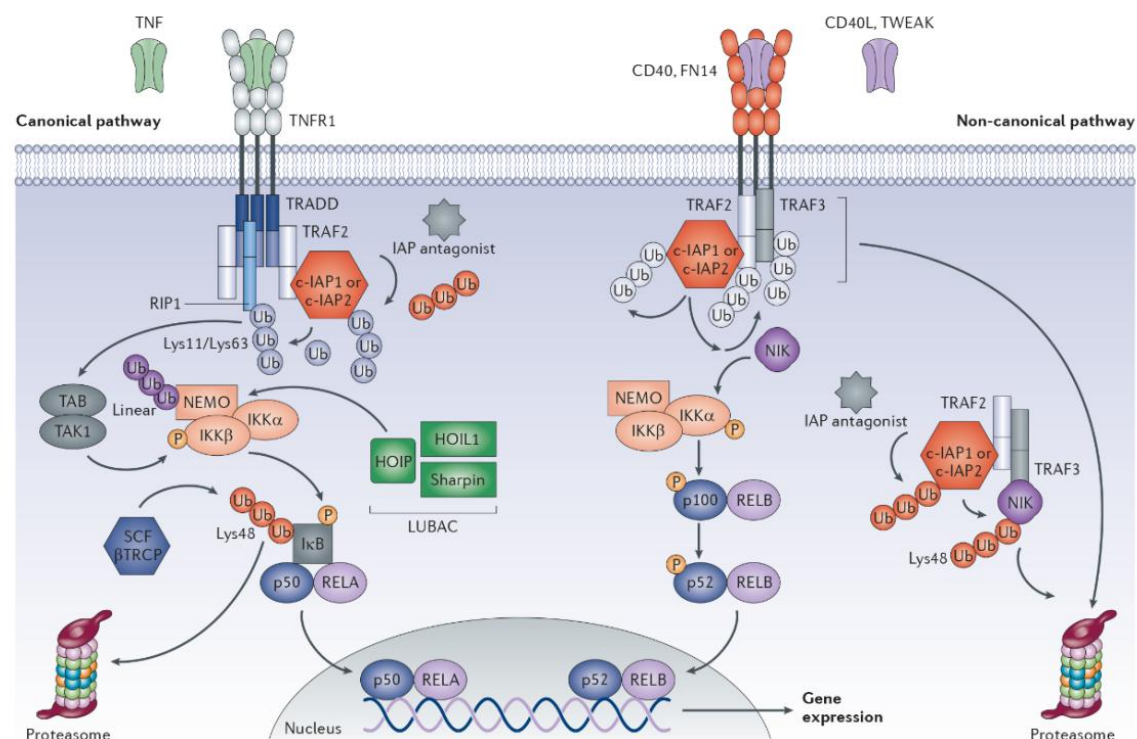


Figure 8. NF- κ B signaling pathways: canonical and non-canonical

Canonical and non canonical NF- κ B pathways. TLRs and proinflammatory cytokines such as TNF α and IL-1 are triggered the canonical pathway and, leading to activation of RelA that regulates expression of proinflammatory and cell survival genes. (IKK) complexes, as well as the linear ubiquitin chain assembly complex (LUBAC), to stimulate the activation of IKK β . This leads to the degradation of the inhibitor of NF- κ B (I κ B) and the translocation of p50 and the transcription factor RELA to the nucleus. The non-canonical NF- κ B pathway is activated by LT β , CD40L, BAFF, and RANKL, but not TNF α , and results in activation of RelB/p52 complexes. Activation of the alternative pathway regulates genes required for lymph-organogenesis and B-cell activation. These pathways are characterized by the differential requirement for IKK subunits. IKK β regulates activation of the canonical pathway through phosphorylation of I κ Bs and requires the IKK γ subunit but not IKK α , whereas IKK α is required for activation of the alternative pathway through the phosphorylation and processing of p100, the precursor for p52, but this is independent of both IKK β and IKK γ .

3.6. SOX18, PROX1 and Lymphatic endothelial cell development

Three principal roles of the lymphatic system are to ensure the maintenance of the tissue and blood volume, to generate immune cells from lymphoid and to transport of dietary fat from the intestine to the liver. In cancer case, metastasizing cancer cells are provided by conduits in the lymphatic vessels to leave the primary tumor site and to establish secondary tumors in regional lymph nodes until they reach distant organs (Albrecht and Christofori, 2011). Lymphatic endothelial cells (LECs) develop molecular mechanism whereas lymphangiogenesis is induced by vascular endothelial growth factor (VEGF-C) (Alitalo, 2011), which has the role on LECs that differentiate from venous endothelial cells at midgestation (Karkkainen, M.J. et al, 2004, Wigle JT et al, 1999). PROX1 is one of the transcription factors which is expressed by LECs. In embryogenesis, lymph endothelial cell differentiation is preceded by an interaction between COUP-TFII venous nuclear receptor with the SOX18 transcription factor which will subsequently express PROX1. (François, M. et al, 2008, Srinivasan, R.S. et al 2010) (Fig.9)

PROX1 belongs to a homeodomain family of transcription factors is a mammalian homolog of the *Drosophila* prospero gene and one of crucial homeobox gene for the development of the mammalian lymphatic vasculature. In addition, PROX1 also acts as a tumor suppressor (Doe CQ et al 1991, Betschinger J et al, 2006). Known as a key player on lymphangiogenesis, PROX1 protein plays an essential role in embryogenesis and in the development of various organs and normal tissues such as lens, heart, liver, kidney, skeletal muscles, pancreas, and central nervous system (Rudzinska M et al, 2017, Srinivasan, R.S. et al 2010).

As a marker of lymphatic endothelial cells, PROX1 also plays a pivotal role in determining the lymphatic endothelial cells characteristics (Wigle JT et al, 1999, Hong YK, et al, 2002). As an upstream regulator of this transcription factor, SOX18 regulates PROX1 expression in lymph endothelial cell (François et al. 2008). The expression of the protein level of PROX1 as well as the transcript level has been found in several types of cancer in humans, such as colon cancer and pancreatic cancer (Rudzinska M et al, 2017).

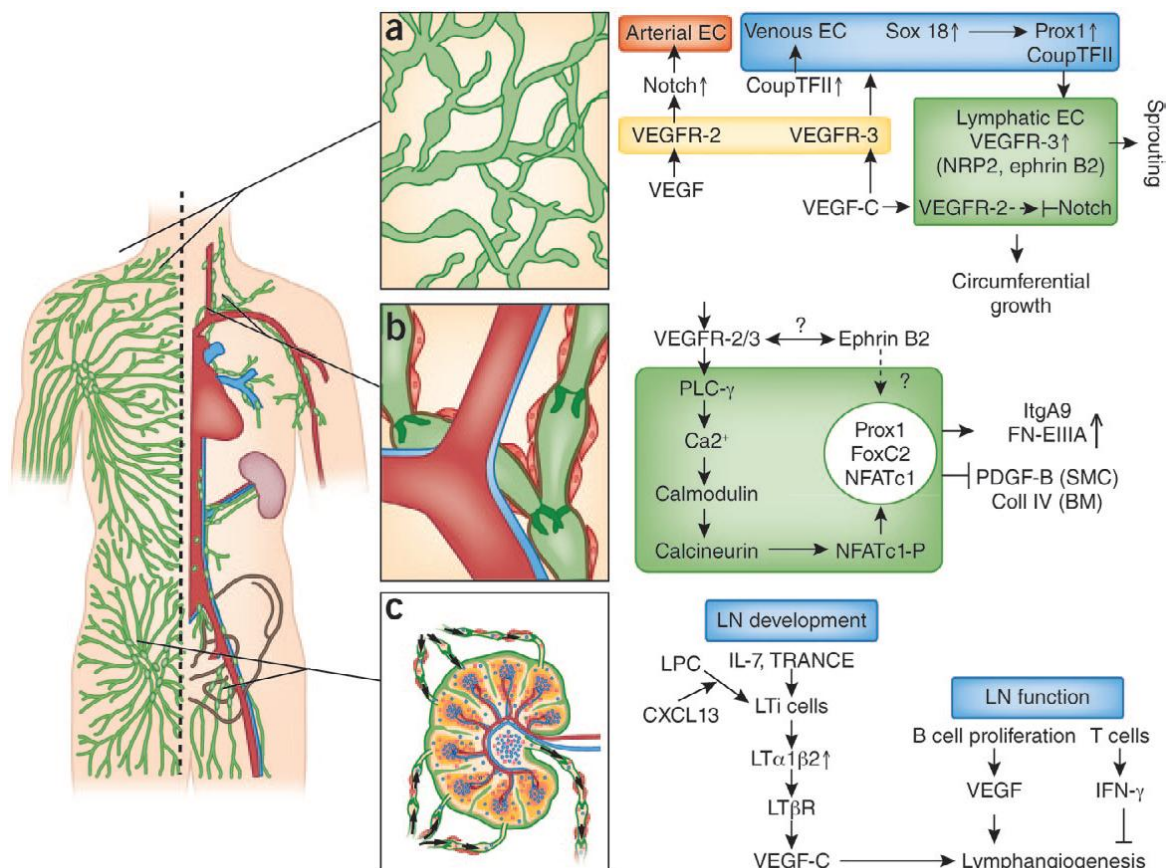


Figure 9. Molecular mechanisms involved in the lymphatic vasculature. (Alitalo, K. Nature Medicine, 2011)

(a) SOX18 activates PROX1 after the arteriovenous differentiation controlled by two transcription factors which are Notch and COUP-TFII. Lymphatic capillaries are derived from venous endothelial cells. PROX1 then interacts with COUP-TFII and induces lymphatic endothelial differentiation involving the elevated expression of VEGFR-3. VEGF-C then induces the sprouting of the LECs to generate new vessels. The size of lymphatic vessels can also increase by VEGF-C and VEGF (b) A calcium-induced signal via phospholipase C-g (PLC-g) and calmodulin to calcineurin that dephosphorylates the NFATc1 transcription factor are needed to form the lymphatic valves, which enters the nucleus and induces valve-specific genes in a complex with FoxC2. VEGFR-2 and VEGFR-3 and ephrin B2 are upstream regulators of the pathways necessary for valve development³². PDGF-B and collagen IV (ColIV) production is inhibited simultaneously. ItgA9, integrin a9; FN-ElIIA, embryonic form of fibronectin; BM, bone marrow. (c) When IL7 start developing lymph nodes and TRANCE stimulate LTi cells that develop from lymphatic precursor cells (LPCs) under the influence of the chemokine CXCL13, then lymphangiogenesis is first induced.

The transcription factor SRY-related box 18 (SOX18) drives lymphatic vessel development in tumour-induced neo-lymphangiogenesis. In a mouse model, genetic disruption of SOX18 established the SOX18 protein as a molecular target to protects from tumour metastasis. SOX18 plays in regulation of lymphangiogenesis (Francois et al, 2008), angiogenesis (Duong T et al, 2014) and hair follicle differentiation (Pennisi D, et al, 2000). The transactivation of

the Prospero homeobox 1 (PROX1) gene is caused by the binding of SOX18 to its proximal promoter (Rudzinska M et al, 2017), the hallmark of LEC identity.

Loss of function in SOX18 is sufficient to change the patterning of the lymphatic vasculature cause lymphatic dysfunction in the human syndrome contributes to the hypotrichosis-lymphoedema-telangiectasia (HLT) (Alitalo, 2011, Francois et al, 2008, Srinivasan, R.S. et al 2010). On the other hand, several cancer types in humans such as ovarian cancer and gastric cancer indicate the overexpression of SOX18 (Betschinger J et al, 2006, Zinovieva RD et al, 1996, Wigle JT et al, 1999).

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5. AIM OF STUDY

Due to the complexity of metastases disease and inadequate test kits, the prevention and treatment of cancer with metastasis are very limited. A 3D in vitro assay, which faithfully resembles intravasation in metastasis, has been recently established and validated by the group of Prof. Dr. Kerjashki (Kerjashki et al, 2011), and the thesis aims to investigate the molecular mechanisms of cancer cell interaction with the stroma, in particular, the intravasation of MDA-MDB231 breast cancer spheroids through the lymph endothelial barrier

Apart from upregulating MMPs and MLC2 in cancer cells, and PROX1 and guidance proteins in endothelial cells, the detailed contribution of SOX18 in endothelial barrier dis-integration (which facilitates tumour intravasation) is entirely unknown. Therefore, the major aim of this investigation attempts to elucidate 12(S)-HETE signaling in LECs focussing on the unexplored gap between SOX18 and NF- κ B, as NF- κ B itself prominently contributes to intravasation through lymphatics. This involves investigating the contribution of the 12(S)-HETE receptors, 12-HETER and BLT2, as well as of the downstream targets PROX1 and ICAM1. Another aim is the investigation of the molecular mechanism by which some screened FDA-approved clinical drugs and flavanoids inhibit intravasation.

Taken together, as breast cancer cells predominantly spread through the lymphatic vasculature, inhibiting cancer cell intravasation may reduce the ability of breast cancer cells to metastasize. Therefore, inhibiting intravasation could be a strategy to develop medicines for anti-metastatic intervention.

6. RESULTS

This cumulative doctoral dissertation is based on the following research articles which developed from the investigation of molecular mechanisms of breast cancer interaction with the stroma, in particular, the intravasation of MDA-MB231 breast cancer spheroids through the lymph endothelial barrier in vitro

6.1. **A Fristiohady**, D Milovanovic , S Krieger , N Huttary , CH Nguyen, J Basilio , W Jäger , R de Martin , G Krupitza. 12(S)-HETE induces lymph endothelial cell retraction in vitro by upregulation of SOX18

Accepted to International Journal of Oncology

6.2. J Hong, **A Fristiohady**, CH Nguyen, D Milovanovic, N Huttary, S Krieger, JQ Hong, S Geleff, P Birner, W Jäger, A Özmen, L Krenn, G Krupitza. Apigenin and Luteolin Attenuate The Breaching of MDA-MB231 Breast Cancer Spheroids Through The Lymph Endothelial Barrier *In Vitro*.

Frontiers in Pharmacology (2018) 9 : 220 <https://doi.org/10.3389/fphar.2018.00220>

6.3. S Holzner, S Brenner, AG Atanasov, D Senfter, S Stadler, CH Nguyen, **A Fristiohady**, D Milovanovic, N Huttary, S Krieger, Z Bago-Horvath, O de Wever, I Tentese, A Özmen, W Jäger, H Dolznig, VM Dirsch, RM Mader, L Krenn, G Krupitza. Intravasation of SW620 colon cancer cell spheroids through the blood endothelial barrier is inhibited by clinical drugs and flavonoids in vitro

Food and Chemical Toxicology (2017) : 114-124 <https://doi.org/10.1016/j.fct.2017.11.015>

6.4. CH Nguyen, N Huttary, AG Atanasov, W Chatuphonprasert, S Brenner, **A Fristiohady**, J Hong, S Stadler, S Holzner, D Milovanovic, VM Dirsch, B Kopp, P Saiko, L Krenn, W Jäger, G Krupitza. Fenofibrate Inhibits Tumour Intravasation By Several Independent Mechanisms In A 3-Dimensional Co-Culture Model.

International Journal of Oncology (2017) 50: 1879-1888, DOI: 10.3892/ijo.2017.3956

6.5. CH Nguyen, S Brenner, N Huttary, Y Li, AG Atanasov, VM Dirsch, S Holzner, S Stadler, J Riha, S Krieger, D Milovanovic, **A Fristiohardy**, I Simonitsch-Klupp, H Dolznig, P Saiko, T Szekeres, B Giessrigl, W Jäger, G Krupitza. 12(S)-HETE increases intracellular Ca²⁺ in lymphendothelial cells disrupting their barrier function in vitro; stabilization by clinical drugs impairing calcium supply.

Cancer Letters 2016: 380(2016) 174-183 doi: 10.1016/j.canlet.2016.06.022

**12(S)-HETE induces lymph endothelial cell retraction in vitro
by upregulation of SOX18**

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de Martin , G Krupitza

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As the first author i contribute to designing the experiments, carrying out all the experiments including cell cultures experiments, CCID assay, siRNA transfection, immunoblotting/western blotting and real time q-PCR. I also performed the statistical analyses of the data and prepared the figures. I took part in the preparation of all chapters of the paper.

12(S)-HETE induces lymph endothelial cell retraction *in vitro* by upregulation of SOX18

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Abstract. Metastasising breast cancer cells communicate with adjacent lymph endothelia, intravasate and disseminate through lymphatic routes, colonise lymph nodes and finally metastasize to distant organs. Thus, understanding and blocking intravasation may attenuate the metastatic cascade at an early step. As a trigger factor, which causes the retraction of lymph endothelial cells (LECs) and opens entry ports for tumour cell intravasation, MDA-MB231 breast cancer cells secrete the pro-metastatic arachidonic acid metabolite, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid [12(S)-HETE]. In the current study, treatment of LECs with 12(S)-HETE upregulated the expression of the transcription factors SRY-related HMG-box 18 (SOX18) and prospero homeobox protein 1 (PROX1), which determine endothelial development. Thus, whether they have a role in LEC retraction was determined using a validated intravasation assay, small

interfering RNA mediated knockdown of gene expression, and mRNA and protein expression analyses. Specific inhibition of SOX18 or PROX1 significantly attenuated *in vitro* intravasation of MDA-MB231 spheroids through the LEC barrier and 12(S)-HETE-triggered signals were transduced by the high and low affinity receptors, 12(S)-HETE receptor and leukotriene B4 receptor 2. In addition, the current findings indicate that there is crosstalk between SOX18 and nuclear factor κ -light-chain-enhancer of activated B cells, which was demonstrated to contribute to MDA-MB231/lymph endothelial intravasation. The present data demonstrate that the endothelial-specific and lymph endothelial-specific transcription factors SOX18 and PROX1 contribute to LEC retraction.

Introduction

Cancer cells undergo several obligatory biochemical/molecular changes to colonise distant organs (1). In this process, tumour intravasation is an early step when cancer cells enter the vasculature (2). Breast cancer cells predominantly spread through lymphatics (3), rather than blood vessels, and lymph node metastasis in patients with breast cancer is an intermediate, yet rate-limiting step, preceding distant organ metastasis (4). Thus, the 'lymph node status' is a prognostic marker (5) and blocking lymph node metastasis is expected to positively correlate with survival. As endothelial vessel formation is crucial for tumour dissemination, the inhibition of endothelial cell growth, cell assembly and disruption of vascular integrity is a strategy to prevent tumour spreading.

SRY-related HMG-box 7 (SOX7), together with SOX18, and a limited number of other transcription factors provoke endothelial cell differentiation from mouse embryonic stem cells (6). SOX18 directly regulates (among other target genes) the transcription of prospero homeobox protein 1 (PROX1), which itself determines the differentiation from blood vessels to lymphatic vessels during embryonic development (7-11). Furthermore, as SOX18 is overexpressed in various cancer entities (12-18), this additionally renders SOX18 a candidate target to block vascularisation and metastasis. Thus, a screening approach was undertaken to identify small molecules

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Abbreviations: 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid, 'S' stereoisomer; 12-HETER, 12(S)-HETE receptor; BLT2, leukotriene B4 receptor 2; CCID, circular chemo repellent induced defect; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; I κ B α , inhibitor of nuclear factor κ -light-chain-enhancer of activated B cells; LEC, lymph endothelial cell; miR, microRNA; NF κ B1, nuclear factor of κ light polypeptide gene enhancer in B cells 1; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; PROX1, prospero homeobox protein 1; RELA, v-Rel avian reticuloendotheliosis viral oncogene homolog A; siRNA, small interfering RNA; SOX18, SRY-related HMG-box 18

Key words: breast cancer, endothelial retraction, SRY-related HMG-box 18, prospero homeobox protein 1, nuclear factor κ -light-chain-enhancer of activated B cells

that inhibit SOX18. In an extract of the marine brown algae *Caulocystis cephalornithos*, lead structures were discovered from which a derivative compound, sm4, was developed (19). In a breast cancer mouse model, sm4 reduced the density of blood vessels within the tumour, tumour-associated lymphatic vessels and metastatic spread (20). Conceivably, these results may lead to a first clinical trial. However, pharmacological inhibition of SOX18 by sm4 is not entirely specific, as the interaction of other SOX family members to their DNA promotor sites is also blocked (19). Thus, the particular contribution of SOX18, as well as mechanistic insight into its mode of action requires further investigation. To this end, we investigated the role of SOX18 and its transcriptional target, PROX1, during tumour intravasation by specifically inhibiting them with small interfering RNAs (siRNAs). A mechanism that breast cancer cells use to traverse the lymph endothelial wall is by secretion of 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid [12(S)-HETE] (4), which causes endothelial cell retraction (21-23). Thus, gaps emerge in the lymph endothelial barrier [termed circular chemorepellent-induced defects (CCIDs)] through which cancer emboli can transmigrate. The results of the current study demonstrated that 12(S)-HETE strongly induced the expression of SOX18 and PROX1, and their inhibition attenuated CCID formation. Thus, the 12(S)-HETE-triggered signal transduction pathway, in addition to recently established cross-talk between SOX18 and nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) (24,25), which also contributes to breast cancer cell intravasation (26-28), were examined.

Material and methods

Antibodies and reagents. Rabbit polyclonal anti-leukotriene B4 receptor 2 (BLT2; cat. no. L7042) and mouse monoclonal anti- β -actin antibody (clone AC-15; cat. no. A3854) were purchased from Sigma-Aldrich (Munich, Germany), rabbit monoclonal anti-PROX1 (clone EPR19273; cat. no. Ab119359), rabbit polyclonal anti-SOX18 (cat. no. Ab23342) and mouse monoclonal anti-intercellular adhesion molecule 1 antibody (ICAM-1; clone MEM111; cat. no. Ab2213) from Abcam (Cambridge, UK), mouse monoclonal anti-v-Rel avian reticuloendotheliosis viral oncogene homolog A (RELA/p65; clone L8F6; cat. no. 4767) from Cell Signaling Technology, Inc. (Danvers, MA, USA), polyclonal rabbit anti-nuclear factor of κ -light polypeptide gene enhancer in B cells 1 (NFKB1/p50; cat. no. SC8414) was bought from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), polyclonal rabbit anti-12(S)-HETE receptor (12-HETER; cat. no. CAYM14083-1) antibody was from Cayman Chemical Company (Ann Arbor, MI, USA), polyclonal rabbit anti-mouse (cat. no. Z0420) and polyclonal swine anti-rabbit IgGs (cat. no. E0353) were from Dako (Agilent Technologies, Inc., Santa Clara, CA, USA). The antibody dilutions used in western blotting were as follows: Anti-BLT2, 1:500; anti-12-HETER, 1:300; anti-SOX18, 1:250; anti-PROX1, 1:500; anti-ICAM-1, 1:300-500; anti-RELA/p65, 1:300; anti-NFKB1/p50, 1:500; anti- β -actin, 1:5,000; and anti-rabbit/mouse secondary antibodies 1:2,000. All antibodies were diluted in PBS containing 5% skim-milk.

The siRNAs targeting human *SOX18* (cat. no. L-019035-00-0005), *BLT2* (cat. no. L-005654-00-0005), 12-HETER

(cat. no. L-005564-00-0005), *NFKB1* (cat. no. L-003520-00-0005 *NFKB2* (cat. no. L-003918-00-0005) and *PROX1* (cat. no. L-016913-005) were from Dharmacon (Lafayette, CO, USA). The siRNA targeting human *RELA* (cat. no. 4390824; ID s11914) and non-targeting control siRNA (*Silencer*R Select Negative Control no. 1 siRNA; cat. no. 4390843) were obtained from Ambion (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All siRNAs were resuspended in RNase-free water to obtain a stock concentration of 20 μ M.

12(S)-HETE was purchased from Cayman Chemical Company. The inhibitor of nuclear factor κ -light-chain-enhancer of activated B cells (I κ B α) phosphorylation inhibitor (E)-3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (Bay11-7082) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

Cell culture. Human MDA-MB231 breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in MEM medium supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin and 1% non-essential amino acids (all from Invitrogen; Thermo Fisher Scientific, Inc.). Microvessel endothelial cells were purchased from Clonetics™ (Lonza Group, Ltd., Basel, Switzerland), immortalized by telomerase, and then human lymph endothelial cells (LECs) were isolated from this TERT-immortalised mixture of human dermal endothelial cells (29,30). LECs were authenticated by analysing expression of the lymph endothelial cell markers PROX1, lymphatic vessel endothelial hyaluronic acid receptor 1 and podoplanin, and the pan-endothelial cell marker CD31 by immunofluorescence and laser scanning microscopy (data not shown). LECs were cultured in EGM2 MV (cat. no. CC-4147; Clonetics™; Lonza Group, Ltd.). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For CCID formation assays, LECs were stained with cytotracker green (Invitrogen; Thermo Fisher Scientific, Inc.).

CCID assay. This assay measures the size of cell-free areas (CCIDs) in the LEC monolayer, which are triggered by MDA-MB231 tumour spheroids. MDA-MB231 cells (input of 6x10³ cells per spheroid) were transferred to 30 ml MEM medium containing 0.3% methylcellulose. Cell suspensions (150 μ l) were transferred to each well of 96-well round bottom microtiter plates to allow spheroid formation within the subsequent 48 h. Then, MDA-MB231 spheroids were washed in PBS and transferred to cytotracker (1 mg/ml final concentration)-stained LEC monolayers that were seeded into 24-well plates in 2 ml EGM2 MV medium 48 h before and cultured to confluence. Following the staining of LECs and 30 min pre-treatment with Bay11-7082 (or with dimethyl sulfoxide for control), the MDA-MB231 spheroids were carefully transferred to the 24-well plates containing LECs. After 4 h of co-incubation, the CCID areas in the LEC monolayers underneath the MDA-MB231 spheroids were imaged using an Axiovert fluorescence microscope (Zeiss GmbH, Jena, Germany) to visualise cytotracker (green)-stained LECs underneath the spheroids. CCID areas were calculated with the Axiovision Re. 4.5 software (Zeiss GmbH). Experiments were performed in triplicate and for each condition, the CCID sizes underneath ≥ 12 were measured.

Transfection of LEC monolayer. LECs were seeded in 24-well plates (1 ml/well) and cultured in EGM2 medium. Transfections were performed when the cells were 70–80% confluent. A total of 0.75 μ g siRNA (3 μ l from 20 μ M stock) and 6 μ l HiPerFect Transfection Reagent (Qiagen GmbH, Hilden, Germany) were mixed in 100 μ l serum-free medium and incubated for 30 min at room temperature to allow the formation of transfection complexes. The old cell culture medium was gently removed and 500 μ l fresh EGM2 medium were added into each well. Then the transfection complexes were added drop-wise to the cells (to a final siRNA concentration of 100 nM) and incubated for 24 h at 37°C. After 24 h, the medium was replaced by fresh medium and cells were incubated for another 24 h to recover. The LECs monolayers were used for CCID assays or isolated RNA for reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. LECs were harvested following transfection (or stimulation) and RNA was isolated using the RNeasy Mini Kit 50 and QIAshredder 50 (Qiagen GmbH). The final RNA concentration was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific Inc.). Total RNA (2 μ g) was reverse transcribed to cDNA using EcoDry Premix at 42°C for 60 min according to the manufacturer's instructions (Clontech Laboratories, Inc., Mountainview, CA, USA) and the resulting cDNA was amplified using TaqMan Gene Expression Master Mix (Applied Biosystems, Vienna, Austria) and TaqMan primer pairs (Applied Biosystems; Thermo Fisher Scientific, Inc.). IDs for the primers were as follows: GAPDH (assay ID, Hs99999905_m1), RELA (p65; assay ID, Hs00153294_m1), NFKB1 (p100; assay ID, Hs00765730_m1), NFKB2 (p105; assay ID, Hs00174517_m1), ICAM-1 (assay ID, Hs00164932_m1), SOX18 (assay ID, Hs00746079_s1) and PROX1 (assay ID, Hs00896293_m1). The cycling profile for DNA amplifications was as follows: 50°C for 2 min (1 cycle); 95°C for 10 min (1 cycle); 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec (40 cycles); and 72°C for 10 min (1 cycle). The PCR products were analysed on the Chromo4 PCR System (Bio-Rad Laboratories, Hercules, CA, USA). qPCR was performed in triplicate for each cDNA template. Gene expression was normalized to GAPDH expression and calculated using the $\Delta\Delta C_q$ method (31).

Western blotting. To monitor knock down of siRNA transfected cells or to follow the activation/inactivation of signalling molecules upon 12(S)-HETE stimulation, western blot analyses were performed. LECs were seeded in 6-well plates (2.5 ml medium/well) and stimulated with indicated concentrations of 12(S)-HETE or for indicated times. Then, cells were washed twice with ice cold PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluorid and protease inhibitor cocktail and the lysate was centrifuged at 13,500 $\times$ g at 4°C for 20 min. The supernatant was stored at -20°C until further analysis. Total protein (20 μ g/well; determined by Pierce bicinchoninic acid protein assay; Thermo Fisher Scientific Inc.) was separated by 10% SDS-polyacrylamide gel electrophoresis and electro-transferred onto Hybond polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA) at 100 V for 1 h at 4°C. To control equal sample loading, membranes were

stained with Ponceau S (Sigma-Aldrich). Following washing with PBS/Tween-20 (PBS/T; pH 7.2) or TBS/T (pH 7.6), membranes were immersed in blocking solution (5% non-fat dry milk in TBS containing 0.1% Tween or in PBS containing 0.5% Tween-20) at room temperature for 1 h. Membranes were washed and incubated with primary antibody (in blocking solution; dilution 1:500–1:1,000) by gently rocking at 4°C overnight or at room temperature for 1 h. Subsequently, the membranes were washed with PBS/T or TBS/T and incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG; dilution, 1:2,000) at room temperature for 1 h. Chemiluminescence was developed by ECL-Plus detection kit (GE Healthcare), and detected using a Lumi Imager F1 Workstation (Roche Diagnostics, Basel, Switzerland). Densitometry of the western blots was analysed with ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Statistical analyses. For statistical analyses Excel 2013 software (Microsoft Corporation, Redmond, WA, USA) and Prism 6 software package (GraphPad Software, San Diego, CA, USA) were used. The values are expressed as the mean $\pm$ standard error, t-test or one-way analysis of variance with Tukey's post-test and were applied to compare differences between control samples and treatment groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

12(S)-HETE induces SOX18. 12(S)-HETE reduces the resilience of the lymph endothelial barrier (4) through activation of NF- κ B (26,27). Reportedly, NF- κ B cross-talks to the endothelia-specific transcription factor SOX18 in human umbilical vein endothelial cells (HUVECs) (24). This prompted investigation of the expression of SOX18 in LECs upon 12(S)-HETE treatment. Treatment with 0.5 and 1 μ M 12(S)-HETE significantly increased SOX18 mRNA (Fig. 1A) and protein expression, which was dependent on dose (Fig. 1B) and time (Fig. 1C).

12(S)-HETE signals to SOX18 through 12(S)-HETE receptor (12-HETER) and BLT2 and contributes to LEC barrier breaching in vitro. 12(S)-HETE increased SOX18 mRNA and protein expression, which was dependent on the high affinity receptor 12-HETER and the low affinity receptor BLT2, as siRNAs targeting 12-HETER and BLT2, inhibited 12(S)-HETE-induced SOX18 mRNA overexpression (Fig. 2A) and SOX18 protein upregulation (Fig. 2B). As 12(S)-HETE causes the retraction of LECs (4), whether SOX18 and BLT2 also contribute to this phenomenon was investigated. LEC retraction was measured in a validated *in vitro* co-culture assay calculating the areas of generated CCIDs in LEC monolayers underneath 12(S)-HETE-secreting MDA-MB231 cell spheroids (4,32). The CCID areas that were triggered by MDA-MB231 spheroids were significantly smaller in those LEC monolayers, which were transfected with siRNAs targeting SOX18 or BLT2 (Fig. 2C and D, respectively). The efficient downregulation of SOX18 and BLT2 by siRNAs was determined by western blotting (Fig. 2E and F, respectively). Thus, the transcription factor SOX18 and the low affinity 12(S)-HETE receptor BLT2

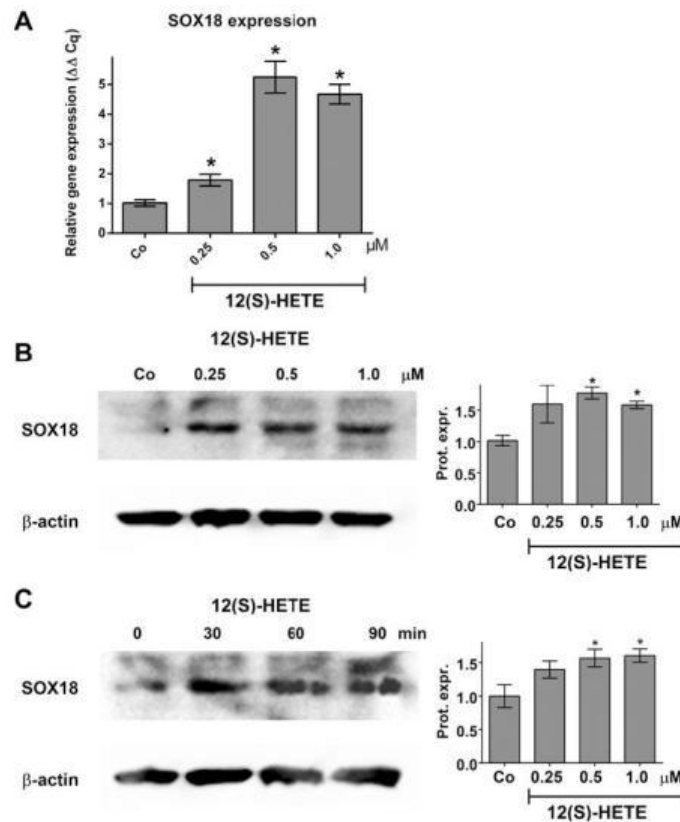


Figure 1. 12(S)-HETE induces SOX18 expression. LECs were treated with solvent (DMSO, Co) or the indicated concentrations of 12(S)-HETE (A) for 15 min when cells were lysed and analysed by reverse transcription-quantitative polymerase chain reaction for *SOX18* mRNA expression (normalised to *GAPDH* mRNA), or (B) for 1 h when proteins were separated by SDS-PAGE and SOX18 expression analysed by western blotting using SOX18 antibody (Ponceau S-staining and β -actin expression served as loading controls). The relative protein expression was quantified by densitometry (three replicates each). (C) LECs were treated with solvent or 0.5 μ M 12(S)-HETE for the indicated times when cells were lysed and SOX18 protein expression was analysed. Values are presented as the mean $\pm$ standard error. * $P < 0.05$ vs. Co (one-way analysis of variance with Tukey's post hoc test). LECs, lymph endothelial cells; SOX18, SRY-related HMG-box 18; 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; Co, control.

contributed to LEC retraction. The role of 12-HETER for LEC retraction was demonstrated previously, as well as the down-regulation of 12-HETER by siRNA in LECs (32,33).

PROX1 contributes to LEC barrier breaching. SOX18 regulates the transcription factor PROX1 (34), which in adults, is expressed in LECs and thus, is a prominent marker of lymph endothelia. Hence, the expression of PROX1 was analysed upon treatment of LECs with 12(S)-HETE. 12(S)-HETE increased *PROX1* mRNA expression, which was suppressed by knockdown of SOX18, 12-HETER and BLT2 (Fig. 3A). In addition, CCID formation was attenuated in PROX1 knock-down LEC monolayers (Fig. 3B). Efficient downregulation of PROX1 protein by siRNA targeting PROX1 was demonstrated by western blot analysis and densitometry (Fig. 3C). Thus, the effects produced by 12(S)-HETE and via 12-HETER/BLT2 were propagated further downstream from *PROX1*, which itself contributed to CCID formation by a mechanism that needs to be established.

SOX18 expression depends on RELA, NFKB1 and NFKB2. Tumour necrosis factor- α treatment inhibits SOX18 expression in HUVECs via NF- κ B/RELA (24) by altered binding of auxiliary factors at NF- κ B promoter/enhancer sites (35). Thus, the interplay between NF- κ B and SOX18 was further investigated in LECs. Treating LECs with the I κ B α /NF- κ B inhibitor Bay11-7082 inhibited 12(S)-HETE-induced SOX18 protein expression. Thus, NF- κ B activity was associated with SOX18 expression in LECs (Fig. 4A) as demonstrated previously in HUVECs. To determine causality, experiments in which RELA, NFKB1 and NFKB2 were knocked down in LECs were performed. The efficacy of the siRNAs, which downregulate RELA, NFKB1 and NFKB2 in LECs, was established previously (28). qPCR analysis demonstrated that *SOX18* mRNA expression in LECs (Fig. 4B), and consequently also the expression of *PROX1* mRNA (Fig. 4C), was significantly inhibited by these siRNAs. As a control, the expression of the well-established NF- κ B target, ICAM-1, was analysed indicating that RELA, NFKB1 and NFKB2 siRNAs repressed

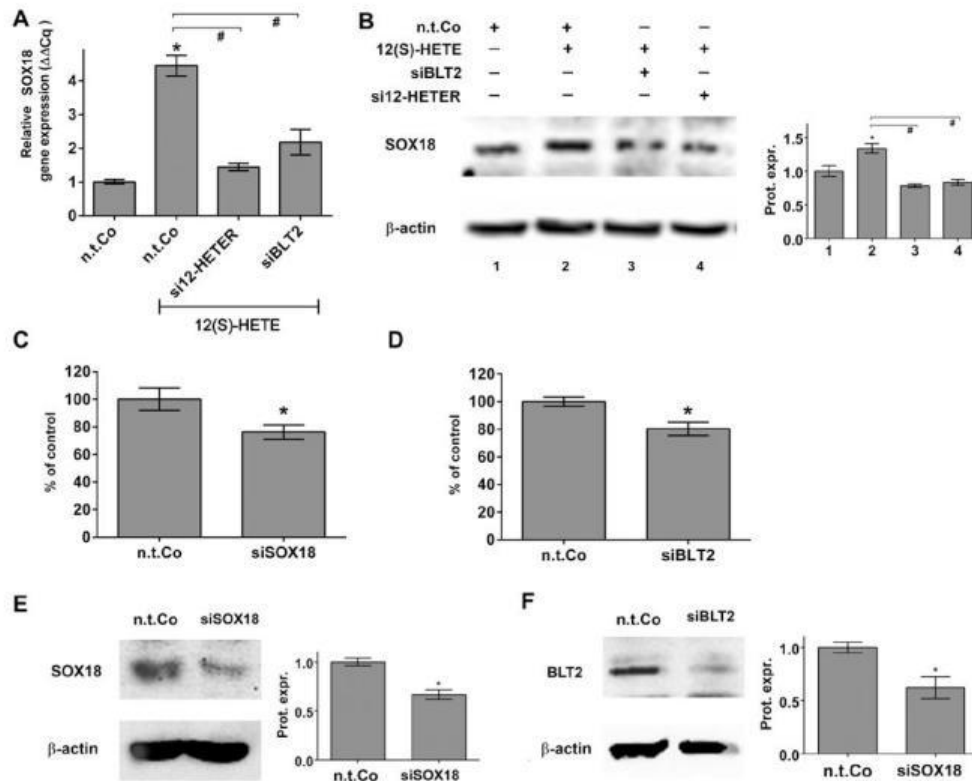


Figure 2. 12(S)-HETE signals to SOX18 through 12-HETER and BLT2 in LEC. LECs were transfected with n.t.Co or siRNA targeting *BLT2* or *12-HETER*. After 24 h cells were stimulated with solvent (DMSO; n.t.Co) or 1 μ M 12(S)-HETE, lysed and analysed (A) by reverse transcription-quantitative polymerase chain reaction for *SOX18* mRNA expression (normalised to *GAPDH* mRNA), or (B) by SDS-PAGE and western blotting for SOX18 protein expression using SOX18 antibody (Ponceau S-staining and β -actin expression served as loading controls). The relative protein expression was quantified by densitometry (three replicates each). LECs were transfected with either (C) n.t.Co or siRNA targeting *SOX18*, or (D) siRNA targeting *BLT2* and after 24 h MDA-MB231 breast cancer cell spheroids were placed on top of confluent LEC monolayers and co-incubated for 4 h when circular chemorepellent-induced defects formation was measured. Three independent experiments with at least 10 replicates were measured. The downregulation of (E) SOX18 protein by siSOX18 or (F) BLT2 protein by siBLT2 was analysed by western blotting. Values are presented as the mean $\pm$ standard error, * $P < 0.05$ vs. n.t.Co (no 12(S)-HETE); # $P < 0.05$ vs. n.t.Co (12(S)-HETE-treatment). LECs, lymph endothelial cells; n.t.Co, non-targeting control RNA; si, small interfering RNA; SOX18, SRY-related HMG-box 18; 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 12-HETER, 12(S)-HETE receptor; BLT2, leukotriene B4 receptor 2.

12(S)-HETE-induced *ICAM-1* mRNA induction (Fig. 4D). Similar to SOX18, the upregulation of *ICAM-1* mRNA and ICAM-1 protein (Fig. 4D and E, respectively) was also mediated by 12-HETER and BLT2. The data confirmed that SOX18 was downstream of NF- κ B signalling.

SOX18 regulates *RELA* and *NFKB1* expression. Reportedly, 12(S)-HETE signals to NF- κ B in LECs [27] and in the former experiments the cross-talk between NF- κ B and SOX18 was demonstrated. Reciprocally, it was examined whether SOX18 has a role in the expression of NF- κ B following stimulation of LECs with 12(S)-HETE. Analysing the mRNA profile in LECs transfected with SOX18-targeting siRNA demonstrated that SOX18 contributed to 12(S)-HETE-induced effects on *RELA* and *NFKB1*, but not on *NFKB2* (Fig. 5A-C, respectively). Furthermore, the suppression of *RELA* and *NFKB1* protein upon SOX18 knock down in LECs suggested the SOX18 has a role upstream of NF- κ B signalling, as the

expression of the established NF- κ B target, ICAM-1, was also inhibited (Fig. 5D).

As SOX18 was also demonstrated to be downstream of NF- κ B (in the previous experiments), a positive feed-back loop between these transcription factors may exist in LECs.

Discussion

When breast cancer cells disseminate through the lymphatic vasculature the first lymph node reached, termed the sentinel, becomes colonised (36). The emerging lymph node metastasis is drained by *de novo* assembled lymphatic vessels, which perforate the neoplastic tissue (4,37,38). As soon as metastatic cancer cells intravasate these *de novo* lymphatics they are transported to the efferent lymphatic sinus and colonise downstream lymph nodes until they reach and infest distant organs (4). Reportedly, 12(S)-HETE, which is secreted by cancer cells, triggers the retraction of

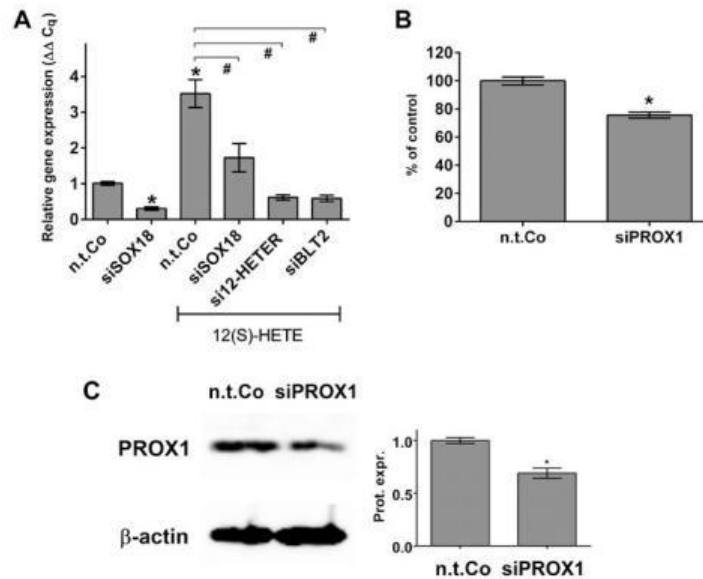


Figure 3. 12(S)-HETE induces PROX1 expression. (A) LECs were treated with solvent (DMSO, Co) or 1.0 μ M 12(S)-HETE for 15 min when cells were lysed and analysed by reverse transcription-quantitative polymerase chain reaction for *PROX1* mRNA expression (normalised to *GAPDH* mRNA; quadruplicates were performed for each experimental condition). (B) LECs were transfected with either n.t.Co or siRNA targeting *PROX1* and after 24 h MDA-MB231 breast cancer cell spheroids were placed on top of confluent LEC monolayers and co-incubated for 4 h when circular chemorepellent-induced defects formation was measured. Three independent experiments with at least 10 replicates were measured. (C) Downregulation of PROX1 protein by siPROX1 was analysed by SDS-PAGE and western blotting using PROX1 antibody (Ponceau S-staining and β -actin expression served as loading controls). The relative protein expression was quantified by densitometry (three replicates each). Values are presented as the mean $\pm$ standard error. * P < 0.05 and # P < 0.05 vs. n.t.Co (12(S)-HETE-treatment). LECs, lymph endothelial cells; n.t.Co, non-targeting control RNA; si, small interfering RNA; SOX18, SRY-related HMG-box 18; 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 12-HETER, 12(S)-HETE receptor; BLT2, leukotriene B4 receptor 2; PROX1, prospero homeobox protein 1.

juxtaposed LECs allowing cancer emboli to intrude through the emerging defect into the lymphatic vasculature (4). To prevent this rate limiting step of breast cancer metastasis a number of potential mechanisms of this phenomenon were investigated and described previously (4,32,39). One mechanism involves 12(S)-HETE-triggered activation of NF- κ B in LECs as a significant contributor of intravasation *in vitro* (26-28). However, due to the versatile roles of NF- κ B in cellular processes, including inflammatory response, tumour cell proliferation, invasion, cell survival and angiogenesis (40,41), the inhibition of NF- κ B as a strategy to specifically combat metastasis has certainly its limitations. It was discovered that NF- κ B co-regulates the transcription factor SOX18 (24,25), which itself regulates PROX1 (9), which is 'the' marker for lymphatic vessels. This recommends these transcription factors as more specific targets for intervention, as they exhibit much more restricted expression profiles (9,10) compared with NF- κ B.

The present data demonstrate that 12(S)-HETE induced CCID formation, which was dependent on SOX18 and its target PROX1. Additionally, the upregulation of RELA, NFKB1 and ICAM-1 were dependent on SOX18. Whether SOX18 regulated NF- κ B expression directly or as a co-factor remains to be established.

The 12(S)-HETE-triggered signal was transduced by the high-affinity and low-affinity receptors, 12-HETER and BLT2, respectively; however, it is unknown how these

receptors induced the transcription of SOX18. Notably, ectopic overexpression of SOX18 cDNA did not upregulate constitutive expression of RELA (data not shown), thus supporting a hypothetical auxiliary function of SOX18 in the regulation of NF- κ B, but not a direct or exclusive one. A negative auxiliary regulation of SOX18 by NF- κ B in HUVECs was already suggested (24,25), whereas the (co-) induction of NF- κ B by SOX18 indicated a positive (auxiliary) feed-back loop between SOX18-NF- κ B-SOX18 in LECs. This mechanism of regulation may be specific for LECs, as 12(S)-HETE did not induce SOX18 expression in HUVECs (data not shown).

siRNA-mediated inhibition of SOX18 and PROX1 attenuated CCID formation to a similar extent as siRNA-mediated inhibition of RELA, NFKB1 or NF- κ B essential modulator, as demonstrated previously (28). This supports a strategy to reduce intravasation into lymphatics based on inhibition of SOX18 or PROX1 signalling (instead of NF- κ B-signalling) as it may attenuate LEC barrier intravasation with higher specificity. The expression of SOX18 and PROX1 is strongly associated with endothelial and lymph-endothelial expression and differentiation, respectively. Therefore, SOX18 mutations (causing loss of function) are associated with the rare diseases hypotrichosis-lymphedema-telangiectasia syndrome and hypotrichosis-lymphedema-telangiectasia renal defect syndrome (42,43). Targeting SOX18/PROX1 would therefore, provide improved specificity in LECs. However, it will remain a matter of dispute whether inhibition of tumour

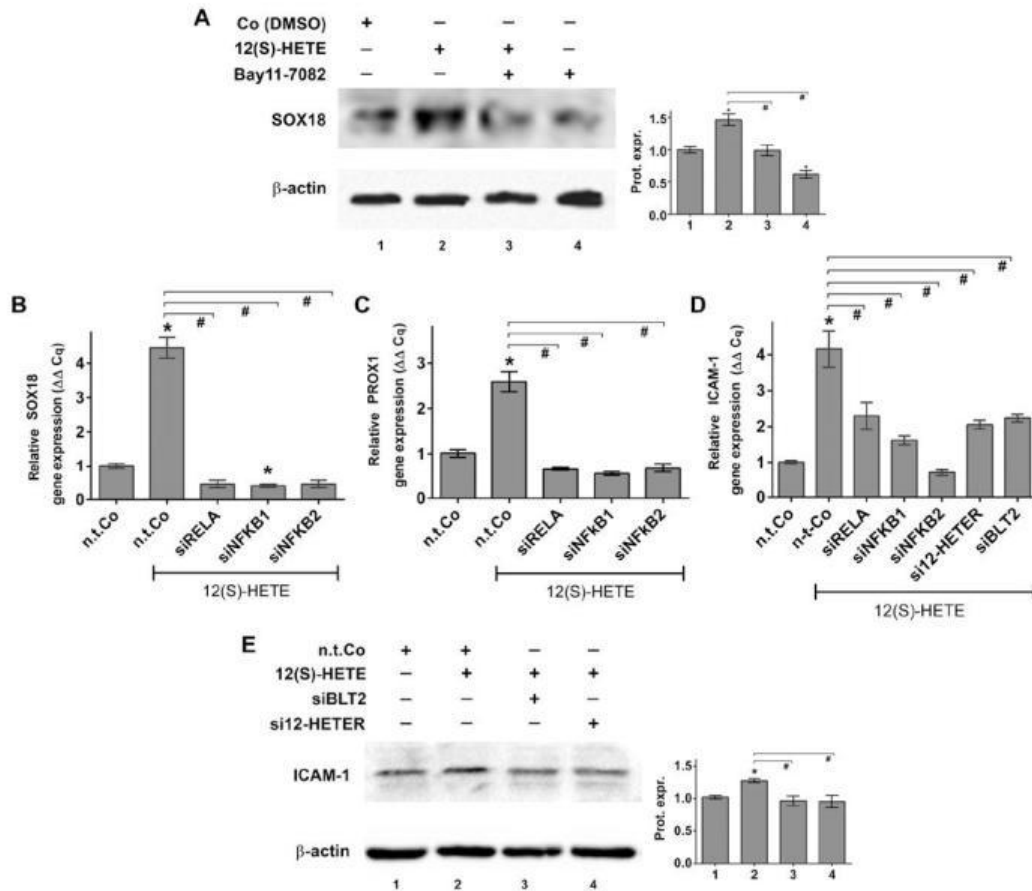


Figure 4. Suppression of NF- κ B in LECs inhibits SOX18 expression. (A) Lymph endothelial cells were pre-treated with Bay11-7082 for 20 min followed by stimulation with either solvent control (DMSO; Co) or with 1 μ M 12(S)-HETE for 15 min. Then, cells were lysed, proteins separated by SDS-PAGE and analysed by western blotting using SOX18 antibody (Ponceau S-staining and β -actin expression served as loading controls). The relative protein expression was quantified by densitometry (three replicates each). LECs were transfected either with n.t.Co or siRELA, siNFKB1 or siNFKB2 and after 24 h cells were stimulated with solvent (DMSO; n.t.Co) or 1.0 μ M 12(S)-HETE for 15 min when the mRNA expression of (B) *SOX18*, (C) *PROX1* and (D) *ICAM-1* was measured by reverse transcription-quantitative polymerase chain reaction (normalised to GAPDH mRNA; assay was performed in quadruplicate). (E) LECs were transfected with n.t.Co or siBLT2 or si12-HETER. After 24 h cells were stimulated with solvent (DMSO; n.t.Co) or 1 μ M 12(S)-HETE, lysed and analysed by SDS-PAGE and western blotting for ICAM-1 protein expression using ICAM-1 antibody (Ponceau S-staining and β -actin expression served as loading controls). Values are presented as the mean $\pm$ standard error, * P < 0.05 vs. n.t.Co (no 12(S)-HETE); # P < 0.05 vs. n.t.Co (12(S)-HETE-treatment). LECs, lymph endothelial cells; Co, control; 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; SOX18, SRY-related HMG-box 18; n.t.Co, non-targeting control RNA; si, small interfering RNA; RELA, v-Relavian reticuloendotheliosis viral oncogene homolog A; NFKB, nuclear factor of κ -light polypeptide gene enhancer in B cells; 12-HETER, 12(S)-HETE receptor; BLT2, leukotriene B4 receptor 2; ICAM-1, intercellular adhesion molecule 1.

intravasation has priority versus a potential induction of these rare syndromes, also involving hair loss and bleeding telangiectasia. The correct dosage may exhibit specificity in attenuating SOX18-dependent metastasis with tolerable side effects. As an example, niflumic acid, a well-known FDA-approved drug used to treat joint and muscular pain due to its cyclooxygenase-2-inhibitory properties, inhibits SOX18 at high μ M concentrations (19). It was recently demonstrated that niflumic acid also inhibited NF- κ B, cytochrome P450 1A1/2 and CCID-formation at much lower concentrations (44) with a wide spectrum of activities and limited specificity for SOX18. To date, no reports regarding the identification and

development of PROX1 inhibitors exist, which may target the lymphatic vasculature more precisely. Thus, future screening approaches in search of SOX18/PROX1 inhibitors are required and may reveal compounds with improved specificity. SOX18 overexpression is observed in different cancer types (12-18) causing enhanced migration, adhesion and invasivity (45). This underscores the importance to determine specific SOX18-targeting strategies (20).

SOX18 is targeted by microRNAs (miRs), miR-7a and miR-24-3p (46). Thus, miR-7a and miR-24-3p may provide another option to interfere with SOX18 activity as a therapeutic concept. Furthermore, PROX1 was also previously

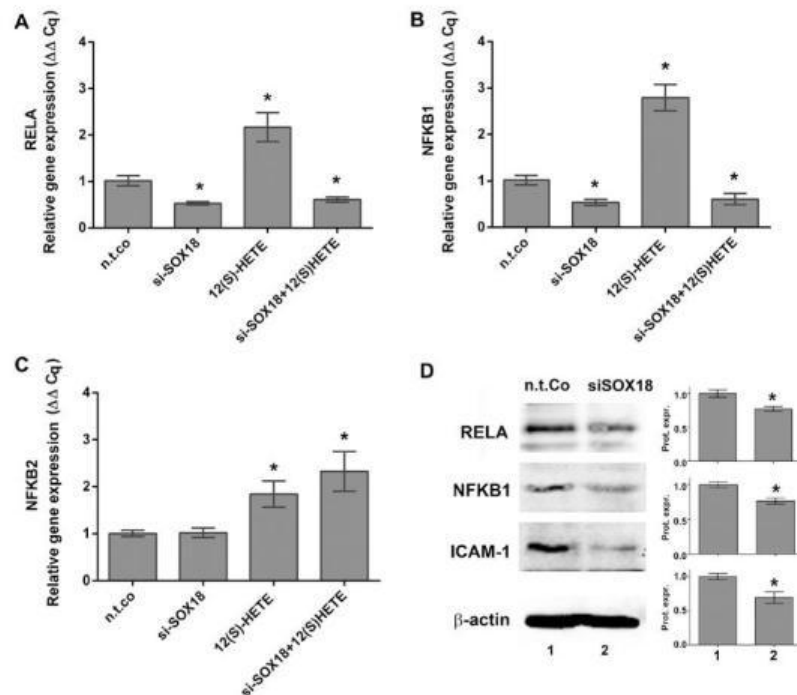


Figure 5. Suppression of SOX18 inhibits expression of RELA and NFKB1. LECs were transfected with either n.t.Co or siSOX18. After 24 h LECs were either treated with solvent (DMSO; n.t.Co) or 1 μ M 12(S)-HETE and the mRNA expression of (A) *RELA*, (B) *NFKB1*, or (C) *NFKB2* was analysed by reverse transcription-quantitative polymerase chain reaction (three replicates each; normalised to *GAPDH* mRNA). (D) LECs were lysed, proteins separated by SDS-PAGE and expression analysed by western blotting using the indicated antibodies (Ponceau S-staining and β -actin expression served as loading controls). The relative protein expression was quantified by densitometry (three replicates each). Values are presented as the mean $\pm$ standard error, * $P < 0.05$ vs. n.t.Co. *RELA*, v-Rel avian reticuloendotheliosis viral oncogene homolog A; n.t.Co, non-targeting control RNA; si, small interfering RNA; SOX18, SRY-related HMG-box 18; 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; NFKB1, nuclear factor of κ -light polypeptide gene enhancer in B cells 1; ICAM-1, intercellular adhesion molecule 1.

demonstrated to be downregulated by miR-181a, thereby re-programming the lymphatic phenotype towards a blood endothelial signature (47). This phenomenon may reduce the ability of breast cancer cells to intravasate, as breast cancer cells predominantly spread through the lymphatic vasculature.

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Availability of data and materials

All data generated or analysed during this study are included in this published article. The datasets used and/or analysed

during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Study concepts: GK, RdM, WJ. Study design: GK, WJ, RdM, AF, DM; Data acquisition: AF, DM, CHN. Quality control of data and algorithms: GK, SK, NH, CHN, JB. Data analysis and interpretation: AF, WJ, RdM, GK. Statistical analysis: AF, DM. Manuscript preparation: GK, AF, RdM, WJ. Manuscript editing: AF, GK, RdM. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Apigenin and Luteolin Attenuate The Breaching of MDA-MB231 Breast Cancer Spheroids Through The Lymph Endothelial Barrier *In Vitro*.

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I carried out the Ethoxyresorufin-O-deethylase (EROD) assay selective for CYP1A1/A2 activity and western blot to investigate inhibition of Tyr397-FAK phosphorylation by apigenin. I also participated in the discussion and preparation of the paper.



Apigenin and Luteolin Attenuate the Breaching of MDA-MB231 Breast Cancer Spheroids Through the Lymph Endothelial Barrier *in Vitro*

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Flavonoids, present in fruits, vegetables and traditional medicinal plants, show anticancer effects in experimental systems and are reportedly non-toxic. This is a favorable property for long term strategies for the attenuation of lymph node metastasis, which may effectively improve the prognostic states in breast cancer. Hence, we studied two flavonoids, apigenin and luteolin exhibiting strong bio-activity in various test systems in cancer research and are readily available on the market. This study has further advanced the mechanistic understanding of breast cancer intravasation through the lymphatic barrier. Apigenin and luteolin were tested in a three-dimensional (3-D) assay consisting of MDA-MB231 breast cancer spheroids and immortalized lymph endothelial cell (LEC) monolayers. The 3-D model faithfully resembles the intravasation of breast cancer emboli through the lymphatic vasculature. Western blot analysis, intracellular Ca^{2+} determination, EROD assay and siRNA transfection revealed insights into mechanisms of intravasation as well as the anti-intravasative outcome of flavonoid action. Both flavonoids suppressed pro-intravasative trigger factors in MDA-MB231 breast cancer cells, specifically MMP1 expression and CYP1A1 activity. A pro-intravasative contribution of FAK expression in LECs was established as FAK supported the retraction of the LEC monolayer upon contact with cancer cells thereby enabling them to cross the endothelial barrier. As mechanistic basis, MMP1 caused the phosphorylation (activation) of FAK at Tyr397 in LECs. Apigenin and luteolin prevented MMP1-induced FAK activation, but not constitutive FAK phosphorylation. Luteolin, unlike apigenin, inhibited MMP1-induced Ca^{2+} release. Free intracellular Ca^{2+} is a central signal amplifier triggering LEC retraction through activation of the mobility protein MLC2, thereby enhancing intravasation. FAK activity and Ca^{2+} levels did not correlate. This implicates that the pro-intravasative contribution of FAK and of Ca^{2+} release in LECs was independent of each other and explains the better anti-intravasative effects

of luteolin *in vitro*. In specific formulations, flavonoid concentrations causing significant anti-intravasative effects, can certainly be achieved *in vivo*. As the therapeutic strategy has to be based on permanent flavonoid treatment both the beneficial and adverse effects have to be investigated in future studies.

Keywords: intravasation, 3D model, flavonoids, FAK, MMP1, CYP1A1, Ca^{2+} release

INTRODUCTION

Species of the *Scrophularia* family are used in ethno-medicine since ancient times (Galindez et al., 2002). In the search for anti-metastatic natural compounds, extracts of six *Scrophularia* species were tested. For a methanolic extract from *S. lucida* a significant anti-intravasative activity was discovered (Giesrigl et al., 2012). The chloroform fraction of the methanolic extract inhibited *in vitro* intravasation of breast cancer spheroids most effectively. The major compound of the most active sub-fraction was hispidulin which, as pure compound, attenuated this early stage of the metastatic process significantly (Lewenhofer et al., 2018, same issue). Therefore, we decided to test two structurally related flavonoids, luteolin and apigenin, in in-depth mechanistic studies. These flavonoids are present in many medicinal plants used in the traditional medicine of Central and Eastern Europe. One of the oldest references to a healing plant in occidental literature can be found in Homer's *Iliad* (book XI, line 822 et seq.; Murray, 1924). There is said that 'Ἀχιλλεύς used a bitter, astringent root to treat wounds. This background in mythology led to the name of a very important medicinal plant: *Achillea*. In fact, this plant is used to treat, among other ailments, bleeding disorders and bruises (Akram, 2013). Later, *Achillea* sp. ("Achilleos") was also listed in *De Materia Medica*; Dioscurides, 1st century A.D. and used by Classical Greeks, in the Roman Empire, the Byzantium, and the Middle Ages up until our present day. *A. millefolium* is very rich in glycosides of luteolin and apigenin, which are easily metabolized into the respective genins (Benedek et al., 2007; Benetis et al., 2008). Many other apigenin- and luteolin-rich plant genera are also listed in *De Materia Medica*: *Cynara*, *Apium*, *Allium*, *Petroselinum*, *Punica*, *Thymus*, *Solidago*, *Chamaemelum*, *Viola*, and *Vitis* (the entire 5th book is dedicated to *Vitis*).

Triple negative breast cancer (TNBC) comprises about 15% of all breast cancer types. In general, TNBC responds well to chemotherapy as long it has not disseminated to other organs. However, as soon as TNBC metastasizes the success of drug treatment is very limited. This implicates that TNBC, in addition to conventional therapy, needs a metastasis-preventive approach to maintain a tumor state associated with a better prognosis. At an early stage of metastasis breast cancer cells intravasate the lymphatic vasculature and colonize lymph nodes. Importantly, the "lymph node status" is a prognostic breast tumor marker (Sobin et al., 2009). Hence, we hypothesized that compounds which prevent intravasation may interfere with tumor progression such as lymph node- and distant organ metastasis. As such a preventive concept for TNBC has to be based on long term drug administration, the therapeutic regimen has to be mild and rather free of side effects to allow continuous

treatment. Luteolin inhibited proliferation and induced apoptosis of the highly metastatic TNBC cell line MDA-MB231 (Attoub et al., 2011). Anti-invasive/anti-metastatic activities of luteolin (Tsai et al., 2016; Lin et al., 2017) and the structurally related analog apigenin (Lindenmeyer et al., 2001; Qin et al., 2016; Zhao et al., 2017) have also been demonstrated in various cancer cell lines. As these flavonoids are also wide-spread constituents in fruits and vegetables they are considered free of adverse effects. This tempted us to investigate luteolin and apigenin in terms of their anti-intravasative properties. In the daily diet, the concentration of flavonoids is too low to show tumor preventive effects (Vogiatzoglou et al., 2015). However, apigenin and luteolin are also commercially available as food supplements with rather high concentrations (40–200 mg apigenin/capsule; 50 mg luteolin/capsule) and may provide a preventive option.

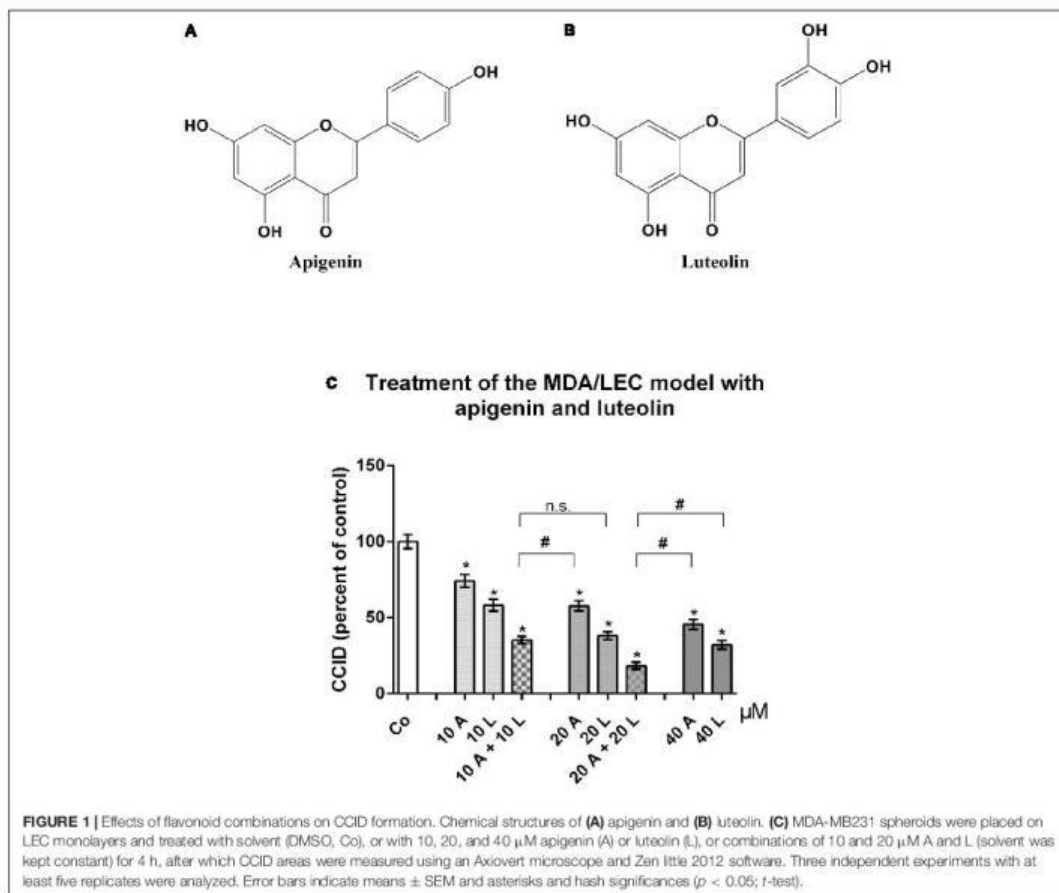
The TNBC cell line MDA-MB231 over-expresses the pro-metastatic matrix metalloprotease 1 (MMP1), and inhibition of MMP1 attenuates tumor intravasation (Nguyen et al., 2015). Furthermore, MDA-MB231 cells secrete the pro-intravasative arachidonic acid metabolite 12(S)-HETE (Nguyen et al., 2016a), which is synthesized by CYP1A1. Therefore, we investigated whether luteolin and apigenin interfered with MMP1 expression and CYP1A1 activity in MDA-MB231 cells.

MMP1 and 12(S)-HETE activate the PAR1 and the 12(S)-HETE receptor, respectively, in LECs, which both trigger the release of Ca^{2+} , causing the rapid retraction of LECs. This opens gaps in the monolayer [circular chemorepellent-induced defects (CCIDs)] through which tumor cells cross the endothelial barrier (Nguyen et al., 2015, 2016b). Hence, the influence of apigenin and luteolin on levels of intracellular Ca^{2+} in LECs was investigated. The expression of FAK in LECs seems to be crucial for rapid cell movement (Desai et al., 2009) as observed during CCID formation. Thus, FAK expression and activity were studied as well.

MATERIALS AND METHODS

Antibodies and Reagents

Polyclonal rabbit anti-focal adhesion kinase (FAK) and polyclonal rabbit anti-phospho-Tyr397-FAK were purchased from Cell Signaling (Danvers, MA, United States), polyclonal rabbit anti-MMP1 from Abcam (Cambridge, United Kingdom) and monoclonal anti- β -actin from Sigma (Munich, Germany). Polyclonal rabbit anti-mouse, anti-goat and anti-rabbit IgGs were acquired from Dako (Glostrup, Denmark). Apigenin and luteolin were purchased from Sigma (Munich, Germany) and human recombinant MMP1 from Sigma-Aldrich (SRP3117,



St. Louis, MO, United States). U73122 was obtained from Calbiochem (Darmstadt, Germany), BAPTA-AM was from Santa Cruz Biotechnology (Heidelberg, Germany).

siRNAs targeting human MLC2 (MYL2; SMART pool, ON-TARGET PLUS, Cat. No.: L-011087000005, siRNAs targeting human FAK (SMART pool, ON-TARGET PLUS, Cat. No.: L-003164000005) were ordered from Dharmacon (Gene Expression and Gene Editing, GE Healthcare, Lafayette, CO, United States). Non-targeting (n.t.) control siRNA (Silencer® Select Negative Control No. 1 siRNA, Cat. No.: 4390843) was purchased from Ambion (Life Technologies, Carlsbad, CA, United States). All siRNAs were re-suspended in RNase-free water to yield a stock concentration of 20 μ M.

Cell Culture

Human MDA-MB231 breast cancer cells from the American Type Culture Collection (ATCC, Rockville, MD, United States) were grown in MEM medium supplemented with 10% fetal

calf serum (FCS), 1% penicillin/streptomycin (PS) and 1% non-essential amino acids (Gibco, Invitrogen, Karlsruhe, Germany). Telomerase immortalized human lymph endothelial cells (LECs) were grown in EGM2 MV (Clonetics CC-4147, Allendale, NJ, United States). The cells were kept at 37°C in a humidified atmosphere containing 5% CO₂. For CCID formation assays, LECs were stained with CellTracker™ green purchased from Invitrogen (Karlsruhe, Germany).

Spheroid Formation

MDA-MB231 cells (input of 6,000 cells per spheroid) were transferred to 30 mL serum free MEM medium containing 6 mL of a 1.6% methylcellulose solution (0.3% final concentration; Cat. No.: M-512, 4000 centipoises; Sigma-Aldrich, Munich, Germany). 150 μ L of cell suspension were transferred to each well of a 96-well plate (Greiner Bio-one, Cellstar 650185, Kremsmünster, Austria) to allow spheroid formation within 48 h.

CCID (Circular Chemorepellent Induced Defect) Assay

In this assay, the sizes of the cell free areas (CCIDs) formed in the endothelial monolayer underneath the tumor spheroids, were measured. MDA-MB231 spheroids were washed in PBS and transferred to CellTracker™ (green)-stained LEC monolayers seeded into 24-well plates (Costar 3524, Sigma-Aldrich, Munich, Germany) in 1 mL EGM2 MV medium. After 4 h of incubation, the CCID areas in the LEC monolayers underneath the MDA-MB231 spheroids were photographed using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope. CCID areas were calculated using the Zen Little 2012 (Zeiss, Jena, Germany). For each treatment and control the CCID size of at least 25 spheroids (unless otherwise specified) was measured.

SDS Gel Electrophoresis and Western Blotting

MDA-MB231 cells and LECs were grown in T-25 tissue culture flasks (Nunc, Roskilde, Denmark) to 80% confluence and then pre-treated with the flavonoids for 0.5, 1, 2, and 4 h. Then, cells were processed for SDS gel electrophoresis and Western blotting as described before (Nguyen et al., 2015). Chemo-luminescence was developed by Amersham ECL prime Kit (GE Healthcare, Freiburg, Germany) and detected using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland). Densitometry of the Western blots was analyzed with the Image-J software (National Institutes of Health, Bethesda, MD, United States).

Ethoxyresorufin-O-Deethylase (EROD) Assay Selective for CYP1A1/A2 Activity

MDA-MB231 cells were grown in phenol red-free DMEM/F12 medium (Gibco, Karlsruhe, Germany) containing 10% FCS and 1% PS (Invitrogen, Karlsruhe, Germany). Before treatment, the cells were transferred to DMEM/F12 medium supplemented with 10% charcoal-stripped FCS (PAN Biotech, Aldenbach, Germany) and 1% PS. After 4 h of treatment with apigenin and luteolin CYP1A1 activity was measured with minor modifications as previously described (Teichmann et al., 2014). Briefly, ethoxyresorufin (final concentration 5.0 μ M, Sigma-Aldrich, Munich, Germany) was added, 0.4 mL aliquots of the medium were collected after 180 min and the formation of resorufin was analyzed by spectro-fluorometry (PerkinElmer LS50B, Waltham, MA, United States) at an excitation wavelength of 530 nm and an emission wavelength of 585 nm.

Intracellular Ca^{2+} Assay

Free intracellular Ca^{2+} levels were measured using the FluoForte™ Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, MI, United States). 8×10^3 LECs/well/100 μ L EGM2 medium were seeded into 96-well black-wall clear-bottom plates (Nunc, Thermo Fisher Scientific, Rochester, NY, United States) and after 24 h LECs were pre-treated with apigenin, luteolin or DMSO for 1 h. Then, medium was removed and 100 μ L FluoForte™ Dye-loading (containing apigenin, luteolin, or DMSO) were added to each well. Cells were further incubated for 45 min at

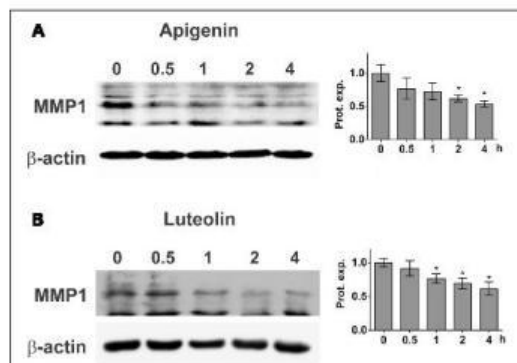


FIGURE 2 | Inhibition of MMP1 expression upon flavonoid treatment. MDA-MB231 cells were grown to ~80% confluence and then treated with solvent (0), or (A) 20 μ M apigenin, or (B) 20 μ M luteolin for 0.5, 1, 2, and 4 h. Then, cells were lysed, proteins separated by SDS gel electrophoresis and subjected to Western blotting using the indicated antibodies. Staining with Ponceau S and immunoblotting with anti- β -actin antibody controlled equal sample loading. Relative protein expression levels (Prot. expr.) are shown to the right of the blots. Densitometries are means $\pm$ SEM from at least three experiments (asterisks indicate significances, $p < 0.05$; t-test) and the Western blot images are representatives for illustration.

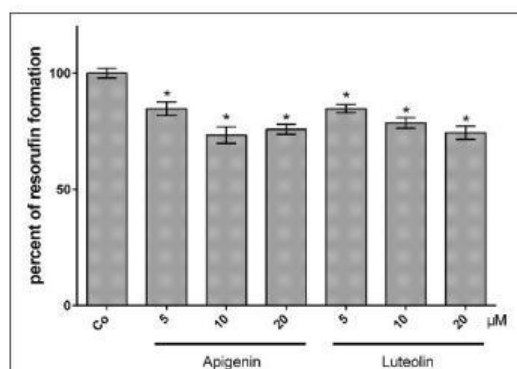
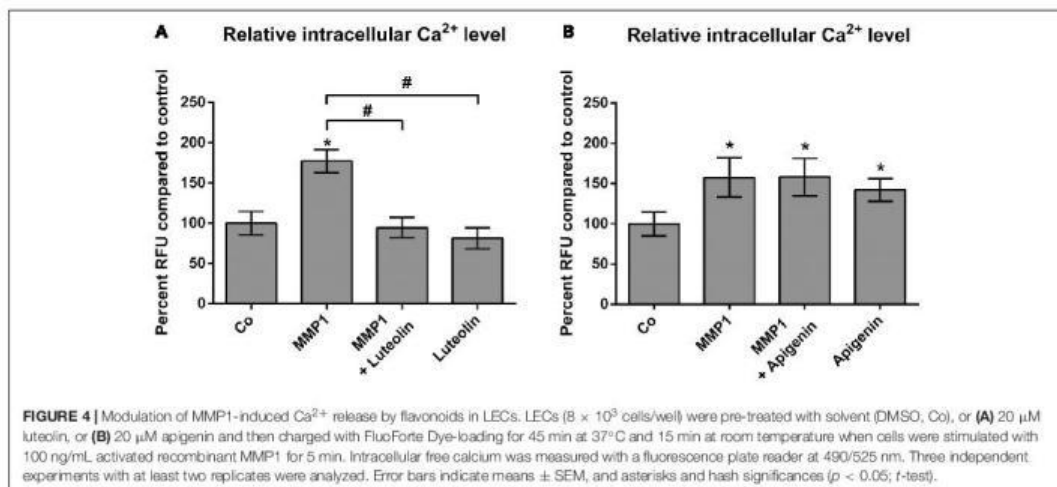


FIGURE 3 | Inhibition of CYP1A1 activity in MDA-MB231 cells by flavonoids. MDA-MB231 cells were treated with solvent (DMSO, Co), or the indicated concentrations of apigenin and luteolin. Ethoxyresorufin (5 μ M) was added after 4 h and the formation of resorufin was measured. Three independent experiments with at least three replicates were analyzed. Error bars indicate means $\pm$ SEM and asterisks significances ($*p < 0.05$; ANOVA together with Tukey's post-test).

37°C followed by 15 min at room temperature. Then, cells were stimulated with 1 μ M 12(S)-HETE for 5 min, and fluorescence was measured with a fluorescence plate reader at 490/525 nm.

Transfection of LEC Monolayer

Lymph endothelial cells were seeded in 24-well plates (1 mL/well) and grown in EGM2 medium. Transfections were performed



at a confluence of 70–80%. 0.75 μg siRNA and 6 μL HiPerFect Transfection Reagent (Cat. No.: 301705; Qiagen, Hilden, Germany) were mixed in 100 μL serum-free medium and incubated for 30 min at room temperature to allow the formation of transfection complexes. Cell culture medium was discarded and 500 μL of fresh EGM2 medium were added into each well. Then, the transfection complexes were added drop-wise to the cells (to a final siRNA concentration of 100 nM) and incubated for 24 h at 37°C. After 24 h, the medium was replaced and cells were incubated for another 24 h to recover. The LEC monolayers were used for CCID assays or isolated RNA for qPCR.

Statistical Analysis

For statistical analyses Excel 2013 software and Prism 6 software packages (GraphPad, San Diego, CA, United States) were used. The values were expressed as mean $\pm$ SEM, and the Student's t -test and ANOVA with Tukey's post-test was used to compare differences between control samples and treatment groups as well as differences among treatment groups. The statistical significance level was set to $p < 0.05$.

RESULTS

Apigenin/Luteolin Inhibit CCID Formation Induced by MDA-MB231 Spheroids

Breast cancer cells have to intravasate the lymphatic barrier to colonize lymph nodes, and the CCID *in vitro* assay realistically resembles the intravasation of tumor spheroids through the lymphatic vasculature (Kerjaschki et al., 2011). Treatment of the three-dimensional MDA-MB231/LEC model with apigenin (Figure 1A) and luteolin (Figure 1B) dose dependently inhibited CCID formation in the LEC monolayer, with luteolin showing a stronger effect than apigenin. Simultaneous treatment with

20 μM luteolin and 20 μM apigenin synergized and inhibited CCID formation significantly stronger than 40 μM luteolin or 40 μM apigenin, respectively, thus suggesting that luteolin and apigenin interfered with distinct pro-intravasative mechanisms (Figure 1C).

Apigenin/Luteolin Inhibit MMP1 Expression and CYP1A1 Activity in MDA-MB231 Cells

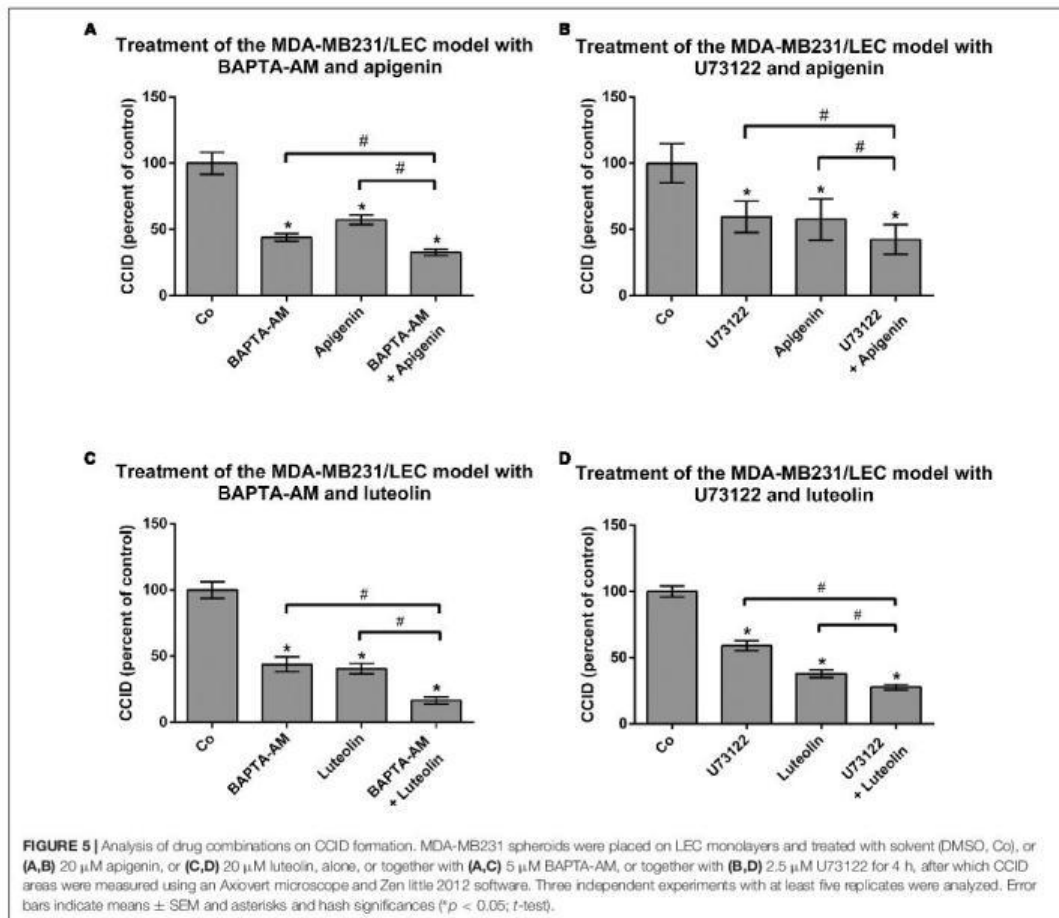
MDA-MB231 cell spheroids express MMP1, which is one mechanism causing CCIDs in the adjacent LEC monolayer (Nguyen et al., 2015). Thus, the expression of MMP1 was investigated after treatment of MDA-MB231 cells with apigenin and luteolin. 20 μM apigenin and 20 μM luteolin caused a significant and time-dependent down-regulation of MMP1 protein (Figures 2A,B).

Another mechanism of breast cancer emboli to cross the endothelial barrier is the secretion of 12(S)-HETE which, in MDA-MB231 cells, is produced by CYP1A1 (Nguyen et al., 2016a). Hence, the activity of CYP1A1 in MDA-MB231 cells was analyzed by EROD assay after treatment with increasing concentrations of apigenin and luteolin. 5–20 μM apigenin and luteolin inhibited CYP1A1 activity significantly within 4 h (Figure 3).

The results proved that apigenin and luteolin inhibited MMP1 expression and CYP1A1 activity, which trigger the retraction of the adjacent LEC barrier thereby enabling MDA-MB231 tumor spheroid intravasation.

Influence of Apigenin and Luteolin on Intracellular Ca^{2+} Release in LEC

The intravasation of breast cancer cells relies on an interplay between cancer emboli and the LEC barrier (Vonach et al., 2011; Viola et al., 2013). Therefore, it was investigated whether apigenin



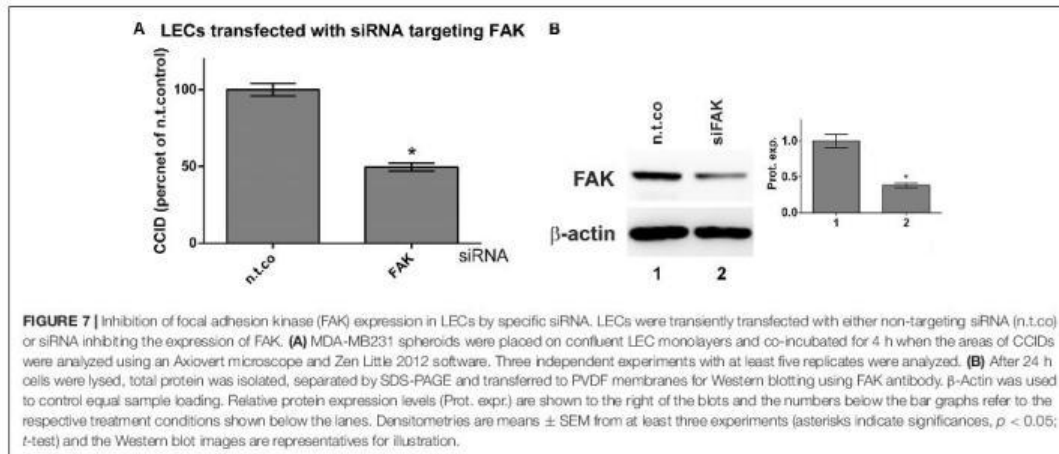
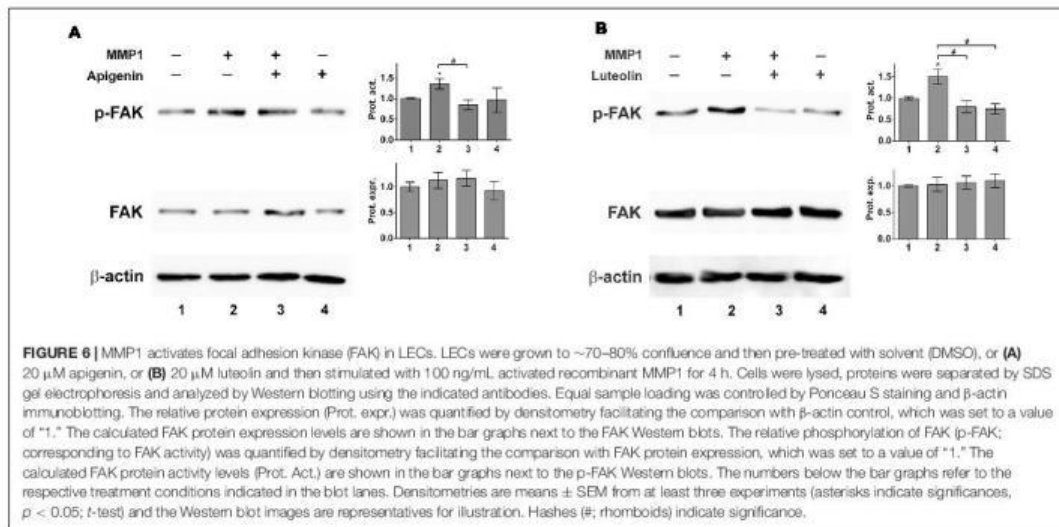
or luteolin would also inhibit the pro-intravasative response of LECs.

The release of Ca^{2+} from intracellular stores plays a central role in MMP1- and 12(S)-HETE-induced CCID formation (Nguyen et al., 2016b; Stadler et al., 2017). Hence, it was tested whether apigenin and luteolin affected intracellular Ca^{2+} release. Luteolin prevented MMP1-induced Ca^{2+} increase (Figure 4A), which is consistent with previous reports (Jiang et al., 2005). In contrast, apigenin did not reduce MMP1-induced Ca^{2+} levels in LECs, but in contrast increased Ca^{2+} release (Figure 4B). This is in agreement with data that apigenin activates potassium channels leading to their hyper-polarization and causing the release of Ca^{2+} (Erdogan et al., 2007). Accordingly, treating the MDA-MB231/LEC model with apigenin and BAPTA-AM (intracellular Ca^{2+} inhibitor) caused a significantly stronger inhibition of CCID formation than apigenin alone, or BAPTA-AM alone (Figure 5A). Ca^{2+} release is dependent

on PLC β activity and, therefore, also the co-treatment with apigenin and U73122 (PLC inhibitor) attenuated CCID formation significantly stronger than the treatment with just apigenin or U73122 (Figure 5B). Luteolin ameliorated the CCID-inhibitory effect of BAPTA-AM (Figure 5C) or U73122 (Figure 5D) even more efficiently than apigenin. Overall, these data support the notion that an additional intravasative mechanism, which was independent of Ca^{2+} signaling, must have been inhibited by apigenin and luteolin as well.

Luteolin/Apigenin Inhibit Phosphorylation of Tyr397-FAK in LEC

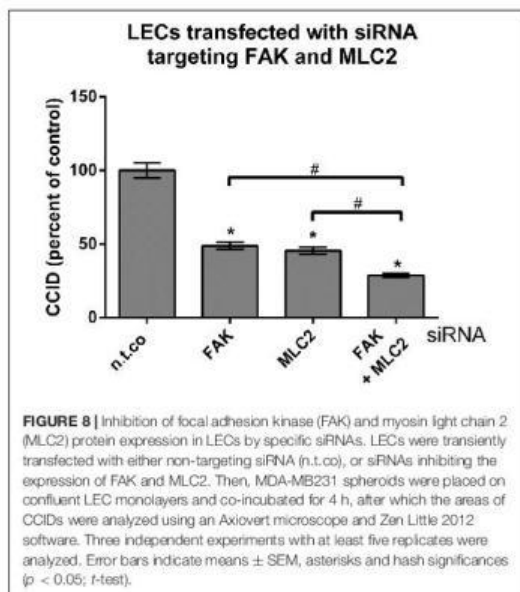
The FAK controls cell-matrix interactions and mobility (Schwock et al., 2010) as observed in CCID formation (Nguyen et al., 2015). Recombinant MMP1 induced the phosphorylation of FAK at Tyr397 indicative for its activation. Pre-treatment



with apigenin or luteolin prevented MMP1-induced, but not constitutive FAK phosphorylation (Figures 6A,B). The inhibition of Tyr397-FAK phosphorylation by apigenin did not correlate with the inhibition of Ca^{2+} release. Therefore, the relevance of FAK for CCID formation was studied by siRNA-mediated knock-down of FAK (siFAK). In fact, siFAK transfection of LECs suppressed MDA-MB231-induced CCID formation by ~50% (Figure 7A). The knock-down of FAK was confirmed by Western blotting (Figure 7B). Hence, FAK significantly contributed to intravasation *in vitro* and apigenin and luteolin inhibited this mechanism.

Earlier work demonstrated that CCID formation through MLC2 depends on intracellular Ca^{2+} release (Nguyen et al.,

2015). Our results show that FAK-dependent CCID formation did not correlate with intracellular Ca^{2+} levels. Although both pathways were induced by MMP1, they, obviously, are independent of each other. Thus, we hypothesized that simultaneous inhibition of MLC2 (Ca^{2+} -dependent) and FAK (Ca^{2+} -independent) by respective siRNAs, should bring about a greater reduction in CCID formation than inhibition of either FAK or MLC2 alone, which was actually confirmed (Figure 8). This supports former data (Figure 1C) showing that luteolin (inhibiting FAK and Ca^{2+} release) and apigenin (inhibiting FAK but not Ca^{2+} release) synergize in CCID attenuation via distinct signaling pathways.



DISCUSSION

We show for the first time that luteolin and apigenin attenuate the intravasation of breast cancer cells *in vitro*. At the tested concentrations, luteolin and apigenin inhibited CYP1A1 activity in MDA-MB231 cells. CYP1A1 synthesizes the pro-metastatic arachidonic acid metabolite 12(S)-HETE and is induced, e.g., by the activated aryl hydrocarbon receptor, the expression of which is high in many cancer entities (Murray et al., 2014) and also in MDA-MB231 cells (Nguyen et al., 2016a). In HepG2 cells with a luciferase reporter fused to the CYP1A1-regulator region, 5 μ M apigenin increased reporter gene expression by threefold. In contrast, the higher concentration of 20 μ M apigenin inhibited the expression by 50% (Allen et al., 2001). In cell-free assays luteolin inhibited CYP1A1 more potently than apigenin, whereas CYP1A2 was inhibited more efficiently by apigenin (Kim et al., 2005). CYP1A2 mediates the formation of flavonoid metabolites with different properties to the genuine compounds. The comparability of flavonoid effects is rather difficult among different cell lines, which depend on the expression and activity profiles of the various CYP isoforms converting these flavonoids to other (more or less active) products (Androutsopoulos et al., 2009). Based on the very different individual expression levels of CYP1A2 in humans, Breinholt et al. (2002) conclude that this may result in different individual sensitivity.

Apigenin and luteolin inhibited also MMP1 expression in MDA-MB231 cells. MMP1 expression is induced by NF- κ B (Lee et al., 2011), which is activated not only in inflamed- but also in cancerous tissue (Karin and Greten, 2005; Karin, 2006). Recently, we provided evidence that specific inhibition of

NF- κ B (by siRELA, siNFKB1, siNEMO) down-regulated MMP1 expression and attenuated CCID formation (Nguyen et al., 2015). Flavones such as apigenin and luteolin inhibited NF- κ B activity and consequently the expression of its target MMP9 (Amrutha et al., 2014). Hence, the inhibition of NF- κ B by apigenin and luteolin may have been the reason for suppressed MMP1 levels in MDA-MB231 cells.

As shown here, recombinant MMP1 significantly induced the phosphorylation of FAK at Tyr397 in LECs. FAK activity contributes to cancer cell migration by establishing loose cell-matrix interactions necessary for rapid cell movement (Desai et al., 2009; Schwock et al., 2010), as described in CCID formation (Kerjaschki et al., 2011). In our experiments suppression of FAK significantly inhibited CCID formation. The activation of FAK (through phosphorylation of Tyr397) is mediated through Kv2.1 channels (Wei et al., 2008). Since FAK as well as potassium channel activity contribute to LEC retraction and CCID formation (Nguyen et al., 2017), the reduction of Tyr397-FAK phosphorylation by apigenin may have been due to the inhibition of potassium channels. This was supported by reports demonstrating that trimethyl-apigenin interfered with cardiac potassium currents (Liu et al., 2012) and the inhibition of potassium channel activity by the flavonoids acacetin, scutellarin, genistein, and naringenin (Teisseyre and Michalak, 2005; Zhu et al., 2008; Wu et al., 2011; Gasiorowska et al., 2012). However, quite to the contrary, apigenin and luteolin exhibited vasodilatory effects, which are discussed to be due to activation (rather than inactivation) of potassium channels (Calderone et al., 2004; Jiang et al., 2005). A more recent research concluded that the vasodilatory properties of apigenin and luteolin were independent of potassium channel inhibition, but due to extracellular calcium influx (Roberts et al., 2013). Therefore, the mechanism by which apigenin and luteolin inhibit FAK phosphorylation remains to be established.

MMP1 also activates the mobility protein MLC2 in LECs, which contributes to CCID formation as well (Nguyen et al., 2015, 2016c). MMP1 binds to PAR1, activates PLC β , triggers Ca^{2+} release and activates the Ca^{2+} -calmodulin kinase MYLK, which phosphorylates MLC2. MMP1 was also shown to cause migration by binding to PAR1 in breast cancer cells (Boire et al., 2005) and blood endothelial cells (Juncker-Jensen et al., 2013). Whereas MMP1-induced MLC2 activation depends on Ca^{2+} release, FAK activation was not found to correlate with intracellular Ca^{2+} levels. This implicates that the pathway activating FAK represents a pro-intravasative mechanism independent of the one activating MLC2. Therefore, the inhibition both of FAK (either by siFAK or apigenin) and of MLC2 (by siMLC2, or BAPTA-AM, or U73122) reduced CCID formation in LEC monolayers to a greater extent than the inhibition of just one pathway.

Summing up, luteolin and apigenin inhibited pro-intravasative mechanisms in MDA-MB231 breast cancer cells at the levels of CYP1A1 activity and MMP1 expression. In LECs, apigenin and luteolin inhibited MMP1-induced, aberrant FAK activity but not constitutive, normal FAK phosphorylation. Only luteolin inhibited Ca^{2+} signaling in LEC, which contributed

to CCID formation (Nguyen et al., 2016b). This may explain why luteolin affected CCID formation more potently than apigenin as well as the synergism between apigenin and luteolin.

The results implicate that luteolin and apigenin maintained the resilience of the endothelial barrier whilst attacking cancer cells and additionally, inhibited the malignant armamentarium of the tumor. Luteolin and apigenin exerted their effects at low μM concentrations which usually cannot be reached by daily dietary intake (Vogiatzoglou et al., 2015). Nevertheless, the daily intake of particular flavonoid subclasses (flavonols, flavan-3-ols, anthocyanidins) significantly correlates inversely with the risk of colorectal cancer (Woo and Kim, 2013). However, no other flavonoid subclasses or total flavonoids are associated with a lower risk of breast cancer in post-menopausal women (Hui et al., 2013). Intake of highly enriched apigenin and luteolin formulations achieved flavonoid concentrations in blood plasma which are comparable to those described herein *in vitro*. In detail, a single oral dose of apigenin delivered via a carbon nanopowder solid dispersion carrier (60 mg/kg) to rats resulted in peak plasma concentrations of up to 3.2 $\mu\text{g/mL}$ which corresponds to 11.8 μM (Ding et al., 2014). Similarly, a single oral dose luteolin in a peanut hull extract (14.3 mg/kg) to rats resulted in peak plasma concentrations of up to 8.3 $\mu\text{g/mL}$ (29.1 μM) (Zhou et al., 2008). The evaluation as to whether these concentrations are free of negative side effects throughout long term treatment requires controlled phase I studies.

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AUTHOR CONTRIBUTIONS

JJH, AF, and DM performed the experiments. CN, NH, and SK analyzed the data. JqH, SG, PB, and WJ were involved in experiment planning and supervision. AO, LK, and GK supervised experimental work, and compiled and interpreted the data. LK and GK wrote the manuscript.

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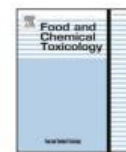
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Intravasation of SW620 Colon Cancer Cell Spheroids Through The Blood Endothelial Barrier is Inhibited by Clinical Drugs and Flavonoids In Vitro

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I carried out the densitometry analyze from western blot result. I also participated in the discussion and preparation of the paper.



Intravasation of SW620 colon cancer cell spheroids through the blood endothelial barrier is inhibited by clinical drugs and flavonoids *in vitro*



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Apigenin

ABSTRACT

Mechanisms how colorectal cancer (CRC) cells penetrate blood micro-vessel endothelia and metastasise is poorly understood. To study blood endothelial cell (BEC) barrier breaching by CRC emboli, an *in vitro* assay measuring BEC-free areas underneath SW620 cell spheroids, so called “circular chemorepellent induced defects” (CCIDs, appearing in consequence of endothelial retraction), was adapted and supported by Western blotting, EIA-, EROD- and luciferase reporter assays. Inhibition of ALOX12 or NF-κB in SW620 cells or BECs, respectively, caused attenuation of CCIDs. The FDA approved drugs vinpocetine [inhibiting ALOX12-dependent 12(S)-HETE synthesis], ketotifen [inhibiting NF-κB], carbamazepine and fenofibrate [inhibiting 12(S)-HETE and NF-κB] significantly attenuated CCID formation at low μM concentrations. In the 5-FU-resistant SW620-R/BEC model guanfacine, nifedipine and proadifen inhibited CCIDs stronger than in the naïve SW620/BEC model. This indicated that in SW620-R cells formerly silent (yet unidentified) genes became expressed and targetable by these drugs in course of resistance acquisition. Fenofibrate, and the flavonoids hispidulin and apigenin, which are present in medicinal plants, spices, herbs and fruits, attenuated CCID formation in both, naïve- and resistant models. As FDA-approved drugs and food-flavonoids inhibited established and acquired intravasative pathways and attenuated BEC barrier-breaching *in vitro*, this warrants testing of these compounds in CRC models *in vivo*.

1. Introduction

A few cancer entities spread and colonise distant organs by intravasating lymphatic- and/or blood vessels first. Intravasation is achieved upon secretion of cancer-derived factors (Uchide et al., 2007; Nguyen et al., 2015), which subsequently trigger the retraction of the adjacent endothelial cell wall (Honn et al., 1994) thereby opening gaps (“circular chemically induced defects”; CCIDs) through which cancer bulks (or single cells) enter the vasculature (Uchide et al., 2007;

Madlener et al., 2010) and metastasise (Kerjaschki et al., 2011). As breast cancer cells spread mainly through lymphatics (Karlsson et al., 2017), the lymph node status, which refers to axillary lymph nodes that are filled with metastatic cancer cells, is a prognostic marker (Sobin et al., 2009). Therefore, intravasation, which is resembled by the validated CCID *in vitro* assay, was studied in three-dimensional (3D) models consisting of breast cancer cell emboli and lymph endothelial cell (LEC) monolayers to elucidate its mechanisms (Vonach et al., 2011; Viola et al., 2013; Nguyen et al., 2016a,b). To this end, the role of ALOX12/

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Abbreviations

3D	3-dimensional	CRC	colorectal cancer
5-FU	5-Fluorouracil	CCID	circular chemorepellent induced defect
12(S)-HETE	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid, "S" stereoisomer	EC	endothelial cell
ALOX12/15	lipoygenase 12/15	FCS	fetal calf serum
Bay11-7082	(E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile	ICAM-1	intercellular adhesion molecule 1
BEC	blood endothelial cell	LEC	lymph endothelial cell
CAF	cancer associated fibroblast	NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
		PS	penicillin/streptomycin
		TCM	Traditional Chinese Medicine

15, its metabolite 12(S)-HETE and its receptor, which triggers the retraction of endothelial cells (Guo et al., 2011) as a prerequisite for breast cancer metastasis (Kerjaschki et al., 2011; Uchide et al., 2007), was established.

Colorectal cancer (CRC) is known to disseminate through lymphatics and promote lymph node metastasis, but also through other routes as lung and liver metastases are independent of the lymph node status. Therefore, it is likely that the blood vasculature is accountable for CRC distribution as well (Knijn et al., 2016). This is in accordance with a recent *in vitro* investigation reporting that colon cancer spheroids penetrate LEC- and blood endothelial cell (BEC) barriers alike (Holzner et al., 2016). Since mechanisms, of how colon cancer emboli breach the blood microvasculature are poorly defined, we studied a model consisting of SW620 cell spheroids and BECs and report on the correlation between tumour intravasation, 12(S)-HETE levels secreted by SW620 cells, and the NF- κ B-dependent response in BECs. Once intravasative mechanisms were identified, tailored intervention measures were taken. For this, FDA approved drugs, which affected these mechanisms, were tested in the SW620/BEC model regarding their anti-intravasative properties. Furthermore, baicalein, which is a *bona fide* inhibitor of ALOX12 activity and 12(S)-HETE synthesis exhibiting anticancer activity *in vitro* and *in vivo* (Hsu et al., 2008), was investigated

regarding its CCID-inhibitory property. Baicalein is a main compound contained in *Scutellaria baicalensis* root (the major component in "Huang-Lian-Jie-Du-Tang") used in TCM and Kampo medicine to treat patients with severe inflammatory conditions (Ma et al., 2005). Therefore, also the structurally related flavonoids apigenin and hispidulin were selected and their effects on CCID formation was analysed. Apigenin is present in many spices, fruits, vegetables and medicinal plants such as *Thymus*, *Petroselinum*, *Punica*, *Vitis*, *Cynara*, *Allium*, *Chamaemelum*, *Solidago*, *Viola* and *Apium* (Sung et al., 2016) and hispidulin is contained in plants used in TCM and Ayurvedic medicine such as *Grindelia argentea*, *Arrabidaea chica*, *Saussurea involucre*, *Crossostephium chinense*, *Scutellaria barbata*, and in traditional European medicine i.e. *Rosmarinus officinalis*, *Artemisia* and *Salvia* species (Patel and Patel, 2017).

Since 5-fluorouracil (5-FU), which is used as CRC standard therapy, induces chemo-resistance (Longley et al., 2003), the effectiveness of these compounds was, moreover, tested in a 5-FU-resistant SW620 (SW620-R) clone.

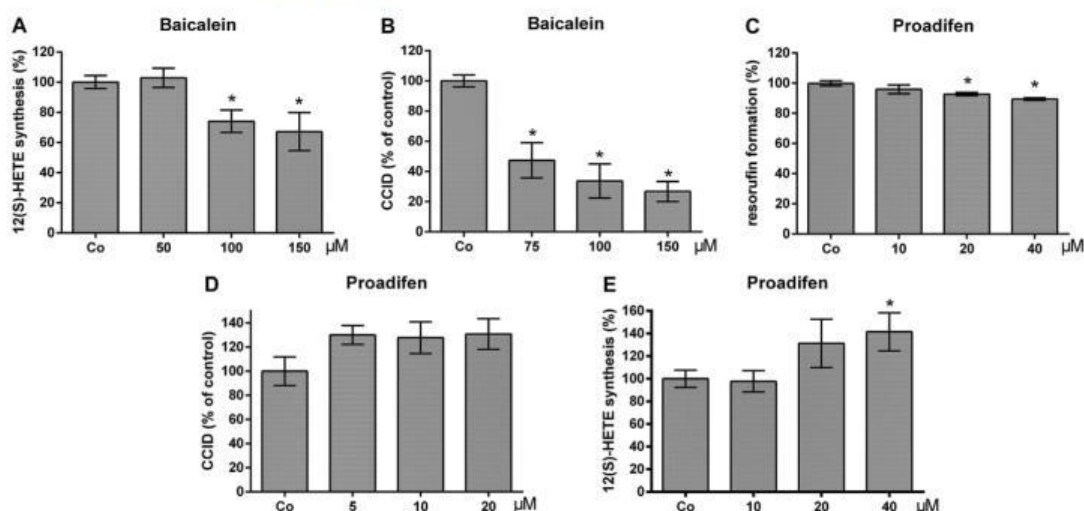


Fig. 1. Specific pathway inhibition. (a, e) SW620 cells were seeded in 3.5-cm dishes, grown to 80% confluence and treated with 10 μM arachidonic acid together with solvent (DMSO (CAS-No. 67-68-5); Co) or the indicated concentrations of (a) baicalein (CAS-No. 491-67-8) and (e) proadifen (CAS-No. 302-33-0) for 4 h. Then, cell culture supernatants were aspirated and 12(S)-HETE was measured. Two independent experiments were performed and for each concentration three replicates were analysed. (b, d) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of (b) baicalein and (d) proadifen. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. (c) SW620 cells were kept under steroid-free conditions and treated with the indicated concentrations of solvent (DMSO; Co) or proadifen. 5 μM ethoxyresorufin was added and after 3 h the formation of resorufin was analysed, which is specific for CYP1A1/1A2 activity. For each experimental point four replicates were analysed. Error bars indicate mean ± S.E.M and asterisks significance (p < 0.05; one-way ANOVA).

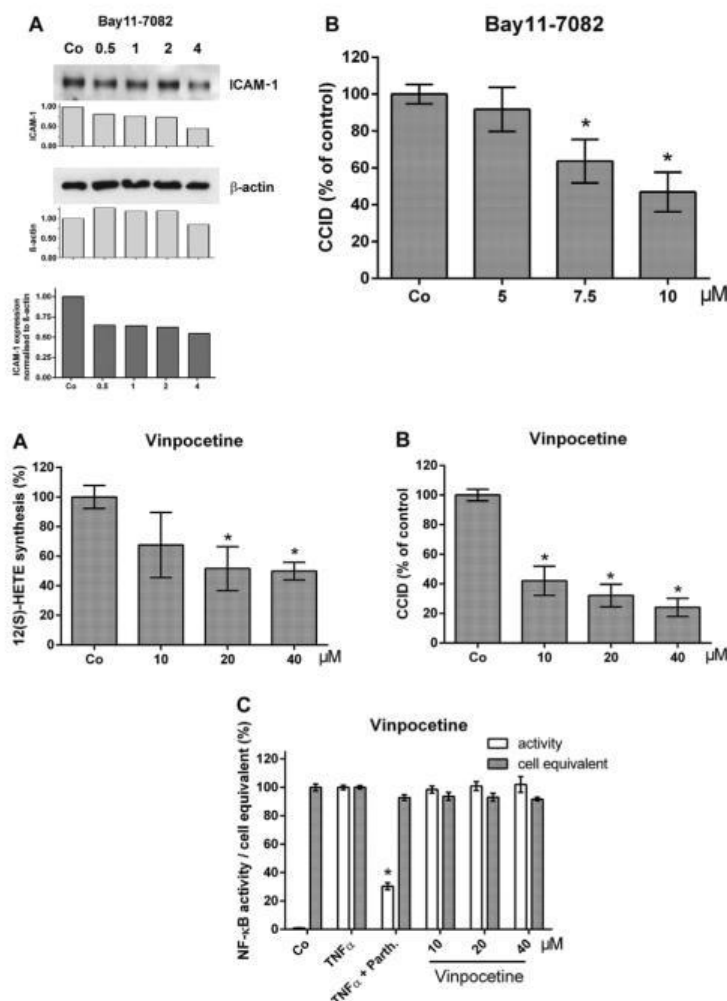


Fig. 2. Bay11-7082 (CAS-No. 19542-67-7). (a) BECs were treated with 10 μM Bay11-7082 for 0.5, 1, 2 and 4 h. Then cells were lysed, proteins separated by SDS gel electrophoresis, transferred to PVDF membrane and subjected to Western blotting using the indicated antibodies. Staining with Ponceau S and immunoblotting with anti-β-actin antibody controlled equal sample loading. Densitometer readings facilitated the comparison of relative ICAM-1- and β-actin protein expression levels with respective controls (which were set as "1"). Then, ICAM-1 expression was normalised to β-actin expression. (b) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of Bay11-7082. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean ± S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

Fig. 3. Vinpocetine (CAS-No. 42971-09-5). (a) SW620 cells were seeded in 3.5-cm dishes, grown to 80% confluence and treated with 10 μM arachidonic acid (CAS-No. 506-32-1) together with solvent (DMSO; Co) or the indicated concentrations of vinpocetine for 4 h. Then, the cell culture supernatant was aspirated and 12(S)-HETE (CAS-No. 54397-83-0) was measured. Two independent experiments were performed and for each concentration three replicates were analysed. (b) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of vinpocetine. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. (c) HEK293^{NF-κB-Luc} cells were transfected with an expression plasmid for green fluorescence protein. After incubation for 6 h, 4×10^4 cells per well were seeded in serum- and phenol red-free DMEM in a 96-transparent-well plate and grown overnight. Then, cells were treated with solvent (DMSO; Co), or 5 μM parthenolide (CAS-No. 20554-84-1), or indicated vinpocetine concentrations for 1 h when cells were stimulated with 2 ng/ml human recombinant TNFα (CAS-No. 94948-59-1) for additional 4 h. The luciferase signal derived from the NF-κB reporter was normalised by the GFP-derived fluorescence to account for differences in cell number. Three independent experiments were performed and for each concentration three replicates were analysed. Error bars indicate mean ± S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

2. Material and methods

2.1. Antibodies and reagents

Mouse monoclonal anti-CD54 (ICAM-1) antibody was from Immunotech (Marseille, France), monoclonal anti-β-actin from Sigma (Munich, Germany) and peroxidase-conjugated polyclonal rabbit anti-mouse-, IgG was from Dako (Glostrup, Denmark). Vinpocetine, ketotifen, guanfacine, nifedipine, carbamazepine, fenofibrate, proadifen, apigenin and hispidulin were purchased from Sigma (Munich, Germany), and Bay11-7082 and baicalein from Biomol (Hamburg, Germany).

2.2. Cell culture

Human SW620 (CCL-227, lymph node metastasis) colon adenocarcinoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin (PS) and L-glutamine (Gibco, Invitrogen, Karlsruhe,

Germany). From this cell line, the resistant subclone SW620-R was generated by continuous addition of 125 μM 5-FU (CAS-No. 51-21-8) over a period of time (Schmidt et al., 2004; Tentes et al., 2010) and it was further cultivated in presence of 5-FU, which was withdrawn several days before this clone was used for experiments.

Telomerase immortalized human dermal microvascular blood endothelial cells (BECs; C-12260, Promocell, Heidelberg, Germany) were grown in EGM2 MV (Clonetics CC-4147, Allendale, NJ, USA) (Schoppmann et al., 2004). Human primary CAF (CT5.3) were isolated from a colorectal adenocarcinoma resection specimen obtained in accordance with the local ethics committee (Ghent University Hospital). In short, tissue fragments were cut in small pieces and transferred into a 6-well plate with FCS. Isolated CT5.3 cells were infected with a pBabe retroviral vector expressing the hTERT open reading frame (De Vlieghere et al., 2015). CT5.3 cells were cultivated in DMEM supplemented with 10% FCS, 1% PS, 1% L-glutamine and 2 μg/ml puromycin. All cells were kept at 37 °C in a humidified atmosphere containing 5% CO₂. For CCID formation assays, BECs or CAFs were stained with CellTracker™ green purchased from Invitrogen (Karlsruhe, Germany).

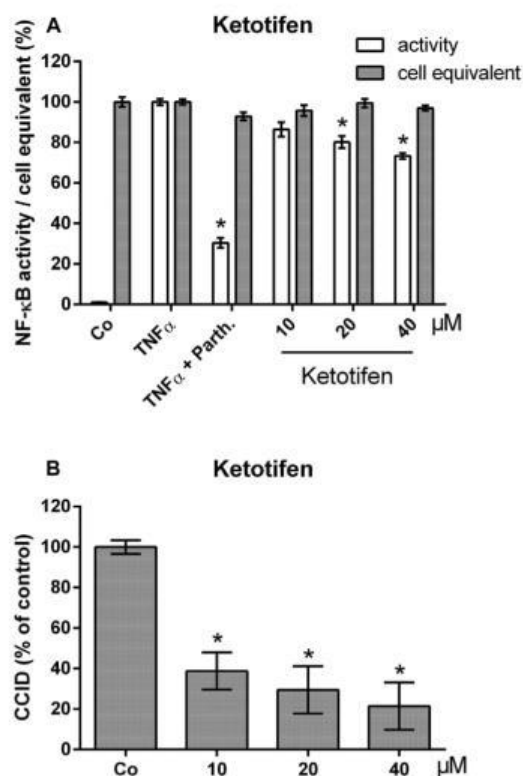


Fig. 4. Ketotifen (CAS-No. 34580-14-8). (a) HEK293^{NF- κ B/Luc} cells were transfected with an expression plasmid for green fluorescence protein. After incubation for 6 h, 4×10^5 cells per well were seeded in serum- and phenol red-free DMEM in a 96-transparent-well plate and grown overnight. Then, cells were treated with solvent (DMSO; Co), or 5 μ M parthenolide, or indicated ketotifen concentrations for 1 h when cells were stimulated with 2 ng/ml human recombinant TNF α for additional 4 h. The luciferase signal derived from the NF- κ B reporter was normalised by the GFP-derived fluorescence to account for differences in cell number. Three independent experiments were performed and for each concentration three replicates were analysed. (b) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of ketotifen. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

2.3. Spheroid formation and drug treatment

SW620 cells (2000 cells per spheroid, unless otherwise specified) were transferred into DMEM (supplemented with 5% FCS, 1% PS and 1% L-glutamine) containing methylcellulose (M-512; Sigma Aldrich) at a final concentration of 0.3%. 150 μ l of this cell suspension was transferred to each well of a 96-well round bottom plate (Greiner Bio-one, Cellstar 650185, Kremsmünster, Austria). The plates were centrifuged for 15 min at 1200 rpm and thereafter incubated at 37 °C, 5% CO₂ for 6 days to allow spheroid formation. Then, spheroids were collected, washed twice with fresh medium to remove the methylcellulose and were pre-treated with different inhibitors for 20 min in 1 ml EGM2V medium. All inhibitor solutions were freshly prepared in DMSO and the final DMSO concentration of 0.1% was kept constant. Afterwards, the incubation medium together with the spheroids were transferred to confluent CellTrackerTM (green)-stained BEC (or CAF) monolayers that were seeded into 24-well plates (Costar 3524, Sigma-

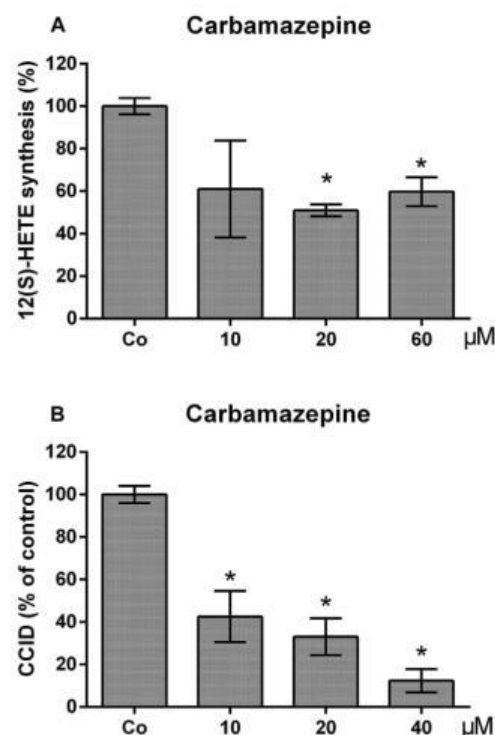


Fig. 5. Carbamazepine (CAS-No. 298-46-4). (a) SW620 cells were seeded in 3.5-cm dishes, grown to 80% confluence and treated with 10 μ M arachidonic acid together with solvent (DMSO; Co) or the indicated concentrations of carbamazepine for 4 h. Then, the cell culture supernatant was aspirated and 12(S)-HETE was measured. Two independent experiments were performed and for each concentration three replicates were analysed. (b) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of carbamazepine. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

Aldrich, Munich, Germany) and co-incubated at cell culture conditions for 4 h.

2.4. CCID (circular chemorepellent induced defect) assay

In this assay, the sizes of the cell free areas (CCIDs) formed directly underneath the tumour cell spheroids within the BEC- or CAF monolayers, were photographed using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope to visualise CellTrackerTM-stained BECs and CAFs underneath the spheroids. CCID areas were calculated with the Zen Little 2012 (Zeiss, Jena, Germany). For each treatment and control the CCID size of at least 12 spheroids (unless otherwise specified) was measured.

2.5. SDS gel electrophoresis and Western blotting

BECs were grown in T-25 tissue culture flasks (Nunc, Roskilde, Denmark) to 80% confluence and then pre-treated with FDA-drugs and flavonoids for 0.5, 1, 2 and 4 h. Then, cells were processed for SDS gel electrophoresis and Western blotting as described before (Nguyen et al., 2015). Chemo-luminescence was developed by Amersham ECL prime

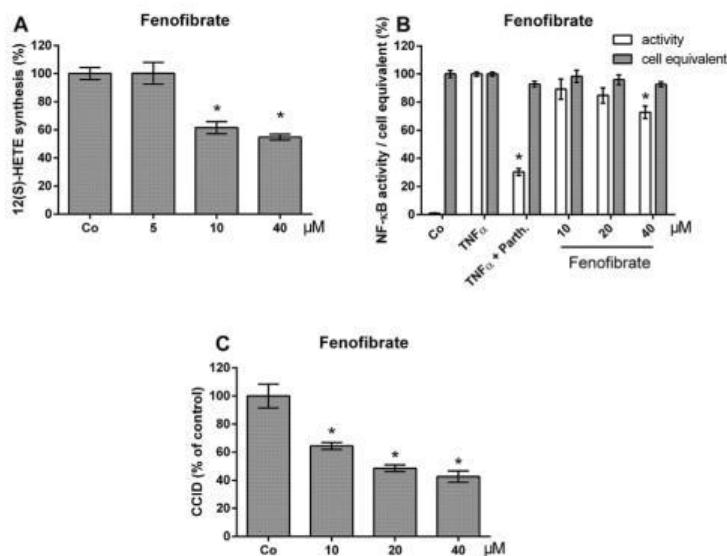


Fig. 6. Fenofibrate (CAS-No. 49562-28-9). (a) SW620 cells were seeded in 3.5-cm dishes, grown to 80% confluence and treated with 10 μM arachidonic acid together with solvent (DMSO; Co) or the indicated concentrations of fenofibrate for 4 h. Then, the cell culture supernatant was aspirated and 12(S)-HETE was measured. Two independent experiments were performed and for each concentration three replicates were analysed. (b) HEK293^{NF-κB/Luc} cells were transfected with an expression plasmid for green fluorescence protein. After incubation for 6 h, 4×10^4 cells per well were seeded in serum- and phenol red-free DMEM in a 96-transparent-well plate and grown overnight. Then, cells were treated with solvent (DMSO; Co), or 5 μM parthenolide, or indicated fenofibrate concentrations for 1 h when cells were stimulated with 2 ng/ml human recombinant TNFα for additional 4 h. The luciferase signal derived from the NF-κB reporter was normalised by the GFP-derived fluorescence to account for differences in cell number. Three independent experiments were performed and for each concentration three replicates were analysed. (c) SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of fenofibrate. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

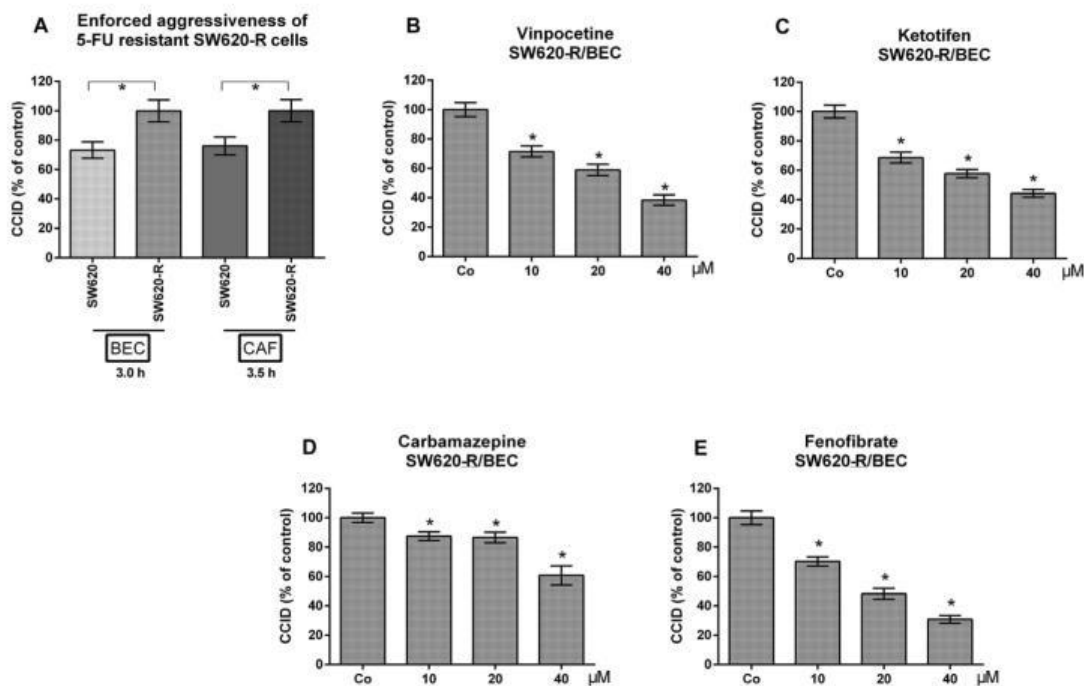


Fig. 7. Distinct aggressiveness and response of 5-FU resistant SW620-R cells. (a) An input of 3000 cells/spheroid were used to grow spheroids for 6 days, which were then placed on confluent BEC or CAF layers for the 3.0 h or 3.5 h (respectively) when CCID areas were measured. CCIDs triggered by resistant SW620-R spheroids were set 100% and related to naive SW620 spheroids. Three parallel experiments were performed and for each cell clone and condition a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance (Significance: $p < 0.05$ analysed by t-test). (b–e) An input of 2000 SW620-R cells/spheroid were used to grow spheroids for 6 days, when they were pre-treated for 20 min with solvent (Co, DMSO) or the indicated concentrations of (b) vinpocetine, (c) ketotifen, (d) carbamazepine, and (e) fenofibrate. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$ analysed by one-way ANOVA).

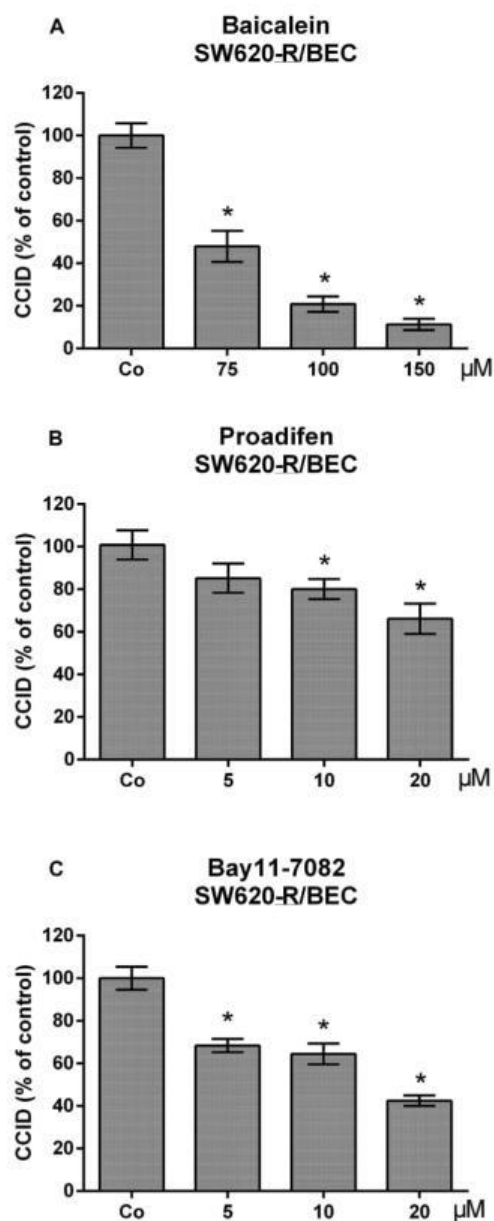


Fig. 8. Specific pathway inhibition. Resistant SW620-R spheroids were pre-treated for 20 min with solvent (Co, DMSO) or the indicated concentrations of (a) baicalein, (b) proadifen and (c) Bay11-7082. Then, spheroids and compounds were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

Kit (GE Healthcare, Freiburg, Germany) and detected using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland). Densitometry of the Western blots was analysed with Image-J software (National

Institutes of Health, Maryland, USA).

2.6. 12(S)-HETE assay

SW620 cells were seeded in 6-well plates and grown in 2.5 ml complete RPMI medium to ~70–80 confluence. Then FCS was removed overnight and cells were treated with 10 μM arachidonic acid (#A3555, Sigma-Aldrich, Munich, Germany) in serum free medium for 4 h. Subsequently test compounds were added for another 4 h, as indicated. Afterwards, medium was aspirated and shortly centrifuged to remove debris. To stabilize eicosanoids in samples butylated hydroxytoluene (BHT, 1:1000) was added to the supernatants and the mixture stored at -80°C . The concentration of 12(S)-HETE in the cellular supernatant was measured with minor modifications as described previously (Teichmann et al., 2014), by using the 12(S)-HETE enzyme immunoassay kit (EIA, # ADI-900-050; Enzo Life Sciences, Lausen, Switzerland). Absorbance was measured with a Wallac 1420 Victor 2 multilabel plate reader (Perkin Elmer Life and Analytical Sciences). The concentration of 12(S)-HETE in the cellular supernatant was normalised to the cell number.

2.7. Ethoxyresorufin-O-deethylase (EROD) assay selective for CYP1A1/1A2 activity

SW620 cells were grown in phenol red-free RPMI 1640 medium (PAN Biotech, Aldenbach, Germany) containing 10% FCS and 1% PS (Invitrogen, Karlsruhe, Germany). Before treatment, the cells were transferred to phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped FCS (PAN Biotech, Aldenbach, Germany) and 1% PS. After 18 h of incubation, ethoxyresorufin (final concentration 5.0 μM, Sigma-Aldrich, Munich, Germany) was added with or without test compounds and 0.4 ml aliquots of the medium were sampled after 180 min and the formation of resorufin, which is indicative for CYP1A1/1A2 activity, was measured by spectrofluorometry (PerkinElmer LS50B, Waltham, MA, USA) with an excitation wavelength of 530 nm and an emission wavelength of 585 nm.

2.8. NF-κB transactivation assay

The transactivation of a NF-κB-driven luciferase reporter gene was quantified in HEK293^{NF-κB/Luc} cells (Panomics, RC0014, Fremont, CA, USA) as previously described (Rozema et al., 2012). In brief, cells were maintained at 37°C and 5% CO_2 , humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 2 mM glutamine, 100 μg/ml hygromycin B, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, and 10% FCS. One day before the experiments 1×10^7 cells were seeded in 20 ml full growth DMEM medium in a 15-cm dish and then, cells were transfected with an expression plasmid for green fluorescence protein (GFP) (pEGFP-N1; Clontech, Mountain View, CA, USA). A total of 30 μl Lipofectamin 2000 (Invitrogen) and 7.5 mg DNA were mixed in 2 ml transfection medium and incubated for 20 min at RT and then added to the cells. After incubation for 6 h in humidified atmosphere containing 5% CO_2 cells were reseeded in 96-well plates at a density of 4×10^4 cells/well in phenol red-free and FCS-free DMEM overnight, and then pre-treated with the test compounds for 1 h prior to stimulation with 2 ng/ml $\text{TNF}\alpha$ for additional 4 h. The final concentration of DMSO in the experiments was 0.1% or lower and an equal concentration of DMSO was used as control. After cell lysis the luminescence of the firefly luciferase and the GFP-derived fluorescence, which was used for normalisation accounting to differences in cell number, was quantified on a GeniosPro plate reader (Tecan, Grodig, Austria). The accepted NF-κB inhibitor parthenolide (Sigma, Munich, Germany) was used as control.

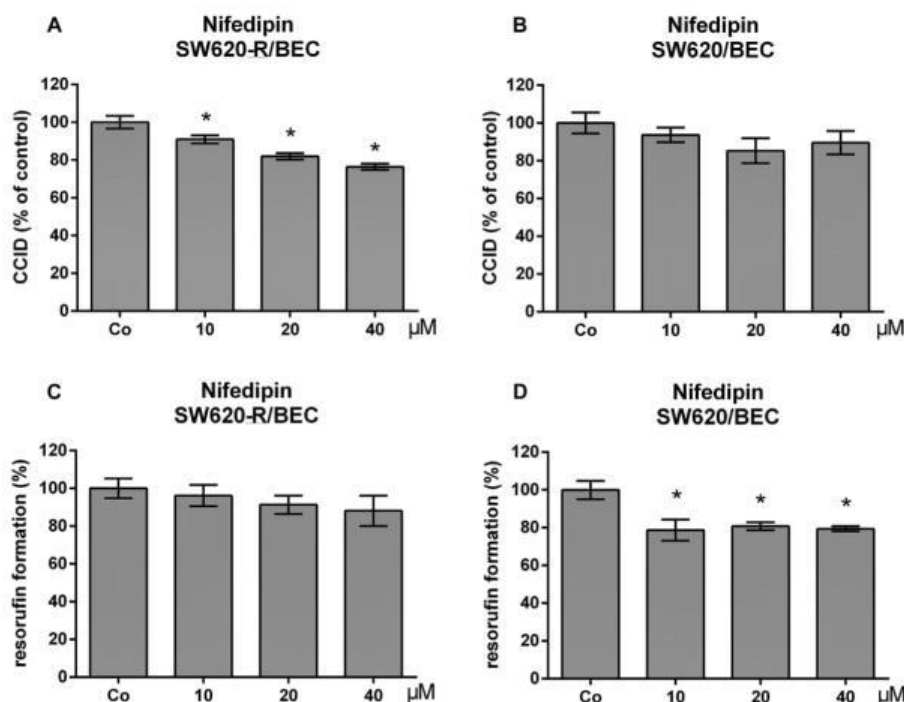


Fig. 9. Effect of nifedipin in resistant SW620-R and naïve SW620 models. (a) Resistant SW620-R- and (b) naïve SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of nifedipin (CAS-No. 21829-25-4). Then, spheroids and compound were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. (c) Resistant SW620-R- and (d) naïve SW620 spheroids were kept under steroid-free conditions and treated with the indicated concentrations of solvent (DMSO; Co) or nifedipin. 5 µM ethoxyresorufin was added and after 3 h the formation of resorufin was analysed, which is specific for CYP1A1/1A2 activity. For each experimental point four replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

2.9. Statistical analysis

For statistical analyses Excel 2013 software and Prism 6 software package (GraphPad, San Diego, CA, USA) were used. The values were expressed as mean $\pm$ S.E.M and t-test and one-way ANOVA were used to compare differences between control samples and treatment groups as well as difference among treatment groups. Statistical significance level was set to $p < 0.05$.

3. Results

3.1. Potential mechanisms of SW620 spheroid intravasation through the BEC barrier

SW620 colon cancer cells express ALOX12 and produce 12(S)-HETE (Klampfl et al., 2012). Therefore, the established ALOX12 inhibitor baicalein significantly reduced 12(S)-HETE levels (Fig. 1a) and attenuated CCID formation (Fig. 1b). 12(S)-HETE is also synthesised by CYP1A1 as a by-product (Nguyen et al., 2016c; El-Sherbeni and El-Kadi, 2016). The pan-CYP inhibitor proadifen suppressed CYP1A1/1A2-specific conversion to resorufin which was measured by EROD assay (Fig. 1c). Interestingly, treatment with proadifen caused an increase in 12(S)-HETE levels in SW620 cells (Fig. 1d), and CCID formation in the SW620/BEC model was not inhibited (Fig. 1e). CYPs and ALOX12 compete for arachidonic acid as their common substrate to synthesise their respective metabolites (CYPs produce mostly epoxides). Inhibition of CYP family members by proadifen attenuated the conversion of

ethoxyresorufin to resorufin and at the same time favoured ALOX12 to metabolise more substrate thereby increasing constitutive 12(S)-HETE levels, as it was observed earlier in MCF-7 cells (Kretschy et al., 2013). CRC cell lines, such as SW620, express hardly any CYP1A1 (Habano et al., 2009). Therefore, CYP1A1 activity was unlikely to account for the 12(S)-HETE levels measured in SW620 cells. Hence, CYP1A2 activity must have been responsible for ethoxyresorufin conversion, which was weakly inhibited by proadifen treatment.

Endothelial ICAM-1 expression is another prerequisite for LEC retraction and CCID formation (Viola et al., 2013; Nguyen et al., 2015). ICAM-1 is regulated by NF- κ B and therefore, we tested its role in the BEC model. Specific inhibition of I κ B α phosphorylation by Bay11-7802 reduced ICAM-1 expression in BECs (Fig. 2a) and treatment of the SW620/BEC model with increasing concentrations of Bay11-7802 significantly attenuated CCID formation (Fig. 2b). Neither proadifen nor baicalein suppressed ICAM-1 protein levels indicating that these inhibitors did not affect NF- κ B activity in BECs (data not shown). Hence, NF- κ B and ALOX12 (but not CYP1A1) contributed to CCID formation in the SW620/BEC model.

3.2. Inhibition of CCID formation by clinically used drugs

Previous studies report that several FDA approved drugs inhibit distinct cellular mechanisms relevant for CCID formation in breast cancer/LEC- and colon cancer/CAF (cancer associated fibroblast) models (Kretschy et al., 2013; Teichmann et al., 2014; Nguyen et al., 2017; Stadler et al., 2017). Hence, we tested some of these drugs in the

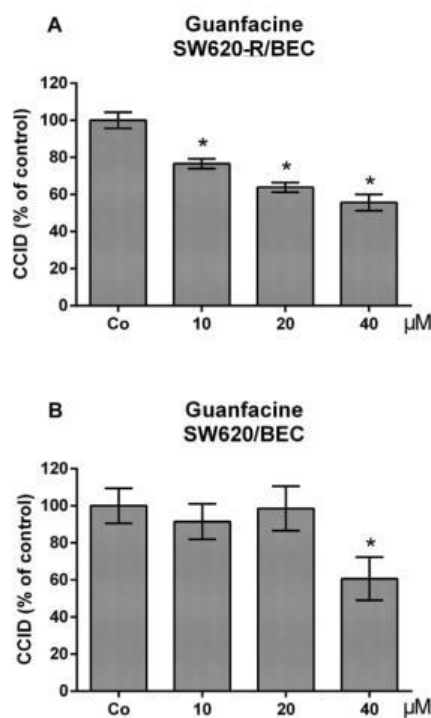


Fig. 10. Effect of guanfacine in resistant SW620-R and naïve SW620 models. (a) Resistant SW620-R- and (b) naïve SW620 spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of guanfacine (CAS-No. 29110-47-2). Then, spheroids and compound were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

SW620/BEC model. Vinpocetine inhibited 12(S)-HETE synthesis (Fig. 3a) and attenuated CCID formation (Fig. 3b), whereas NF- κ B activity remained unaffected (Fig. 3c). Conversely, ketotifen inhibited NF- κ B activity (Fig. 4a) and CCID formation (Fig. 4b), whereas 12(S)-HETE levels remained unchanged in SW620 cells (data not shown). Carbamazepine decreased both, 12(S)-HETE production (Fig. 5a) and NF- κ B activity (Teichmann et al., 2014), and attenuated CCID formation (Fig. 5b). Similarly, fenofibrate inhibited 12(S)-HETE synthesis (Fig. 6a), NF- κ B activity (Fig. 6b) and CCID formation (Fig. 6c). Neither compound affected conversion of ethoxyresorufin (data not shown). Hence, FDA approved drugs, which inhibited NF- κ B and/or 12(S)-HETE synthesis, attenuated SW620 breaching through the BEC barrier *in vitro*.

3.3. Inhibition of CCID formation in a 5-FU-resistant SW620-R/BEC model

5-FU is a standard clinical drug for the treatment of CRC. 5-FU-resistant SW620 (SW620-R) spheroids exhibited superior intravasative

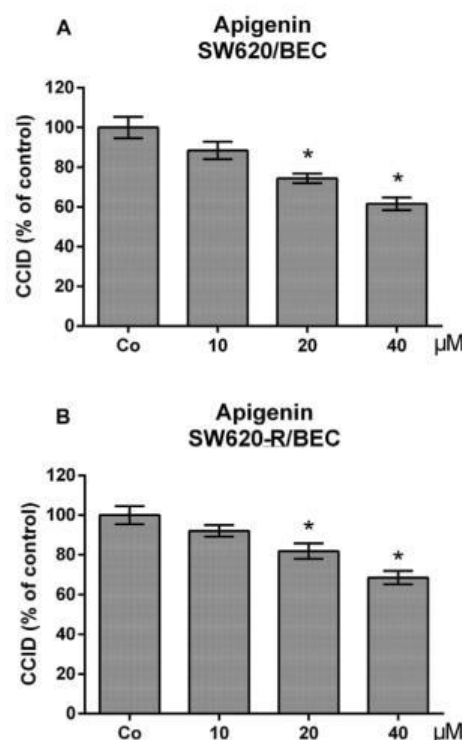


Fig. 12. Effect of apigenin in naïve SW620 and resistant SW620-R models. (a) Naïve SW620- and (b) resistant SW620-R spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of apigenin. Then, spheroids and compound were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

potential as they formed larger CCIDs in BEC- or fibroblast barriers than naïve SW620 spheroids (Fig. 7a). The inhibition of CCIDs by vinpocetine, ketotifen and carbamazepine was less efficient in the resistant SW620-R/BEC model as compared to the naïve test system indicating the development of cross-resistance in the SW620-R clone, whereas fenofibrate was similarly active in both 3D models (Fig. 7b, c, 7d, 7e; respectively). To elucidate whether similar mechanisms were responsible for CCID formation in the 5-FU-resistant setting, the SW620-R/BEC model was treated with the inhibitors baicalein (Fig. 8a), proadifen (Fig. 8b) and Bay11-7802 (Fig. 8c). Baicalein and Bay11-7802 were similarly active in the SW620-R/BEC and SW620/BEC models. Unexpectedly, proadifen exhibited much stronger anti-intravasative properties in the SW620-R/BEC model than in the naïve model (compare to Fig. 1c). This implicated that a hitherto silent pro-intravasative mechanism became manifest throughout the acquisition of 5-FU-resistance, which was then targetable by proadifen. We

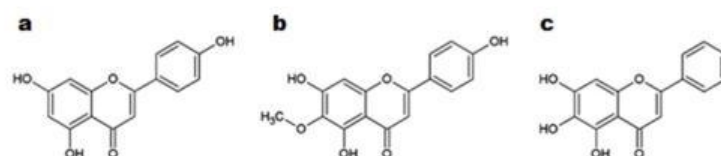


Fig. 11. Chemical structures. (a) apigenin (CAS-No. 520-36-5), (b) hispidulin (CAS-No. 1447-88-7) and (c) baicalein (CAS-No. 491-67-8).

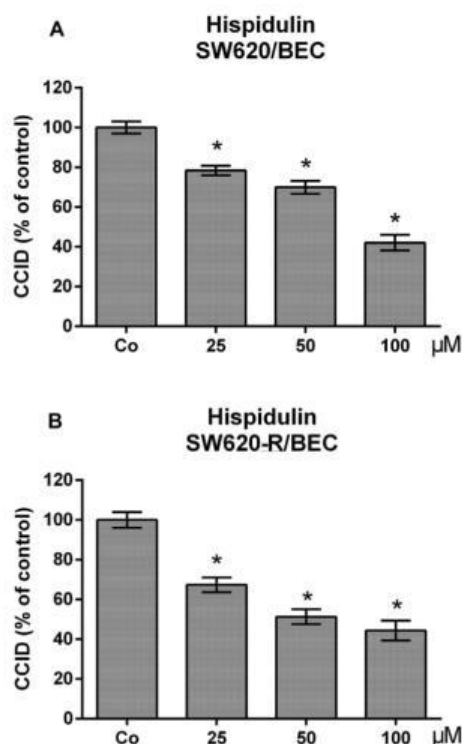


Fig. 13. Effect of hispidulin in naive SW620 and resistant SW620-R models. (a) Naive SW620- and (b) resistant SW620-R spheroids were pre-treated for 20 min with solvent (DMSO; Co) or the indicated concentrations of hispidulin. Then, spheroids and compound were placed on top of BEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three parallel experiments were performed and for each concentration a total of at least 12 replicates were analysed. Error bars indicate mean $\pm$ S.E.M and asterisks significance ($p < 0.05$; one-way ANOVA).

hypothesized that in the SW620-R clone CYP1A1 may have become over-activated and tested this possibility by EROD assay. However, the activity of CYP1A1/1A2 was not higher in resistant SW620-R cells as compared to naive SW620 cells (data not shown). Therefore, proadifen, besides CYP1A1/1A2, must have inhibited also a different mechanism in the SW620-R clone. It awaits to be investigated whether other CYP family members contribute to tumour intravasation as well. The hypothetical up-regulation of previously silent intravasation mechanisms was substantiated by experiments testing nifedipine and guanfacine, because both drugs inhibited CCID formation stronger in the chemo-resistant SW620-R/BEC model, than in the naive SW620/BEC model (Figs. 9a, b and 10a, b; respectively). Interestingly, nifedipine inhibited resorufin formation in the naive SW620/BEC model (Fig. 9d), but not in the resistant SW620-R/BEC model (Fig. 9c) and guanfacine did not inhibit resorufin formation in either model (data not shown). This further discouraged a role of CYP1A1 in the enforcement of the intravasative property of SW620-R cells during the acquisition of 5-FU-resistance. Hence, the mechanisms which render SW620-R cells accessible to therapy regimens using proadifen, nifedipine and guanfacine remain to be elucidated.

3.4. Inhibition of CCID formation by flavonoids contained in fruits and herbs

The ALOX12 inhibitor baicalein was tested against metastatic breast cancer in a phase I/II trial (<http://tinyurl.com/2oyohu>; Rugo et al., 2007). As baicalein exhibited anti-intravasative activity, we tested two other plant flavonoids, apigenin and hispidulin, which are structurally closely related to baicalein (Fig. 11a, b, c; respectively) and contained in medicinal plants, herbs, fruits and vegetables. Apigenin inhibited CCID formation in naive SW620/BEC- and 5-FU-resistant SW620-R/BEC model with quite similarly efficiency (Fig. 12a, b) and hispidulin was slightly more effective in the SW620-R/BEC model (Fig. 13a, b) at concentrations that might be achieved *in vivo*.

4. Discussion

Metastasising CRC invades the surrounding stroma, intravasates lymphatic- and blood vessels and colonises distant organs along these routes. Here, we investigated potential mechanisms how colon cancer cells breach the BEC barrier *in vitro*. SW620 colon cancer cells secrete 12(S)-HETE (Klampfl et al., 2012), which induces endothelial- and fibroblast retraction and thus, intravasation and invasion, respectively (Senfter et al., 2015; Stadler et al., 2017). 12(S)-HETE is produced by ALOX12/15 (Funk, 2006) and CYP1A1 (Nguyen et al., 2016c) and hence, the mechanisms leading to 12(S)-HETE generation may differ. To the contrary, inhibition of CYP1A1 even induced 12(S)-HETE levels due to non-consumed arachidonic acid now available for ALOX12-metabolism, and did not reduce the intravasative potential of SW620 cells.

Another mechanism that tumour intravasation relies on is the response of the microenvironment to cancer cell-secreted 12(S)-HETE, as this triggers NF- κ B-dependent retraction of LECs (Viola et al., 2013; Nguyen et al., 2016a,b) and BECs. NF- κ B regulates ICAM-1 expression in LECs, which is responsible for CCID formation in a neutrophil model (Rigby et al., 2015) and in breast cancer models (Viola et al., 2013; Nguyen et al., 2015), and also in BECs ICAM-1 and CCID formation was regulated by NF- κ B. Therefore, SW620 cells use similar mechanisms to adopt control over their microenvironment and weaken its resilience, e.g. of adjacent stroma, lymph- or blood endothelial vasculature. Importantly, FDA approved drugs either strengthened BEC resilience by inhibiting NF- κ B, or reduced cancer cell aggressiveness by inhibiting 12(S)-HETE synthesis, or did both. In the 5-FU-resistant SW620-R/BEC model vinpocetine, ketotifen and carbamazepine exhibited reduced anti-intravasative properties indicating the development of cross-resistance. In contrast, proadifen, guanfacine and nifedipine gained anti-intravasative potential in the resistant model that was hardly observed in the naive one. This indicated the activation of genes, which were silent before resistance-acquisition and that became now targetable by these drugs. Yet, the hypothesis which suspected a “gain-of-CYP1A1-function” throughout chemo-resistance-development had to be dismissed, despite the inhibition of SW620-R cell aggressiveness by proadifen. Reportedly, proadifen inhibits also glibenclamide-sensitive $K^{(+)}$ channels, and also nifedipin (which, as well, exhibited activity-gaining in the chemo-resistant model) blocks the binding sites of calcium channel receptors, which causes an influx inhibition of exogenous calcium. As the activation of $Ca^{(2+)}$ and $K^{(+)}$ channels during acquisition of chemo-resistance (Liu et al., 2015; He and Ma, 2016; Mahmood et al., 2014), and their participation in CCID formation was demonstrated (Nguyen et al., 2017), the gain of ion channel activity throughout the development of drug resistance is feasible. Therefore, the emergence of previously silent targets (that await to be elucidated) may also happen throughout chemo-resistance development *in vivo* and should be considered in individualised therapy concepts.

Most anti-cancer treatments are targeting cell division mechanisms, whereas the drugs tested herein were neither developed against cell cycle- nor metastatic protagonists, but are directed against entirely different ailments. Hence, the here reported anti-intravasative

properties were unintended “side effects”.

Like baicalein, the flavonoids apigenin and hispidulin attenuated *in vitro* intravasation in the naïve and 5-FU-resistant models. Modes of apigenin and hispidulin activity include the inhibition of COX2 and NF- κ B (Wang et al., 2014; Clavin et al., 2007) explaining their anti-inflammatory/anti-proliferative properties. Apigenin is contained in parsley, spinach, celery, chamomile, thyme, pomegranate, etc. and hispidulin is a constituent of traditional medicinal plants such as *Artemisia*, *Salvia* and *Eupatorium* species. Apigenin and hispidulin were active at 20–25 μ M and although these concentrations may be reached *in vivo*, this cannot be achieved just through dietary intake (Vogiatzoglou et al., 2015). Apigenin is on the market as an inexpensive, isolated compound, whereas the availability of hispidulin is scarce. This may argue for apigenin as a preventive strategy strengthening BEC barrier resilience blocking off aggressive SW620 and SW620-R cells. There exist even more active flavonoid subclasses (flavonols, flavan-3-ols, anthocyanidins) in the diet and their frequent consumption indeed inversely correlates with CRC risk (Woo and Kim, 2013).

In summary, the use of FDA approved drugs, especially those applied in long term treatment schemes (i.e. fenofibrate or carbamazepine), should be investigated in meta-analyses regarding cancer- and survival rates. Particularly carbamazepine reaches blood concentrations in patients of around 50 μ M and beyond, which may reveal preventive effects. When considering apigenin supplementation as a preventive strategy adverse effects cannot be excluded and therefore, apigenin needs to be tested in appropriate CRC *in vivo* models.

Conflicts of interest

The authors declare no conflict of interest.

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Fenofibrate Inhibits Tumour Intravasation By Several Independent Mechanisms In A 3-Dimensional Co-Culture Model.

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As the co-author, I carried out the Gap assay (CCID) to investigate the ability of Chlorotoxin and Oubain inhibiting MDA-MB231 breast cancer spheroids intravasation through LECs monolayers. I also contributed in the discussion and preparation of the paper.

Fenofibrate inhibits tumour intravasation by several independent mechanisms in a 3-dimensional co-culture model

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Abstract. Lymph node metastasis of breast cancer is a clinical marker of poor prognosis. Yet, there exist no therapies targeting mechanisms of intravasation into lymphatics. Herein we report on an effect of the antidiabetic drug fenofibrate with vasoprotective activity, which attenuates breast cancer intravasation *in vitro*, and describe the potential mechanisms. To measure intravasation in a 3-dimensional co-culture model MDA-MB231 and MCF-7 breast cancer spheroids were placed on immortalised lymphendothelial cell (LEC) monolayers. This provokes the formation of circular chemorepellent induced defects (CCIDs) in the LEC barrier resembling entry ports for the intravasating tumour. Furthermore, the expression of adhesion molecules ICAM-1, CD31 and FAK was investigated in LECs by western blotting as well as cell-cell adhesion and NF- κ B activity by respective assays. In MDA-MB231 cells the activity of CYP1A1 was measured by EROD assay. Fenofibrate inhibited CCID formation in the MDA-MB231/LEC- and MCF-7/LEC models and the activity of NF- κ B, which in turn downregulated ICAM-1 in LECs and the adhesion of cancer cells to LECs. Furthermore, CD31 and the activity of FAK were inhibited. In MDA-MB231 cells, fenofibrate attenuated CYP1A1 activity. Combinations with other FDA-approved drugs, which reportedly inhibit different ion

channels, attenuated CCID formation additively or synergistically. In summary, fenofibrate inhibited NF- κ B and ICAM-1, and inactivated FAK, thereby attenuating tumour intravasation *in vitro*. A combination with other FDA-approved drugs further improved this effect. Our new concept may lead to a novel therapy for cancer patients.

Introduction

The colonisation of distant organs by disseminating breast cancer cells is the main cause for cancer death in women. Targeting neo-vascularisation with tailored drugs may prevent the spreading of those cancer entities that expand through the blood stream. In the early phases of breast cancer metastasis however, cancer cells disperse through lymphatics. Before metastases colonise organs the sentinel and post-sentinel lymph nodes fill up with breast cancer cells and hence, the axillary lymph node status is a major prognostic marker for clinical outcome (1). As blocking of intravasation would improve prognosis, understanding of mechanistic details of breast cancer intravasation into lymphatic vessels is crucial to develop treatment strategies preventing lymph node metastasis. Although prognostic markers exist, no predictive markers have been identified to date, which could be targeted to combat intravasation. Nevertheless, a few mechanisms supporting the transmigration of cancer cells through the lymphatic barrier were described (2-4). Intravasation is an interplay among cancer cells and the endothelial barrier. Importantly, not only cancer cells play an active role in this process but also lymph endothelial cells (LECs) remarkably contribute to lymph node metastasis i.e. by providing niches for cancer cell settlement (5). To further study the role of LECs at an early metastatic step, a quantitative assay based on a three-dimensional (3D) cell model was developed consisting of breast cancer spheroids placed on top of lymphendothelial monolayers, which resembles the contact zone between the

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tumour and lymphatics (6). This 3D *in vitro* model is validated in scid mice and in tissue sections of human patients (7) and was here utilised to measure tumour cell-induced retraction of LECs, which enables cancer cells to transmigrate the lymphatic barrier. Focussing on the regulation and maintenance of the endothelial barrier function several mechanisms of LECs, which are relevant to withstand tumour intravasation, were found to become weakened by signals of adjacent cancer cells (3,4). Notably, LECs - upon activation of specific receptors - 'invite' cancer cells to intravasate by reducing the resilience of the endothelial wall (2,8,9) through a process called endothelial-to-mesenchymal transition. In turn, LECs acquire a migratory phenotype and open gates for cancer cell transit as a rate limiting step of lymph node metastasis (7). Hence, strengthening vascular integrity and tone may improve resistance to intravasating cancer cells.

As a pro-migratory mechanism ion channels modulate adhesion and mobility of tumour cells and their dysregulation is currently discussed to play a role in cancer metastasis (10). For example, the overexpression of voltage gated K^+ channels was observed in breast-, colon- and prostate cancer and medullablastoma (11,12) and their inhibition attenuates neuroblastoma and endothelial cell migration (13,14). Consistently, the Na^+/K^+ -ATPase inhibitor ouabain (also known as g-strophanthin) attenuates medullablastoma cell migration and the localisation of Tyr397-phosphorylated FAK to lamellipodia (15). Furthermore, the voltage gated $K^+_{v1.2}$ channel induces directional migration of mesenchymal bone marrow or CHO cells through phosphorylation of Tyr397-FAK (16) as well as wound healing in an animal model (17). The antidyslipidemic drug fenofibrate closes K^+ ATP channels (18,19) and voltage gated K^+ channels (20), enhances endothelial barrier function and integrity (5,21) and is therefore, vasoprotective.

Furthermore, fenofibrate exhibits anticancer effects (22). Independent of K^+ channel inhibition, fenofibrate binds to and activates peroxisome proliferator-activated protein alpha (PPAR α), which directly binds to the I κ B α promoter, induces I κ B α protein expression (23) and in turn inhibits NF- κ B (24,25). NF- κ B plays a role in tumour progression and intravasation through lymphendothelial barriers (3,4). Hence, both activities of fenofibrate, PPAR α activation, which was shown to suppress cancer progression (26,27), as well as K^+ channel inhibition (10,18,20) may attenuate adhesion and cell mobility (28,29). In the clinic fenofibrate is used to eliminate triglycerides from the blood stream (30), thereby reducing hypercholesterolemia and the risk of cardiovascular diseases. Therefore, fenofibrate and a few more clinically used drugs with reported ion channel inhibitory properties were here investigated regarding their potential activities inhibiting triple-negative (MDA-MB231) and estrogen receptor-positive (MCF-7) breast cancer cell intravasation *in vitro*. Such drugs would be directly available to patients.

Materials and methods

Antibodies and reagents. Polyclonal rabbit anti-focal adhesion kinase (FAK) and polyclonal rabbit anti-phospho-Tyr397-FAK were from Cell Signaling (Danvers, MA, USA). Polyclonal goat anti-CD54 (ICAM-1) antibody was from

R&D System (clone BBA-17, Minneapolis, MN, USA) and monoclonal anti- β -actin from Sigma (Munich, Germany). Monoclonal mouse anti-CD31 (JC70A), polyclonal rabbit anti-mouse, anti-goat and anti-rabbit IgGs were from Dako (Glostrup, Denmark).

The I κ B α phosphorylation inhibitor (E)-3-[(4-methylphenylsulfonyl)-2-propenenitrile (Bay11-7082) was purchased from Biomol (Hamburg, Germany). Bepridil hydrochloride (bepridil), niflumic acid, fenofibrate, cisapride monohydrate (cisapride), primaquine, proadifen, digoxin, chlorotoxin and ouabain octahydrate (ouabain) were from Sigma. Dyclonine was from Abcam (Cambridge, UK).

Cell culture. Human MDA-MB231 and MCF-7 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in MEM medium supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin (PS) and 1% non-essential amino acids (Gibco/Invitrogen, Karlsruhe, Germany). Telomerase immortalized human lymph endothelial cells (LECs) were grown in EGM2 MV (Clonetics CC-4147, Allendale, NJ, USA). The cells were kept at 37°C in a humidified atmosphere containing 5% CO₂. For CCID formation assays, LECs were stained with CellTracker™ green purchased from Invitrogen (Karlsruhe, Germany).

Spheroid formation. MDA-MB231 cells (input of 6,000 cells per spheroid) and MCF-7 cells (input of 3,000 cells per spheroid) were transferred to 30 ml serum-free MEM medium containing 6 ml of a 1.6% methylcellulose solution (0.3% final concentration; cat. no.: M-512, 4000 centipoises; Sigma-Aldrich, Munich, Germany). Cell suspension (150 μ l) were transferred to each well of a 96-well plate (Greiner Bio-one, Cellstar 650185, Kremsmünster, Austria) to allow spheroid formation within 48 h.

CCID (circular chemorepellent induced defect) assay. In this assay the sizes of the cell-free areas (CCIDs), which are formed in the endothelial monolayer directly underneath the tumour spheroids, were measured (7). MDA-MB231 spheroids were washed in PBS and transferred to CellTracker (green)-stained LEC monolayers that were seeded into 24-well plates (Costar 3524, Sigma-Aldrich) in 1 ml EGM2 MV medium. After 4 h of incubation, the CCID areas in the LEC monolayers underneath the MDA-MB231 spheroids were photographed using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope to visualise CellTracker-stained LECs underneath the spheroids. CCID areas were calculated with the Zen Little 2012 (Zeiss). For each condition the CCID size of at least 25 spheroids (unless otherwise specified) was measured.

SDS gel electrophoresis and western blotting. LECs were grown in T-25 tissue culture flasks (Nunc, Roskilde, Denmark) to 80% confluence and then pre-treated with indicated clinical drugs or inhibitors for 0.5, 1, 2 and 4 h. Then, cells were processed for SDS gel electrophoresis and western blotting as described before (4). Chemo-luminescence was developed by Amersham ECL Prime kit (GE Healthcare, Freiburg, Germany) and detected using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland). Densitometry of the western blots

was analysed with Image-J software (National Institutes of Health, Bethesda, MD, USA).

Ethoxyresorufin-O-deethylase (EROD) assay selective for CYP1A1 and CYP1A2 activity. MDA-MB231 cells were grown in phenol red-free DMEM/F12 medium (Gibco, Karlsruhe, Germany) containing 10% FCS and 1% PS (Invitrogen, Karlsruhe, Germany). Before treatment, the cells were transferred to DMEM/F12 medium supplemented with 10% charcoal-stripped FCS (PAN Biotech, Aldenbach, Germany) and 1% PS. After 24 h of treatment CYP1A1 activity was measured with minor modifications as previously described (31). Briefly, ethoxyresorufin (final concentration 5.0 μ M, Sigma-Aldrich) was added and 0.4 ml aliquots of the medium were sampled after 180 min and the formation of resorufin was analysed by spectrofluorometry (PerkinElmer LS50B, Waltham, MA, USA) with an excitation wavelength of 530 nm and an emission wavelength of 585 nm. The pan-CYP inhibitor proadifen (Sigma-Aldrich) was used as a positive control.

NF- κ B transactivation assay. The transactivation of a NF- κ B-driven luciferase reporter was quantified in HEK293/NF- κ B-luc cells (Panomics, RC0014, Fremont, CA, USA) as previously described (32). In brief, cells were maintained at 37°C and 5% CO₂, humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 2 mM glutamine, 100 μ g/ml hygromycin B, 100 U/ml benzylpenicillin, 100 μ g/ml streptomycin, and 10% FCS. One day before the experiments the cells were stained in serum-free medium supplemented with 2 μ M CellTracker Green CMFDA (C2925; Invitrogen) for 1 h. Then, cells were reseeded in 96-well plates at a density of 4x10⁴ cells/well in phenol red-free and FCS-free DMEM overnight, and pre-treated with the indicated compounds for 30 min prior to stimulation with 2 ng/ml TNF α for 4 h. The final concentration of DMSO in the experiments was 0.1% or lower and an equal concentration of DMSO was used as control. After cell lysis the luminescence of the firefly luciferase and the fluorescence of the CellTracker Green CMFDA were quantified on a GeniosPro plate reader (Tecan, Grodig, Austria). The luciferase-derived signal from the NF- κ B reporter was normalized by the CellTracker Green CMFDA derived fluorescence to account for differences in the cell number. The known NF- κ B inhibitor parthenolide (Sigma-Aldrich, Vienna, Austria) was used as a positive control.

Adhesion assay. MDA-MB231 (40,000 cells/well) were seeded in serum-free medium (DMEM containing 0.5% BSA, 2 mM CaCl₂ and 2 mM MgCl₂). Then 500X CellTracker (500X CellTracker Solution from CytoSelect™ Tumor-endothelium Adhesion Assay from Cell Biolabs, Inc., San Diego, CA, USA, CBA-215) was added to the cell suspension (2 μ l CellTracker to 1 ml suspension), incubated for 1 h at 37°C and centrifuged at 1,000 rpm for 2 min. Then the medium was aspirated, the cell pellet was washed x3 with serum-free medium (DMEM containing 0.5% BSA, 2 mM CaCl₂ and 2 mM MgCl₂) and then the cell pellet was re-suspended in EGM2 MV medium. The CellTracker-stained MDA-MB231 cell suspension was treated either with Bay11-7082 as a positive control, indicated compounds, or DMSO and incubated for 10 min at room temperature. After incubation, the medium was aspirated from

the 48-well plate and 200 μ l of the pre-treated MDA-MB231 cell suspension was added to LECs grown in monolayers and incubated for 30 min at 37°C. Then the medium was aspirated and cells were washed 3x with 250 μ l 1X Wash Buffer (10X Wash Buffer was from CytoSelect Tumor-endothelium Adhesion Assay). Before the third wash, cells were inspected for morphological changes under the microscope. After the final wash the plate was tapped on a flint-free paper towel and 150 μ l of 1X Lysis Buffer (4X Lysis Buffer were from CytoSelect Tumor-endothelium Adhesion Assay) was added to each well and cells were lysed by the shearing forces through a pipette tip. Lysate (100 μ l) was transferred to 96-well black-wall clear bottom plates (Nunc, Thermo Scientific, Rochester, NY, USA) and the fluorescence was measured with a fluorescence plate reader at 485/530 nm.

Statistical analysis. For statistical analyses Excel 2013 software and Prism 6 software package (GraphPad, San Diego, CA, USA) were used. The values were expressed as mean $\pm$ SEM and the Student's t-test and ANOVA with Tukey's post-test was used to compare differences between control samples and treatment groups as well as difference among treatment groups. Statistical significance level was set to P<0.05.

Results

Fenofibrate inhibits NF- κ B, ICAM-1, FAK, adhesion and CCID formation in LECs. Fenofibrate inhibited TNF α -induced NF- κ B activation (Fig. 1A) and transiently downregulated expression of the adhesion molecules ICAM-1 and CD31 in LECs (Fig. 1B). In addition, Bay11-7802 (a specific NF- κ B inhibitor) suppressed ICAM-1 (Fig. 1C) and both compounds inhibited the adhesion of MDA-MB231 cells to LECs (Fig. 1D), which is a prerequisite for intravasation and CCID formation (3). Accordingly, fenofibrate attenuated CCID formation in the MDA-MB231/LEC model (Fig. 1E) and in the MCF-7/LEC model (Fig. 1F). The inhibition of CCID formation by fenofibrate was achieved at concentrations as low as 5 μ M. However, the inhibition of NF- κ B and of cell adhesion was observed at higher concentrations indicating that also another mechanism must have been involved in the CCID-inhibitory effect of fenofibrate. It was shown that fenofibrate inhibits cell migration and stabilises HUVEC barrier function, which is accompanied by the downregulation of FAK activity (5). FAK activity is required for cell-matrix adhesion and directional migration (33,34) and adhesion and migration is also necessary for CCID formation (7). In LEC fenofibrate inhibited the phosphorylation of Tyr397-FAK (Fig. 1G), which is indicative for its activity.

Bona fide K⁺ channel inhibitors attenuate CCID formation. To investigate whether potassium channels contribute to CCID formation, the MDA-MB231/LEC model was treated with ouabain and digoxin, which are used to specifically inhibit Na⁺/K⁺-ATPase (35,36). Ouabain and digoxin dose-dependently attenuated MDA-MB231-triggered CCID formation (Fig. 2A and B; respectively). Ouabain inhibits cell migration through inactivation of FAK (37) and also digoxin inhibits cell migration (38). Similarly, cisapride (a serotonin 5-HT₄ receptor agonist used to treat gastroesophageal reflux

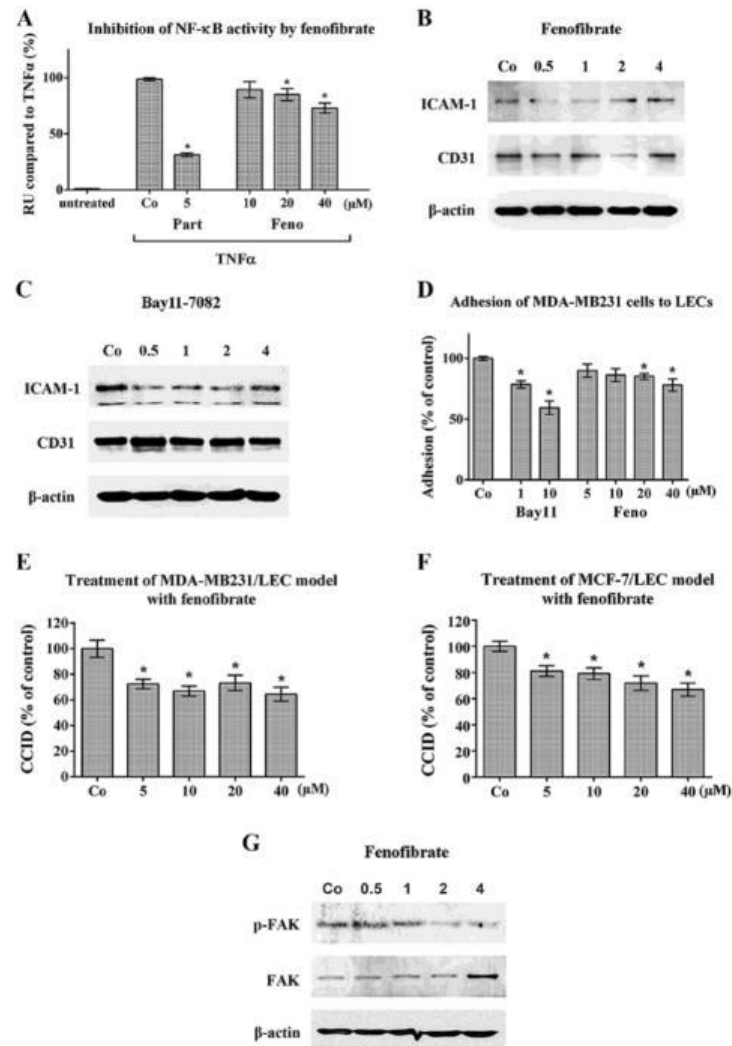


Figure 1. Inhibition of NF-κB activity, adhesion and CCID formation upon fenofibrate treatment. (A) HEK293-NFκB-Luc cells were pretreated with 5 μM parthenolide (Part) as a specific inhibitor of NF-κB or with increasing concentrations of fenofibrate (Feno) or solvent (Co-DMSO) for 1 h. Then, cells were stimulated with 2 ng/ml human recombinant TNFα for an additional 4 h when NF-κB activity was measured. Three independent experiments with 4 replicates were analysed. (B) LECs were grown to ~80% confluence and then pretreated with solvent (Co) or 20 μM fenofibrate or (C) 5 μM Bay11-7082 as a specific inhibitor of NF-κB for 0.5, 1, 2 and 4 h. Then, cells were lysed, proteins separated by SDS gel electrophoresis and subjected to western blotting using the indicated antibodies. Staining with Ponceau S and immunoblotting with anti-β-actin antibody controlled equal sample loading. (D) MDA-MB231 cells were pre-treated with Bay11-7082 (Bay11) or with increasing concentrations of fenofibrate, or solvent (Co) and then placed on confluent LEC monolayers, which were also pre-treated with respective compounds. After 30 min the adhesion of MDA-MB231 cells to LECs was measured as described in Materials and methods. Experiments were performed in triplicate. (E) MDA-MB231 spheroids or (F) MCF-7 spheroids were pre-treated for 30 min with solvent (Co) or the indicated fenofibrate concentrations. Then, spheroids were placed on top of LEC monolayers and co-cultivated for 4 h when CCIDs were analysed. Three independent experiments with at least 8 replicates were analysed. (G) LECs were grown to ~80% confluence and then pretreated with solvent (Co) or fenofibrate for 0.5, 1, 2 and 4 h when cells were lysed, proteins separated by SDS gel electrophoresis and subjected to western blotting using the indicated antibodies. Error bars indicate means ± SEM and asterisks significance (P<0.05; ANOVA together with Tukey's post-test).

disease) and primaquine (used to treat malaria through binding to Plasmodium DNA), which were both reported to inhibit hERG-K⁺ channel (39,40), inhibited MDA-MB231-triggered CCID formation (Fig. 2C and D; respectively) and MCF-7-triggered CCID formation (Fig. 2E and F; respectively). As

the treatment with known K⁺ ATPase- and hERG-K⁺ channel inhibitors resulted in significant inhibition of CCID formation, it was concluded that the activity of K⁺ channels contributes to breast cancer intravasation *in vitro*. Based on these results fenofibrate seems to inhibit CCID formation through inhibition

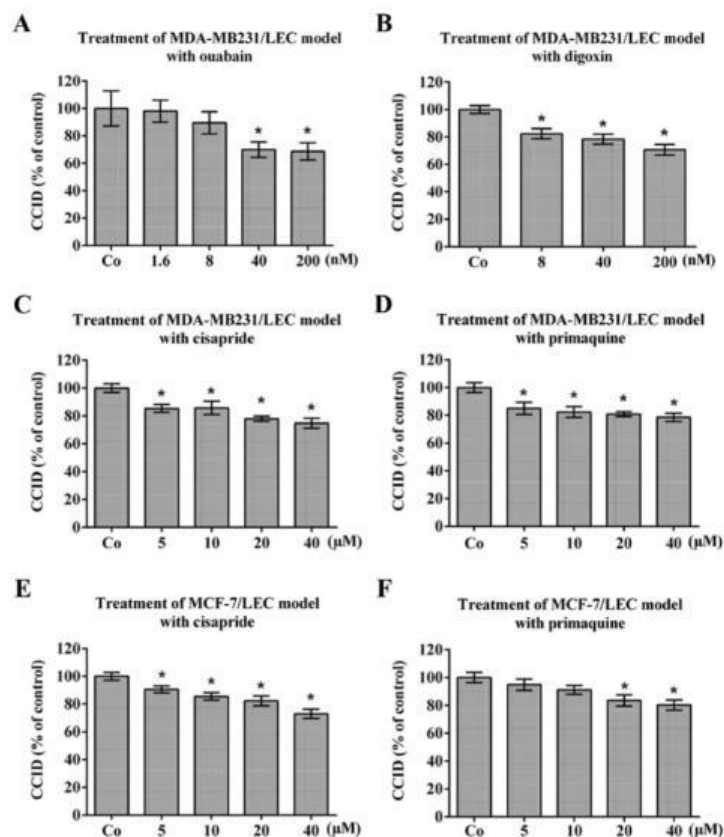


Figure 2. Inhibition of CCID formation in LEC monolayers by bona fide K^+ channel inhibitors and clinical drugs. (A-D) MDA-MB231 spheroids or (E and F) MCF-7 spheroids were pre-treated for 30 min with solvent (Co-DMSO) or the indicated concentrations of (A) ouabain, (B) digoxin, (C and E) cisapride, (D and F) primaquine. Then, spheroids were placed on top of LEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three independent experiments with at least 8 replicates were analysed. Error bars indicate means $\pm$ SEM and asterisks significance ($P < 0.05$; ANOVA together with Tukey's post-test).

of NF- κ B activity as well as of K^+ channels. Reportedly, the delayed rectifier $K^{v2.1}$ channel induces the phosphorylation of Tyr397-FAK (16,17). Therefore, fenofibrate may have inhibited FAK phosphorylation through inhibition of $K^{v1.2}$ channel. Furthermore, primaquine, but not cisapride inhibited the phosphorylation of Tyr397-FAK (data not shown).

Niflumic acid, dyclonine and bepridil hydrochloride inhibit CCID formation. As K^+ -channels, also Cl^- , Na^+ , and Ca^{2+} -channels are dis-regulated during carcinogenesis (10). Therefore, the 3D-models were treated with the reported Cl^- -channel inhibitors chlorotoxin and niflumic acid (41,42) (Fig. 3A, B and E, respectively), the Na^+ -channel inhibitor dyclonine (43) (Fig. 3C and F) and the Ca^{2+} -channel inhibitor bepridil hydrochloride (44) (Fig. 3D and G), which inhibited MDA-MB231- and MCF-7- triggered CCID formation in LEC barriers.

Fenofibrate, primaquine, cisapride, dyclonine, bepridil and niflumic acid inhibit CYP1A1 in MDA-MB231 cells.

Intravasation is an interplay between tumour emboli and the vessel endothelium beneath. In the former experiments the effect of fenofibrate was investigated in LECs. Herein, the effect of fenofibrate and the other clinical drugs was studied in MDA-MB231 breast cancer cells. Recently, it was shown that MDA-MB231 cell intravasation depends on NF- κ B- and CYP1A1-activity of the cancer cells (4,45). Niflumic acid, bepridil and primaquine, but not dyclonine or cisapride, inhibited TNF α -induced NF- κ B activity in HEK293/NF- κ B-luc cells (Fig. 4A). Furthermore, the treatment of MDA-MB231 cells with fenofibrate, primaquine, dyclonine, bepridil, niflumic acid and cisapride significantly inhibited the formation of resorufin indicating the inhibition of CYP1A1 activity (tested by EROD assay; Fig. 4B). Hence, the here tested drugs also exerted their anti-intravasative properties by attenuating CYP1A1 activity in the tumour cells and some, additionally, by inhibiting NF- κ B.

Drug combinations further reduce intravasation. The drugs at 20 μ M of fenofibrate, cisapride, primaquine, niflumic

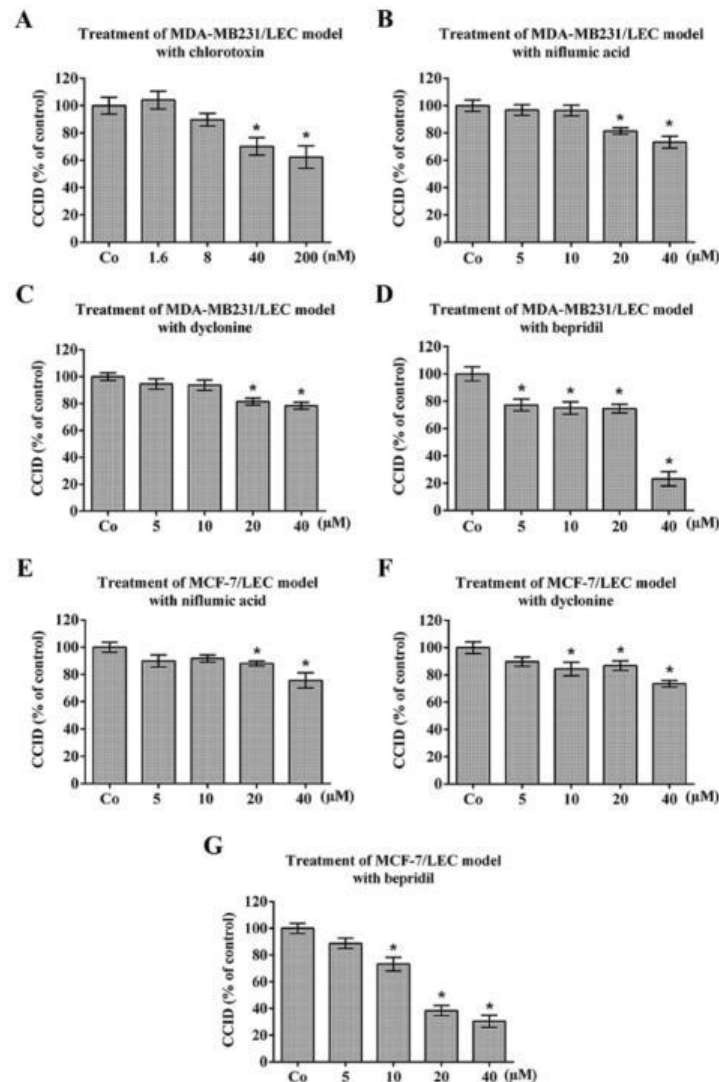


Figure 3. Inhibition of CCID formation in LEC monolayers by clinical drugs. (A-D) MDA-MB231 spheroids or (E-G) MCF-7 spheroids were pre-treated for 30 min with solvent (Co-DMSO) or the indicated concentrations of (A) chlorotoxin, (B and E) niflumic acid, (C and F) dyclonine and (D and G) bepridil. Then, spheroids were placed on top of LEC monolayers and co-cultivated for 4 h when CCIDs were measured. Three independent experiments with at least 8 replicates were analysed. Error bars indicate means $\pm$ SEM and asterisks significance ($P < 0.05$; ANOVA together with Tukey's post-test).

acid, bepridil and dyclonine acid inhibited CCID formation by ~20%. Therefore, we investigated whether drug combinations may improve the CCID-inhibitory effect. Combining only bona fide K^+ channel inhibitors or K^+ channel inhibitors together with the Na^+ channel inhibitor dyclonine did not exhibit improved effects (Table I). In contrast, the combinations of bepridil hydrochloride and primaquine, or bepridil hydrochloride and dyclonine, or bepridil hydrochloride and niflumic acid, or niflumic acid and cisapride, or niflumic acid and primaquine inhibited CCID formation additively. Synergistic effects were achieved when combining bepridil

hydrochloride and cisapride, or bepridil hydrochloride and fenofibrate, or niflumic acid and fenofibrate, or niflumic acid and dyclonine (Fig. 5). Since different drug combinations resulted in additive, synergistic, or unchanged inhibition of CCID formation, this implicated that the treatment with these drugs affected intravasative mechanisms non-randomly.

We searched for potential drug-drug interactions (DDIs) such as between fenofibrate, cisapride, and niflumic acid, which are recorded in the data base of the -Austria-Codex Fachinformation (Oesterreichische Apotheker-Verlagsgesellschaft m.b.H., date of release: 01/09/2016) regarding known cross reactions.

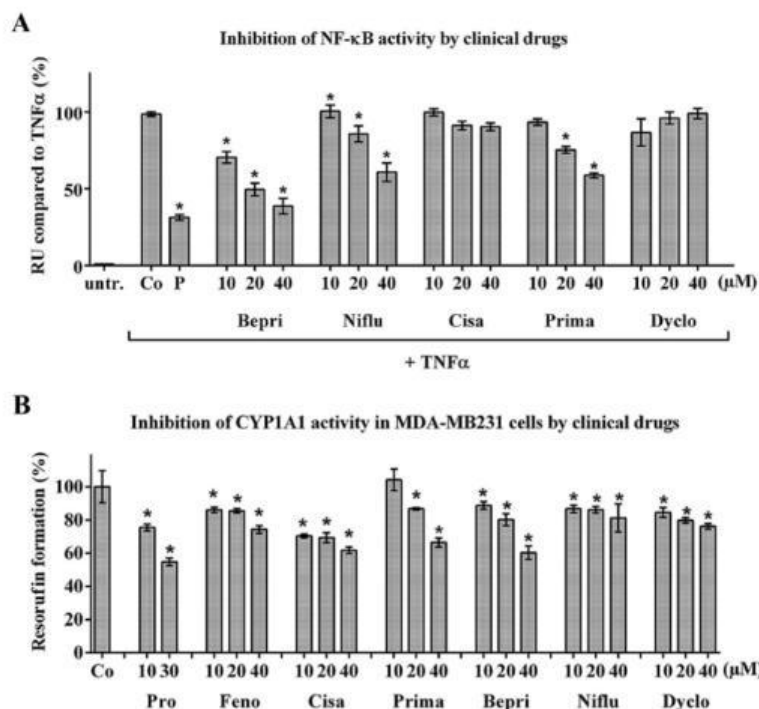


Figure 4. Inhibition of NF-κB activity by clinical drugs. (A) HEK293-NFκB-Luc cells were pretreated with 5 μM parthenolide (P) as a specific inhibitor of NF-κB or with increasing concentrations of the indicated drugs (bepridil-Bepri; niflumic acid-Niflu; cisapride-Cisa; primaquine-Prima; dyclonine-Dyclo) or solvent (DMSO-Co) for 1 h. Then, cells were stimulated with 2 ng/ml human recombinant TNFα for an additional 4 h when NF-κB activity was measured or were left TNFα-untreated (untr.). Three independent experiments with at least 4 replicates were analysed. Inhibition of CYP1A1 activity in MDA-MB231 cells by clinical drugs. (B) MDA-MB231 cells were treated with the indicated concentrations of clinical drugs (fenofibrate-Feno, etc.) or solvent. Ethoxyresorufin (5 μM) was added and after 180 min the formation of resorufin was measured. The pan-CYP-inhibitor proadifen (Pro) was used as a control. Three independent experiments with at least 3 replicates were analysed. Error bars indicate means ± SEM and asterisks significance (*P<0.05; ANOVA together with Tukey's post-test).

Moreover, DDIs between these drugs and standard chemotherapeutic drugs used in breast cancer were screened, yet no dangerous DDIs were retrieved. Bepridil, dyclonine, and primaquine were not listed as these drugs are not licensed in Austria and Germany.

Discussion

Adhesion of breast cancer cells to the LEC wall is obligatory to enable subsequent intravasation into the vessel lumen (3). It was shown that cancer cell adhesion (10) and adhesion of endothelial cells to monocytes depends on K⁺ channel activity (46). Reportedly, the antidiabetic and therefore, vasoprotective drug fenofibrate inhibits K⁺-ATPase and voltage gated K⁺ channels (19,20) and tumour invasivity (5). As dysregulated ion channels assist tumour progression (10), this tempted us to investigate whether fenofibrate and other ion channel inhibitors that are in clinical use attenuate breast cancer cell intravasation. Fenofibrate modulates LEC adhesion (5) by activating PPARα which in turn inhibits NF-κB (24,25) and by inhibiting K⁺ channel activity (20). Herein, we demonstrate a novel property of fenofibrate as an

inhibitor of CCID formation within the LEC barrier, which correlated with the inhibition of NF-κB, the downregulation of ICAM-1 in LECs and subsequent attenuation of adhesion of cancer cells.

Almost all phosphorylation-regulated ion channels, or K⁺ channels containing RGD sequences, may contribute to signalling networks (47) thereby modulating FAK activity. The activity of FAK is necessary for loose adhesion to the matrix and rapid directional migration (33,34) as observed in CCID formation (7) and in a current study establishing the role of FAK in this process we find that knockdown of FAK substantially inhibits CCIDs (Hong *et al*, unpublished data). The inhibition of FAK-phosphorylation in LECs by the Na⁺ channel inhibitor carbamazepine (48) correlates with the inhibition of CCID formation (31) and this supports the notion that also other ion channels contribute to endothelial barrier disintegration and cancer progression. To this end, it was shown that voltage gated Na⁺ channels are differently expressed in astrocytoma and breast cancer cells (49,50). Therefore, the inactivation of FAK, which was most likely achieved through the inhibition of K⁺-ATPase (37) was another, and NF-κB-independent mechanism of fenofibrate compromising metastatic outspread.

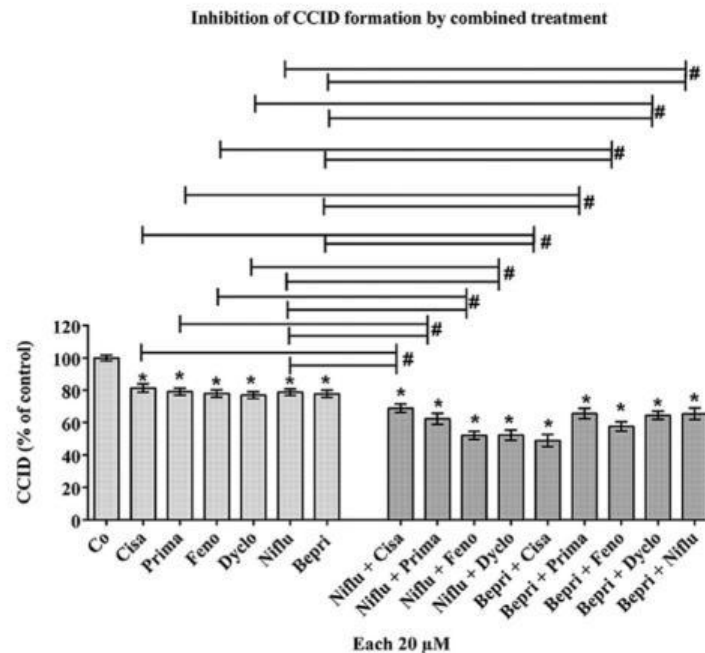


Figure 5. Analysis of drug combinations on CCID formation. MDA-MB231 spheroids were placed on LEC monolayers and treated with solvent (DMSO-Co), or with the indicated drugs (fenofibrate-Feno; cispripide-Cisa; primaquine-Prima, bepridil-Bepri; niflumic acid-Niflu; dyclonine-Dyclo), or drug combinations (solvent was kept constant) for 4 h when CCID areas were measured using an Axiovert microscope and Zen lite 2012 software. Three independent experiments with at least 8 replicates were analysed. Error bars indicate means $\pm$ SEM and asterisks and rhomboids significance ($P < 0.05$; t-test).

Table I. Analysis of drug combinations on CCID formation.

Drugs	Concentration (μ M)	CCID formation (% of control $\pm$ SD)	P-value compared to control
Cisa	20	79.97 $\pm$ 10.60	<0.0001
Prima	20	81.04 $\pm$ 9.70	<0.0001
Feno	20	79.21 $\pm$ 21.85	0.0089
Dyclo	20	81.50 $\pm$ 13.75	<0.0001
Cisa + Prima	20/20	84.43 $\pm$ 21.20	0.0305
Cisa + Feno	20/20	85.51 $\pm$ 19.31	0.0309
Cisa + Dyclo	20/20	84.85 $\pm$ 13.23	0.0006
Prima + Feno	20/20	90.97 $\pm$ 17.96	0.0305
Prima + Dyclo	20/20	84.75 $\pm$ 12.32	0.0009
Feno + Dyclo	20/20	82.32 $\pm$ 16.17	<0.0001

The effect of combination treatment versus single-drug treatment on CCID inhibition was analysed by t-test ($P < 0.05$: significant).

Furthermore, the central role of Ca^{2+} signalling and the relevance of Ca^{2+} channels as central mediators and amplifiers of migration and CIDD formation in LECs and cancer associates fibroblasts (CAFs) was reported (8,51). Therefore, the Ca^{2+} channel inhibitor bepridil hydrochloride was tested in

combination with other bona fide ion channel inhibitors. Some drug combinations improved the CCID inhibitory effect in an additive and even synergistic manner.

Whereas fenofibrate inhibited FAK activity and ICAM-1 expression in LECs, the activity of CYP1A1 was inhibited in MDA-MB231 cancer cells. CYP1A1 was demonstrated to significantly contribute to breast cancer intravasation *in vitro* (45). CYP1A1 resides in heterogenous membrane regions of the endoplasmatic reticulum (52-54) and interactions between P450 molecules and juxtaposed proteins within the lipid layer (such as ion channels) (55) are discussed to have profound effects on CYP function (56). In fact, some studies put CYP function upstream of Na^{+} - and K^{+} channels demonstrating that CYP epoxigenase activity mediates arachidonic acid-triggered sodium and potassium channel inhibition (57,58). Furthermore, epoxyeicosatrienoic acids, which are endothelium-derived CYP metabolites of arachidonic acid, relax vascular smooth muscle by calcium-activated potassium channel activation (59) and CYP-omega-hydroxylation-dependent metabolites inhibit 10 pS chloride channel (60). Directionally migrating cancer cells regulate their cell volume by Cl^{-} channels at their leading edge and inhibiting CIC-3 channel by shRNA affects glioma cell migration (61).

Nevertheless, fenofibrate-mediated inhibition of K^{+} channels and inhibition of CYP1A1 in MDA-MB231 cells could have been entirely separate events. The comparison of MCF-7 (estrogen-dependent cell line)- and MDA-MB231 (triple-negative cell line)-based 3D models revealed differences in

the drug activities on the inhibition of CCID formation, which can only be explained by their impact on the respective breast cancer cells but not on LECs. The mechanisms behind these differences await to be elucidated.

Besides the usage of a few anti-angiogenic therapeutics, no anti-intravasative drugs are currently available and none of the here tested drugs have been designed to inhibit malignant tumour progression. However, pharmaceutically used drugs exhibit side effects and these unintended activities might inhibit steps of the metastatic cascade. Importantly, all tested drugs are directly available to patients and the used concentrations can be reached in humans. Our new concept may lead to an improved therapy of metastasis in cancer patients.

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12(S)-HETE increases intracellular Ca²⁺ in lymphendothelial cells disrupting their barrier function in vitro; stabilization by clinical drugs impairing calcium supply.

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I carried out the preparation of 12(S)-HETE and cell culture experiment. I also participated in the discussion and preparation of the paper.



Original Articles

12(S)-HETE increases intracellular Ca^{2+} in lymph-endothelial cells disrupting their barrier function *in vitro*; stabilization by clinical drugs impairing calcium supply



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ABSTRACT

Secretion of 12(S)-HETE by breast cancer emboli provokes “circular chemorepellent induced defects” (CCIDs) in the adjacent lymphatic vasculature facilitating their intravasation and lymph node metastasis which determines prognosis. Therefore, elucidating the mechanism of lymph endothelial cell (LEC) wall disintegration may provide cues for anti-metastatic intervention. The role of intracellular free Ca^{2+} for CCID formation was investigated in LECs using MCF-7 or MDA-MB231 breast cancer cell spheroids in a three-dimensional cell co-culture model. 12(S)-HETE elevated the Ca^{2+} level in LEC by activating PLC/IP₃. Downstream, the Ca^{2+} -calmodulin kinase MYLK contributed to the phosphorylation of Ser19-MLC2, LEC contraction and CCID formation. Approved clinical drugs, lidoflazine, ketotifen, epiandrosterone and cyclosporine, which reportedly disturb cellular calcium supply, inhibited 12(S)-HETE-induced Ca^{2+} increase, Ser19-MLC2 phosphorylation and CCID formation. This treatment strategy may reduce spreading of breast cancer through lymphatics.

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Introduction

Metastatic primary breast cancer cells invade the surrounding stroma and eventually migrate toward the lymphatic vasculature, intravasate, successively fill up lymph nodes generating lymph node metastases, extravasate and invade the brain, lungs and bone marrow. There, tumor cells may form colonies known as distant metastases

destroying the structure and function of these organs. Thus, an early step of the metastatic outgrowth is the breaching of the endothelial barrier and attenuation of this step might prevent subsequent steps. Very few of the plethora of (proto-) oncogenes are held responsible for causing the crossing of basal membranes or vascular vessels. Oncogenes positively select overexpressing cells, whereas pro-metastatic genes seem to exert less selection pressure advancing cellular sustainment. This fundamental difference in cell proliferation versus cell spreading is the reason why currently used anti-cancer drugs that counteract rapid proliferation are inefficient in stopping metastatic outgrowth; that is, proteases, which remodel the matrix and docking platforms (consisting of a multitude of peptides), are direct protagonists of metastatic spreading. Other mechanisms driving intravasation rely on the activation of inflammation-related genes [1] such as arachidonic acid lipoxygenases (ALOXs). The ALOX12/15 metabolite 12(S)-HETE is physiologically secreted by neutrophils and monocytes, and causes the retraction of adjacent endothelial cells (ECs), thereby facilitating their entry into the vasculature [2,3]. Cancer emboli co-opt this mechanism [4,5]

Abbreviations: Ser19, serine 19; MLC2, myosin light chain 2; CCID, circular chemorepellent induced defect; MYLK, (nonmuscular) myosin light chain kinase; IP₃, inositol trisphosphate; DAG, diacyl-glycerol; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate or PtdIns(4,5)P₂; 12(S)-HETE, 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid, “S” stereoisomer; 12-HETER, 12(S)-HETE receptor (syn. GPR31); PKC, protein kinase C; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ALOX, arachidonate lipoxygenase; EC, endothelial cell; LEC, lymph-endothelial cell; RHO, Ras homolog gene family; ROCK, Rho-associated protein kinase; ICAM-1, intercellular adhesion molecule 1.

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and force the endothelium to form wide entry gates within vessel walls, so called “circular chemically induced defects (CCIDs)”. This allows the intravasation of trans grafted breast cancer cells in SCID-mice, and consistently, CCIDs and the subsequent colonization of breast cancer cells in distant organs are inhibited when 12(S)-HETE production is suppressed [6]. Therefore, understanding details of the intracellular signaling pathway(s) in ECs upon 12(S)-HETE stimulation may allow the development of anti-metastatic intervention strategies. Recently, we found that MLC2, which regulates myosin contraction and therefore cell mobility, is an executing protein of CCID formation [7] and 12(S)-HETE secreted by SW620 colon cancer cell spheroids causes retraction of microenvironment cells by a similar mechanism [8] (unpublished data). 12(S)-HETE binds to GPR31 [9] with high affinity, which was consequently renamed 12(S)-HETE receptor (12-HETER). 12(S)-HETE/12-HETER activates NF- κ B in prostate cancer cells [9] and in LECs [10]. In turn, NF- κ B induces the expression of ICAM-1 in LECs, which enforces their adhesion to cancer cells as a prerequisite of CCID formation [11]. Hence, suppression of NF- κ B or ICAM-1 inhibits tumor intravasation *in vitro* [7,10–12] and also inhibition of ALOX12/15 limiting 12(S)-HETE synthesis attenuates CCID formation [6,11]. However, neither of the experimental ALOX12/15- and NF- κ B inhibitors (sh-, siRNAs, baicalin, parthenolide, or compounds alike) are approved for their use in humans. 12(S)-HETE reportedly elevates free Ca^{2+} levels [13,14] through the low affinity receptor BLT2 [15] and activates L-type calcium channels [16]. Here we describe that 12(S)-HETE-triggered MLC2 activation and formation of CCIDs occurs through Ca^{2+} signaling. Notably, there exist clinical drugs affecting cellular calcium supply, such as lidoflazine and epiandrosterone inhibiting L-type channels [17,18], ketotifen antagonizing several calcium entry mechanisms [19] and cyclosporine interfering with Ca^{2+} transport and calcineurin signaling [20]. Therefore, these drugs were studied regarding their effect on intracellular Ca^{2+} levels in LECs and CCID formation *in vitro*.

Materials and methods

Antibodies and reagents

Polyclonal rabbit anti-phospho-myosin light chain 2 (Ser19) and polyclonal rabbit anti-myosin light chain 2 were purchased from Cell Signaling (Danvers, MA, USA), and polyclonal rabbit anti-GPR31 (anti-12-HETER) from Cayman Chemical (Ann Arbor, MI, USA), and monoclonal mouse anti-MYLK (sc-365352) from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal mouse anti- β -actin (clone AC-15) was from Sigma-Aldrich (Munich, Germany) and polyclonal rabbit anti-mouse and polyclonal swine anti-rabbit IgGs were from Dako (Glostrup, Denmark).

U73122 was purchased from Calbiochem (Darmstadt, Germany), BAPTA-AM was from Santa Cruz Biotechnology (Heidelberg, Germany). Edelfosine, ketotifen, lidoflazine, cyclosporine, epiandrosterone and KN62 were ordered from Sigma-Aldrich (Munich, Germany).

12(S)-HETE was purchased from Cayman Chemical (Ann Arbor, MI, USA). si-RNAs targeting human MYLK (SMART pool “ON-TARGET plus”, cat. no.: L-005351-00-0005) were ordered from Dharmacon (Gene Expression and Gene Editing, GE Healthcare, Lafayette, CO, USA). Non targeting (n.t.) control si-RNA (Silencer® Select Negative Control No. 1 si-RNA, cat. no.: 4390843) was from Ambion (Life Technologies, Carlsbad, CA, USA). All si-RNAs were re-suspended in RNase-free water to make a stock concentration of 20 μM .

Cell culture

Human MDA-MB231 and MCF-7 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in MEM medium supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (PS) and 1% non-essential amino acids (Gibco, Invitrogen, Karlsruhe, Germany). Telomerase immortalized human lymph endothelial cells (LECs) were grown in EGM2 MV (Clonetics CC-4147, Allendale, NJ, USA). The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2 . For CCID formation assays, LECs were stained with Cytotracker-green purchased from Invitrogen (Karlsruhe, Germany).

Spheroid formation

MDA-MB231 cells (input of 6000 cells per spheroid; when used in experiments, the average spheroid diameter was $\sim 320 \mu\text{m}$ overcasting a LEC area of $\sim 99,218 \mu\text{m}^2$) and MCF-7 cells (input of 3000 cells per spheroid; when used in

experiments, the average spheroid diameter was $\sim 470 \mu\text{m}$ overcasting a LEC area of $\sim 179,449 \mu\text{m}^2$) were transferred to 30 mL serum free MEM medium containing methylcellulose (0.3% final concentration; Cat. No.: M-512, 4000 cP; Sigma-Aldrich, Munich, Germany). 150 μL of cell suspension was transferred to each well of a 96-well plate (Greiner Bio-one, Cellstar 650185, Kremsmünster, Austria) to allow spheroid formation within 48 h.

CCID (circular chemorepellent-induced defect) assay

In this assay the sizes of the cell free areas (circular chemorepellent induced defects; CCIDs), which are formed in the endothelial monolayer directly underneath the tumor spheroids, were measured [21]. MDA-MB231 and MCF-7 spheroids were washed and transferred to Cytotracker-stained LEC monolayers that were seeded into 24-well plates (Costar 3524, Sigma-Aldrich, Munich, Germany) in 1 mL EGM2 MV medium. Confluent cytotracker-stained LEC monolayers were pre-treated with indicated inhibitors for 30 min and then spheroids were placed on the top of monolayers. In those experiments using lidoflazine, ketotifen, epiandrosterone and cyclosporine the breast cancer cell spheroids were pre-treated with these drugs for 30 min before spheroids were added to the LEC monolayers. After 4 h of incubation, the CCID areas in the LEC monolayers underneath the MDA-MB231 and MCF-7 spheroids were photographed using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope to visualize cytotracker green-stained LECs underneath the spheroids. CCID areas were calculated with the Zen Little 2012 (Zeiss, Jena, Germany). For each condition the CCID size of 20 or more spheroids (unless otherwise specified) was measured.

Transfection of LEC monolayer

LECs were seeded in 24-well plates (1 mL/well) and grown in EGM2 medium. Transfections were performed when the cell cultures reached a confluence of 70–80%. 0.75 μg siRNA and 6 μL HiPerfect Transfection Reagent (Qiagen, cat. no.: 301705) were mixed in 100 μL serum-free medium and incubated for 30 min at room temperature to allow the formation of transfection complexes. The old cell culture medium was discarded and 500 μL of fresh EGM2 medium was added into each well. Then, the transfection complexes were added drop-wise to the cells (to a final siRNA concentration of 100 nM) and incubated for 24 h at 37 °C. After 24 h, the medium was replaced by fresh medium and cells were incubated for another 24 h to recover. The LEC monolayers were used for CCID assays or isolated RNA for qPCR.

Quantitative RT-PCR

LECs were harvested after transfection and RNA was isolated using the RNeasy Mini Kit 50 and QIAshredder 50 (QIAGEN, Hamburg, Germany). The final RNA concentration was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). An amount of 2.0 μg of total RNA was reverse transcribed using RNA to cDNA EcoDry™ Premix Protocol-At-A-Glance (Clontech, 2 Saint-Germain-en-Laye, France), the resulting cDNA was amplified using TaqMan Gene Expression Master Mix (Applied Biosystems, Vienna, Austria). The PCR products were analyzed on the Chromo4 PCR System (Bio-Rad, Hercules, CA, USA). The following TaqMan probes were used: GAPDH (Hs99999905_m1) and MYLK (Hs00364926_m1). qPCR was performed in triplicate for each cDNA template. Gene expression normalized to GAPDH expression (glyceraldehyde 3-phosphate dehydrogenase) was calculated using the $\Delta\Delta\text{C}_t$ method.

SDS gel electrophoresis and Western blotting

LECs were grown in T-25 tissue culture flasks (Nunc, Roskilde, Denmark) to 80% confluence. Twenty-four hours before treatment, the medium was replaced by fresh serum-free medium. Cells were pre-treated with indicated inhibitors for 1 h and then stimulated with 1 μM 12(S)-HETE for 15 min. Afterwards, cells were washed twice with ice cold PBS and lysed in buffer containing 50 mM Tris-HCl (pH 6.8), 6% SDS, 20% glycerol, 1.85 mM EDTA, phosphatase inhibitor cocktail and protease inhibitor cocktail. For complete cell lysis, the mixture was sonicated 5–10 times on ice. Equal protein amounts were separated by SDS-polyacrylamide gel electrophoresis and electro-transferred onto Amersham Hybond-P PVDF transfer membranes (GE Healthcare, Freiburg, Germany) at 100 V for 1 h in cold transfer buffer (20 mM Tris-base, 150 mM glycine, 20% v/v methanol, pH 8.5). Membranes were stained with Ponceau S (Sigma-Aldrich, Munich, Germany) to control transfer efficiency and equal sample loading. After washing with TBS/T (Tris buffered saline/Tween 20, pH 7.6), membranes were immersed in blocking solution (5% non-fat dry milk in TBS containing 0.1% Tween) at room temperature for 1 h. Membranes were washed and incubated with primary antibodies (in blocking solution; dilution 1:500–1:1000) by gently rocking at 4 °C overnight. Thereafter, the membranes were washed with TBS/T and incubated with second antibodies (peroxidase-conjugated swine anti-rabbit IgG – dilution 1:5000, or rabbit anti-mouse IgG – dilution 1:10000) at room temperature for 1 h. Chemo-luminescence was developed by Amersham ECL prime Kit (GE Healthcare, Freiburg, Germany) and detected using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland). Densitometry of the Western blots was analyzed with Image-J software (National Institutes of Health, Maryland, USA).

Intracellular Ca^{2+} assay

Free intracellular Ca^{2+} levels were measured using the FluoForte™ Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, MI, USA). 8×10^3 LECs/well/100 μL EGM2 medium were seeded into 96-well black-wall clear-bottom plates (Nunc, Thermo scientific, NY, USA) and after 24 h LECs were pre-treated with indicated inhibitors, FDA-drugs or DMSO for 1 h. Then, the medium was removed and 100 μL FluoForte™ Dye-loading (containing inhibitors, FDA-drugs or DMSO where applicable) was added to each well. Cells were further incubated for 45 min at 37 °C followed by 15 min at room temperature. Then, cells were stimulated with 1 μM 12(S)-HETE for 5 min, and the fluorescence was measured with a fluorescence plate reader at 490/525 nm. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]$) was calculated using the formula $[\text{Ca}^{2+}] = K_d(F - F_{\min}) / (F_{\max} - F)$ [22,23]. F is relative fluorescent unit (RFU) at 490/525 nm, whereas $F_{\min}$ and $F_{\max}$ are relative fluorescent unit of the lowest and the highest Ca^{2+} concentration, which were determined for each experiment by adding 10 μM EDTA and 10 μM ionomycin calcium salt, respectively. K_d (effective dissociation constant) of the FluoForte- Ca^{2+} complex is 389 nM at 22 °C.

NF- κB transactivation assay

The transactivation of a NF- κB -driven luciferase reporter was quantified in HEK293/NF- κB -luc cells (Panomics, RCD014) as previously described [24]. In brief, cells were maintained at 37 °C and 5% CO_2 cell culture incubators in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 2 mM glutamine, 100 $\mu\text{g}/\text{mL}$ hygromycin B, 100 U/mL benzylpenicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10% FCS. One day before the experiments the cells were stained in serum-free medium supplemented with 2 μM Cell Tracker Green CMFDA (C2925; Invitrogen) for 1 h. Then, cells were reseeded in 96-well plates at a density of 4×10^4 cells/well in phenol red-free and FCS-free DMEM overnight, and pre-treated with the indicated compounds for 30 min prior to stimulation with 2 ng/mL TNF- α for 4 h. The final concentration of DMSO in the experiments was 0.1% or lower and an equal concentration of DMSO was used as control. After cell lysis the luminescence of the firefly luciferase and the fluorescence of the Cell Tracker Green CMFDA were quantified on a GeniosPro plate reader (Tecan, Grödig, Austria). The luciferase-derived signal from the NF- κB reporter was normalized by the Cell Tracker Green CMFDA-derived fluorescence to account for differences in the cell number. The known NF- κB inhibitor parthenolide (Sigma-Aldrich, Vienna, Austria) was used as a positive control.

12(S)-HETE assay

MDA-MB231 and MCF-7 cells, which were cultivated in MEM medium (Gibco # 10370-047) supplemented with 10% FCS, were seeded in 3.5 cm dishes and grown in 2.5 mL complete MEM medium. The next day, the medium was changed for FCS-free medium and cells were kept at 37 °C for 24 h. Then, cells were treated with 10 μM arachidonic acid (#A3555, Sigma-Aldrich, Munich, Germany) and simultaneously with different concentrations of the indicated compounds for 4 h when the supernatants were aspirated, centrifuged at 2,000 rpm at 4 °C for 5 min. The concentration of 12(S)-HETE in the cellular supernatant was measured with minor modifications as described previously [25] using the 12(S)-HETE enzyme immunoassay kit (EIA, # ADI-900-050; Enzo Life Sciences, Lausen, Switzerland). Absorbance was measured with a Wallac 1420 Victor 2 multilabel plate reader (Perkin Elmer Life and Analytical Sciences). The concentration of 12(S)-HETE in the cellular supernatant was normalized to cell number to account for differences in the cell number.

Statistical analysis

For statistical analyses, Excel 2013 software and Prism 6 software package (GraphPad, San Diego, CA, USA) were used. The values were expressed as mean $\pm$ SEM and the Student t-test and ANOVA were used to compare differences between control samples and treatment groups. Statistical significance level was set to $p < 0.05$.

Results

12(S)-HETE triggers Ca^{2+} -release, MLC2 activation and CCID formation

The treatment of primary- and TERT-immortalized LECs with 12(S)-HETE increased their intracellular Ca^{2+} level from 70 to ~100 nM and from 90 to ~110 nM within 10 min, respectively (Fig. 1A and B). Conversely, the specific cell permeable Ca^{2+} chelator BAPTA-AM (1.25–5 μM) decreased 12(S)-HETE-induced Ca^{2+} levels in primary and in immortalized LECs to that of untreated control, and reduced also constitutive Ca^{2+} level in immortalized LECs (Fig. 1C and D, respectively). MCF-7- and MDA-MB231 breast cancer cells secrete 12(S)-HETE. This causes the retraction of LECs and the formation of CCIDs enabling the cancer cells to traverse the lymphatic

vasculature [6]. 12(S)-HETE dose dependently activates MLC2, which was shown to be required for cell migration [26,27] and to contribute to ~30% of MDA-MB231-induced CCID formation [28]. To this end, we tested whether Ca^{2+} signaling was involved in 12(S)-HETE-mediated Ser19-phosphorylation of MLC2 (phosphorylation at this site indicates activation of MLC2) and CCID formation. Therefore, LECs were pre-treated with 5 μM BAPTA-AM, which completely prevented 12(S)-HETE-triggered phosphorylation of MLC2 (Fig. 1E), and inhibited ~25% of CCID formation, which was induced by either MDA-MB231- or MCF-7 spheroids (Fig. 1F and G; respectively, Supplementary Fig. S1A and B show formation of CCIDs induced by MDA-MB231 spheroids when the LEC monolayers were pre-treated with DMSO or BAPTA-AM, respectively). Hence, a 20–30 nM increase in intracellular Ca^{2+} seemed sufficient to significantly contribute to CCID formation at least when this increase was sustained for 10 min or more.

Inhibition of PLC inhibits Ca^{2+} release, MLC2 activation and CCID formation

Ca^{2+} release from intracellular stores is due to inositol-3-phosphate (IP_3), which is metabolized from phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase-C-beta ($\text{PLC}\beta$). To test whether PLC-generated IP_3 was contributing to the phosphorylation of MLC2 and to CCID formation, LECs were pre-treated with the specific inhibitor U73122. Consistent with the role of Ca^{2+} in LEC migration and lymph endothelial barrier breaching, 2.5 μM U73122 reduced constitutive as well as 12(S)-HETE-induced intracellular Ca^{2+} levels (~30% induction) below that of untreated control (Fig. 2A) and completely prevented 12(S)-HETE-induced MLC2 phosphorylation (Fig. 2B). Furthermore, 2.5 μM U73122 inhibited CCID formation by ~30%, which was triggered either by MDA-MB231 spheroids or by MCF-7 spheroids (Fig. 2C and D, respectively), and also the PLC inhibitor edelfosine dose-dependently attenuated CCID formation triggered by either MDA-MB231- or MCF-7 spheroids (Fig. 2E and F, respectively). Thus, there is a strong correlation between 12(S)-HETE-induced $\text{PLC}\beta/\text{IP}_3$ activity, Ca^{2+} level, MLC2 phosphorylation and CCID formation, and inhibiting $\text{PLC}\beta$ signaling interfered with breast cancer spheroid intravasation *in vitro*.

The Ca^{2+} -calmodulin kinase MYLK contributes to CCID formation

Subsequent to its release, intracellular Ca^{2+} binds to calmodulin activating Ca^{2+} -calmodulin-dependent kinases. The Ca^{2+} -calmodulin-dependent kinase subtype non-muscular myosin light chain kinase (MYLK) participates in inflammation and migration [29,30] and phosphorylates Ser19-MLC2 [31]. Thus, to study the contribution of MYLK to the formation of CCIDs, siRNA (siMYLK) was transiently transfected into LECs to inhibit MYLK expression. The knock-down of MYLK (80% inhibition of mRNA expression and 60% inhibition of protein expression; Supplementary Fig. S2A and B, respectively) significantly attenuated MDA-MB231-triggered CCID formation by ~40% and MCF-7-triggered CCID formation by ~25% (Fig. 3A and B, respectively). Furthermore, 12(S)-HETE-triggered phosphorylation of Ser19-MLC2 was completely abolished by siMYLK (Fig. 3C). As siMYLK inhibited MDA-MB231-triggered CCID formation more potently than other inhibitors, we tested whether MYLK may influence Ca^{2+} levels through a feed-back mechanism. However, neither constitutive nor 12(S)-HETE-induced Ca^{2+} levels were significantly altered upon transfection of siMYLK into LECs (Fig. 3D) and this suggested that the difference between MCF-7- and MDA-MB231-triggered CCID formation (which was not significant) had no mechanistic background but was correlative.

In a parallel investigation we found that CAMKII contributes to fibroblast junction retraction and CCID formation (unpublished data). Therefore, we tested whether CAMKII is also involved in CCID

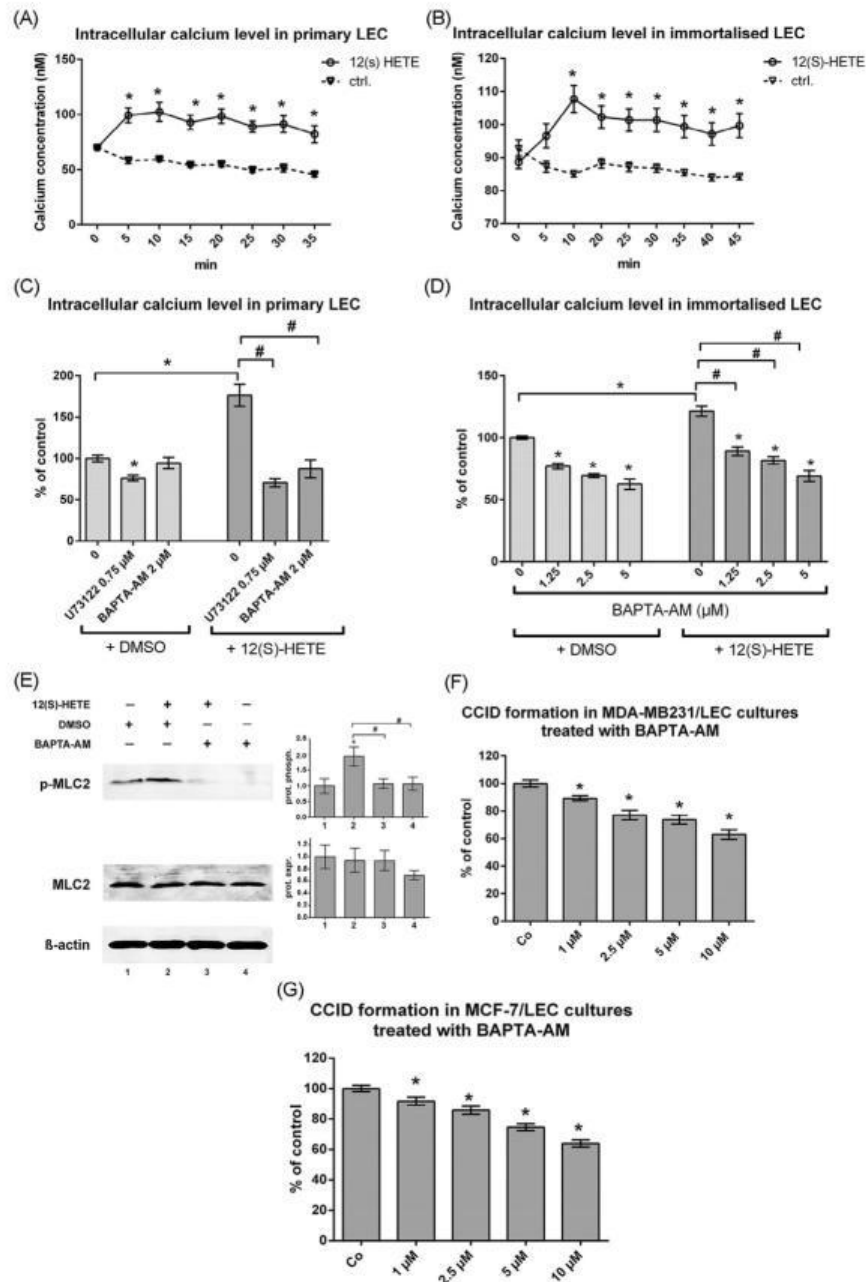


Fig. 1. A 12(S)-HETE-triggered signal is transduced by free intracellular calcium. (A) Primary LECs and (B) TERT-immortalized LECs were stimulated with 1 μM 12(S)-HETE and free intracellular calcium was measured after the indicated time points. Experiments were performed in sextuplicate. (C) Primary LECs or (D) immortalized LECs were pre-treated with the indicated concentrations of BAPTA-AM or U73122, stimulated with 1 μM 12(S)-HETE and intracellular free calcium was measured. Experiments were performed in sextuplicate. (E) Immortalized LECs were pre-treated with 5 μM BAPTA-AM or solvent (DMSO) for 1 h, then stimulated with 1 μM 12(S)-HETE for another 15 min when cells were lysed and proteins separated by SDS gel electrophoresis and analyzed by Western blotting using the indicated antibodies. Relative protein phosphorylation/expression levels (prot. phosph./prot. expr.) are shown at the right side of the blots and the numbers below the bar graphs refer to the respective treatment conditions indicated in the lanes. Experiments were performed in triplicate. (F) MDA-MB231 or (G) MCF-7 spheroids were placed on top of immortalized LEC monolayers, which were pre-treated with increasing concentration of BAPTA-AM or solvent (DMSO), and co-incubated for 4 h when CCID areas were measured. Error bars indicate means ± SEM, and asterisks and rhomboids significance ($p < 0.05$; t-test or ANOVA).

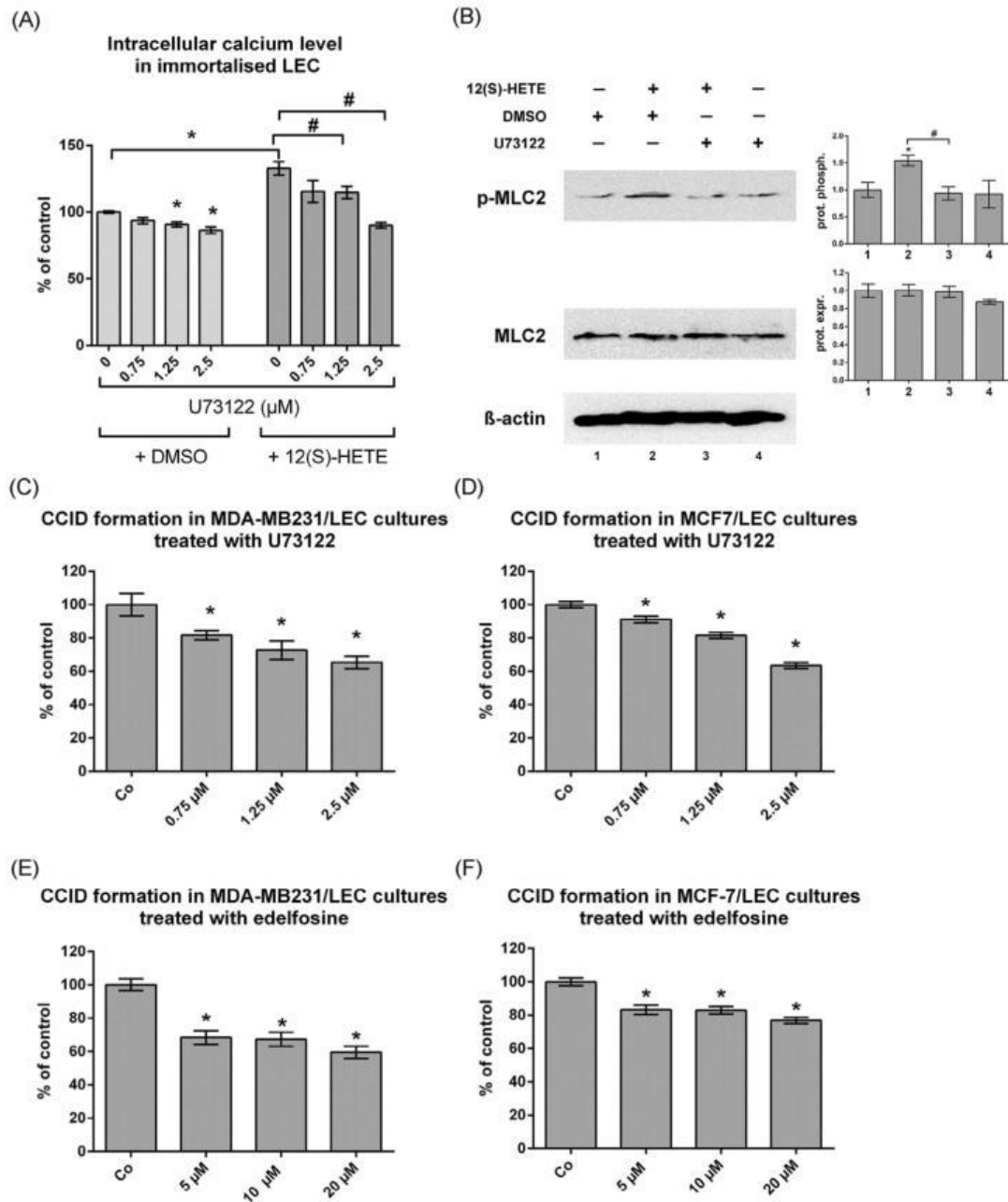


Fig. 2. 12(S)-HETE-triggered CCID formation and MLC2 activation are transduced by PLC/IP₃. (A) Immortalized LECs were pre-treated with the indicated concentration of U73122, stimulated with 1 μM 12(S)-HETE and intracellular free calcium was measured. Experiments were performed in sextuplicate. (B) LECs were pre-treated with 2.5 μM U73122 or solvent (DMSO) and then stimulated with 1 μM 12(S)-HETE for another 15 min when cells were lysed, proteins separated by SDS gel electrophoresis and analyzed by Western blotting using the indicated antibodies. Relative protein phosphorylation/expression levels (prot. phosph./prot. expr.) are shown at the right side of the blots and the numbers below the bar graphs refer to the respective treatment conditions indicated in the lanes. Experiments were performed in septuplicate. (C–F) Immortalized LECs were pre-treated with increasing concentrations of (C, D) U73122 or (E, F) edelfosine and then (C, E) MDA-MB231 or (D, F) MCF-7 spheroids were placed on top of LECs monolayers and co-incubated for 4 h when CCID areas were measured. Error bars indicate means ± SEM, and asterisks and rhomboids significance ($p < 0.05$; t-test or ANOVA).

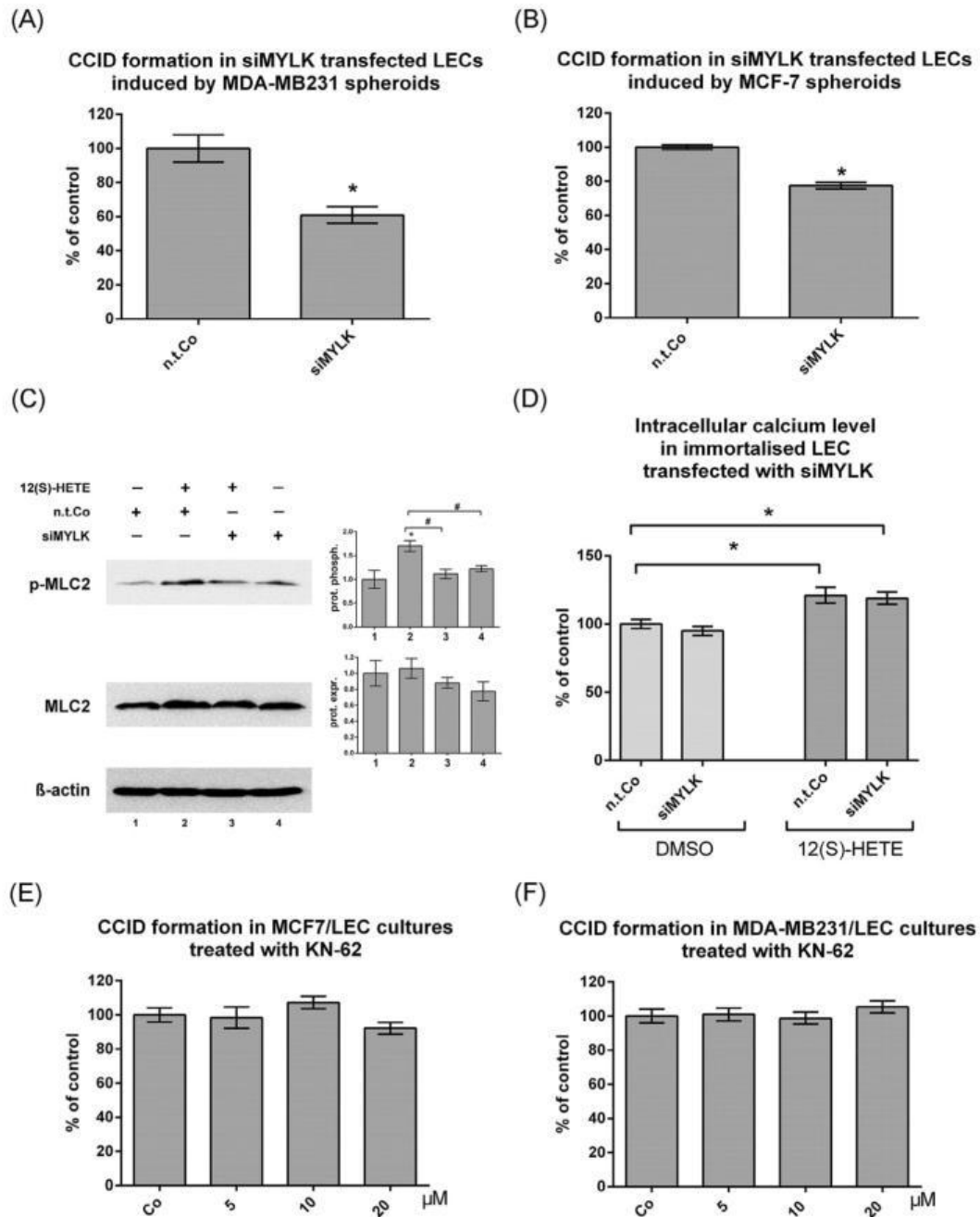


Fig. 3. MYLK transduces the 12(S)-HETE signal to MLC2 and CCID formation. (A) MDA-MB231 or (B) MCF7 spheroids were placed on top of LECs, which were transfected with either non-targeting control RNA (n.t.Co) or siRNA targeting MYLK (siMYLK), and co-incubated for 4 h when CCID areas were measured. (C) LECs, which were transfected with either n.t.Co RNA or siMYLK, were stimulated with 1 μM 12(S)-HETE for 15 min when cells were lysed, proteins separated by SDS gel electrophoresis and analyzed by Western blotting using indicated antibodies. Relative protein phosphorylation/expression levels (prot. phosph./prot. expr.) are shown at the right side of the blots and the numbers below the bar graphs refer to the respective treatment conditions indicated in the lanes. Experiments were performed in triplicate. (D) Immortalized LECs, which were transfected with either n.t.Co or siMYLK, were stimulated with 1 μM 12(S)-HETE and intracellular free calcium was measured. Experiments were performed in sixuplicate. (E) MCF-7 spheroids or (F) MDA-MB231 spheroids were placed on top of LEC monolayers, which have been pretreated with indicated concentrations of KN-62, and after 4 h of co-incubation CCID areas were measured. Error bars indicate means ± SEM, asterisks and rhomboids significance ($p < 0.05$; t-test).

formation of LEC. However, the treatment of LEC with the specific inhibitor KN62 did not inhibit CCID formation regardless of whether it was induced by MCF-7- or MDA-MB231 spheroids (Fig. 3E and F).

Taken together, the data evidence that 12(S)-HETE triggered a signaling cascade involving the expression of Ca^{2+} -calmodulin kinase MYLK (not however, the activity of CAMKII), which correlated with the phosphorylation of Ser19-MLC2 as an effector of cell movement, and with the disintegration of the LEC barrier.

PLC β / Ca^{2+} signaling of 12(S)-HETE is independent of 12-HETER

It was reported that signaling through the 12(S)-HETE high affinity receptor, 12-HETER, did not trigger the release of intracellular Ca^{2+} in prostate carcinoma cells [9]. Therefore, we tested whether 12-HETER contributed to 12(S)-HETE triggered Ca^{2+} increase and to CCID formation in LEC. Consistently, the inhibition of 12-HETER (by siRNA; si12-HETER) did not reduce 12(S)-HETE-triggered Ca^{2+} induction (Fig. 4A; the proper downregulation of 12-HETER by si12-HETER transfection was monitored by Western blotting, see Supplementary Fig. S3). Nevertheless, the knock-down of 12-HETER expression attenuated the formation of CCIDs. Furthermore, simultaneous inhibition of 12-HETER expression (by si12-HETER) together with the inhibition of Ca^{2+} signaling (by BAPTA-AM), or together with the inhibition of IP_3 (by U73122), attenuated CCID formation additively (Fig. 4B). This indicated that two independent signal transduction pathways, which are both triggered by 12(S)-HETE, caused the destabilization of LEC barriers. Recently, the signal transduction of 12(S)-HETE in LEC through 12-HETER-RHO/ROCK to induce CCID formation was reported [28].

Inhibition of CCID formation by clinical modulators of calcium uptake

The increase in intracellular Ca^{2+} upon treatment with 12(S)-HETE lasted over 35–45 min (Fig. 1A and B) and even thereafter the Ca^{2+} level seemed to remain above normal. It was not unlikely that this depleted intracellular stores and that their re-filling was required to maintain elevated intracellular Ca^{2+} levels. To attenuate potential replenishment, we tested FDA (Food and Drug

Administration of the United States) approved clinical drugs that reportedly affect extracellular calcium supply by antagonizing L-type Ca^{2+} channels, store-operated Ca^{2+} channels and calcium homeostasis such as lidoflazine, ketotifen, epiandrosterone and cyclosporine [17–20]. Here, we confirmed that these drugs reduced the constitutive and 12(S)-HETE-induced levels of free intracellular Ca^{2+} in LECs (Fig. 5A) as well as phosphorylation of Ser19-MLC2 (Supplementary Fig. S4). To challenge the hypothesis that Ca^{2+} signaling in LECs is relevant for tumor intravasation *in vitro*, these drugs were tested regarding their effect on CCID formation. In fact, lidoflazine, ketotifen, epiandrosterone and cyclosporine significantly attenuated CCID formation triggered by MDA-MB231- and MCF-7 spheroids (Fig. 5B and C). Compared to the other drugs, lidoflazine was the most potent inhibitor of CCIDs, although it was least potent in inhibiting constitutive Ca^{2+} level and equally potent in inhibiting 12(S)-HETE-triggered Ca^{2+} levels. As there was no direct correlation between reduction of free intracellular Ca^{2+} levels and attenuation of CCIDs, we investigated whether lidoflazine, ketotifen, epiandrosterone and cyclosporine affected also NF- κB , which is another signaling pathway relevant for CCID formation. All four drugs down-regulated NF- κB activation in HEK293 cells stably transfected with a NF- κB luciferase reporter (Fig. 5D) and the extent of NF- κB inhibition roughly corresponded to Ca^{2+} reduction. Therefore, Ca^{2+} increase and NF- κB might have been along the same signal transduction pathway and drug treatment affected still another CCID-forming pathway. Finally, the integrate net-outcome of the signals would define the migratory response of LECs and CCID formation.

Discussion

12(S)-HETE, produced by ALOX12/15, is secreted by neutrophils and breast cancer spheroids and triggers CCID formation in LEC monolayers enabling their transmigration into the lymphatic vasculature [3,6]. Besides, ALOX5 activity in neutrophils supports lung metastasis of breast cancer cells [32]. It was shown that 12(S)-HETE increases the intracellular Ca^{2+} level independent of extracellular calcium level [14,16,33] suggesting that Ca^{2+} contributes to the transduction of a 12(S)-HETE-triggered signal. This *in vitro* study aimed to elucidate whether the 12(S)-HETE-generated intracellular Ca^{2+} increase leads to the disintegration of LEC barriers.

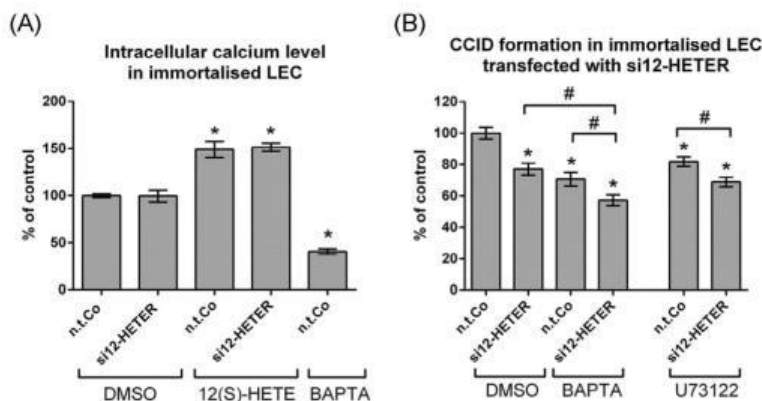


Fig. 4. 12(S)-HETE-triggered calcium increase is independent of 12-HETER expression. Immortalized LECs, which were transfected with either non-targeting control RNA (n.t.Co) or siRNA targeting 12(S)-HETE receptor (si12-HETER), were (A) stimulated with 1 μM 12(S)-HETE and then intracellular free calcium was measured. BAPTA-AM was used as positive control. Experiments were performed in sixuplicate. (B) Transfected LECs were pretreated with 1 μM BAPTA-AM or 1 μM U73122 and then, MDA-MB231 spheroids were placed on top of confluent LEC monolayers, and co-incubated for 4 h when CCID areas were measured. Error bars indicate means $\pm$ SEM, and asterisks and rhomboids significance ($p < 0.05$; t-test or ANOVA).

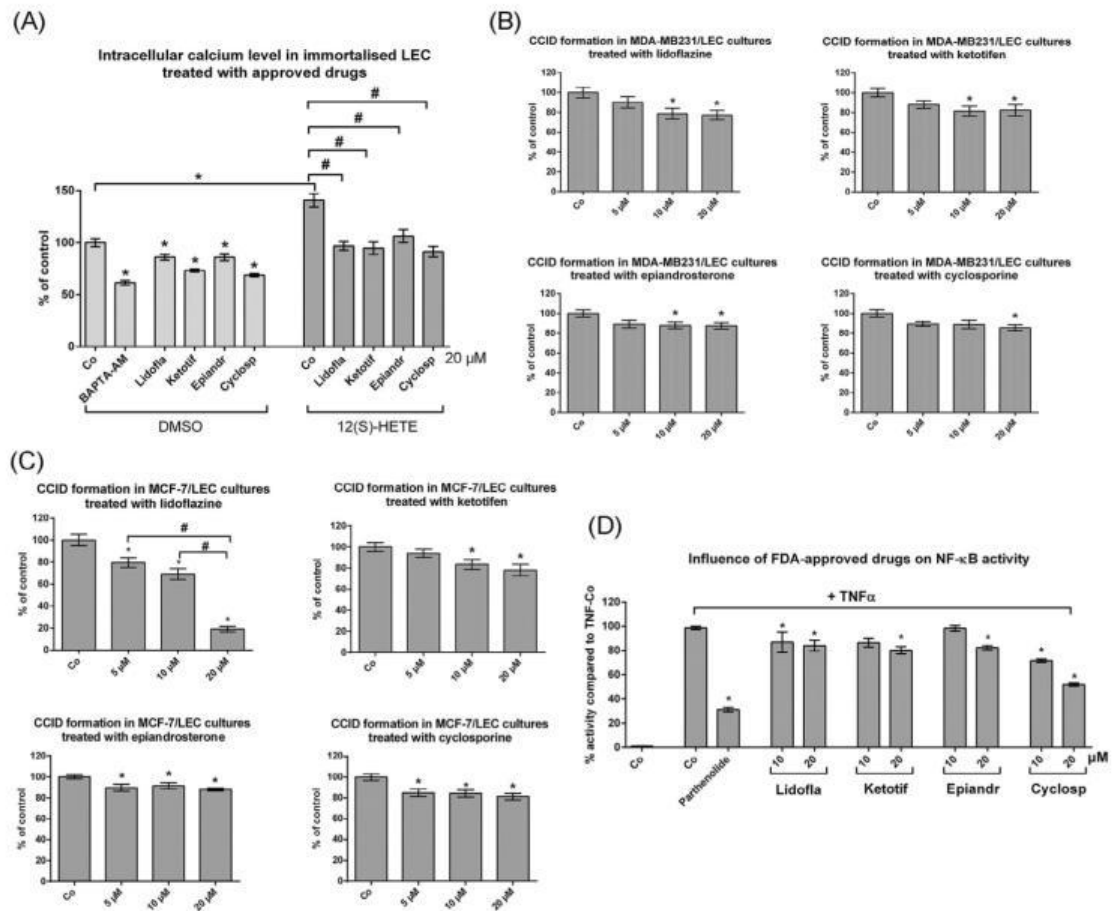


Fig. 5. Decrease of free intracellular calcium levels, NF- κ B activity and CCID formation by clinical drugs. (A) Immortalized LECs were pre-treated with 20 μ M of indicated drugs (lidoflazine, ketotifen, epiandrosterone, cyclosporine) or solvent (Co), stimulated with 1 μ M 12(S)-HETE and then intracellular free calcium was measured. BAPTA-AM was used as positive control. Experiments were performed in sextuplicate, error bars indicate means $\pm$ SEM, and asterisks and rhomboids indicate significance ($p < 0.05$; ANOVA). (B) MDA-MB231 and (C) MCF-7 spheroids were placed on top of confluent LEC monolayers, which were pre-treated with increasing concentrations of indicated drugs, and co-cultivated for 4 h when CCID areas were measured. Error bars indicate means $\pm$ SEM and asterisks significance ($p < 0.05$; ANOVA). (D) HEK293-NF κ B-Luc cells were pretreated with 5 μ M parthenolide as a specific inhibitor of NF- κ B or with 10 and 20 μ M of the indicated drugs for 1 h or solvent (Co). Then, cells were stimulated with 2 ng/mL human recombinant TNF α for an additional 4 h when NF- κ B activity was measured. Experiments were performed in quadruplicate, error bars indicate means $\pm$ SEM and asterisks significance ($p < 0.05$; ANOVA).

We provide evidence that 12(S)-HETE treatment and concomitant Ca^{2+} increase caused the phosphorylation of Ser19-MLC2 and CCID formation. Notably, MLC2 was shown to promote cell migration [27,34] mandatory for CCID formation in LEC monolayers [28]. Ca^{2+} binds to calmodulin and associates with Ca^{2+} -calmodulin-dependent kinases such as MYLK, which directly phosphorylates Ser19-MLC2, thereby weakening endothelial barrier function [31]. This is in agreement with the observation that MYLK mediates intravasation of breast cancer cells through endothelial cells [35]. Knock-down of MYLK and chelation of intracellular Ca^{2+} both inhibited Ser19-MLC2 phosphorylation and CCID formation implicating a strong correlation, if not straight connection, between Ca^{2+} increase, MYLK function and MLC2 activity. In contrast, inhibition of CAMK2 by KN-62 had no effect on CCID formation, although two independent studies report that CAMK2D and CAMK2G are expressed

in human lymphatic endothelial cells. Furthermore, both studies detected the mRNA expression of CAMK3, MYLK and MYLK2 [36] (GSE79160 detected also CAMK1B), whereas Johnson et al. [37] (GSE6257) detected also the expression of CAMK1D, CAMK4 and MYLK3. Therefore, it cannot be ruled out that these CAMKs may play a role in breast cancer spheroid-induced CCID formation in LEC monolayers.

Ca^{2+} -release is triggered by binding of IP_3 to specific receptors at intracellular store membranes. The secondary messengers IP_3 and diacyl glycerol (DAG) are simultaneously produced from phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLC β . Here, the contribution of IP_3 as mediator of 12(S)-HETE-triggered Ca^{2+} increase and Ser19-MLC2 phosphorylation was demonstrated, as well as the protagonist role of IP_3 and PLC in CCID formation. It was shown that IP_3 -triggered Ca^{2+} release induces PKC α [38,39] and DAG induces PKC β

and other PKC isoenzymes [40,41], and both PKC α and PKC β activate NF- κ B [42,43]. Activation of NF- κ B is another inflammatory pathway inducing CCID formation in LECs [10] through ICAM-1 synthesis, which increases their adhesion to cancer cells [7,11]. Thus, upon initial splitting of the PLC β -signal into Ca²⁺ and DAG, the downstream cascades again unite at the level of NF- κ B/adhesion/migration [42,44,45] whereas another splitting of the IP₃/Ca²⁺ signal, which is here described, entertained the MYLK/MLC2 signaling pathway. 12(S)-HETE-triggered Ca²⁺ increase was independent of the high affinity receptor 12-HETER. Nevertheless, knock-down of 12-HETER reduced CCID formation indicating the existence of additional signal transduction pathways in LECs independent of PLC β /IP₃/Ca²⁺/MYLK. Recently, we found that the 12-HETER pathway signaled through RHO/ROCK [28] and therefore, ROCK certainly contributed to 12(S)-HETE-triggered CCID formation, but was independent of the here described signaling pathway. This investigation demonstrates that 12(S)-HETE induced also Ca²⁺ release and this caused CCID formation as well, but was not the only pathway. The implication of additional CCID-inducing pathways, which were triggered by 12(S)-HETE, is evidenced by the fact that the inhibition of the various steps of the here deciphered pathway inhibited CCID formation only partly. In an earlier study it was shown that 12(S)-HETE inhibits VE-cadherin expression and that cell–cell junctions are disrupted [12]. Whether 12(S)-HETE-triggered VE-cadherin down-regulation depends also on Ca²⁺ signaling was not investigated in this model. However, it was shown that increased Ca²⁺ levels activate signaling pathways that not only mediate the cytoskeletal reorganization through MLC-dependent contraction, but also cause the disassembly of VE-cadherin at adherence junctions [38]. This response of endothelial cells to increasing intracellular Ca²⁺ levels is therefore, established. On the other hand, depletion of Ca²⁺ also reduces junction stability and the subsequent replenishment of Ca²⁺ again increases trans endothelial electrical resistance and therefore stabilizes adherence [46]. Hence, Ca²⁺ oscillations, regardless whether they are up or down from steady state, may affect cell–cell junctions. The question arises: if Ca²⁺ increase as well as Ca²⁺ decrease may create signals interfering with junction stability, why not all intracellular Ca²⁺ fluctuations (and there are many) result in CCID formation? A hypothetical and mechanistic answer to this may be the possibility that the 12(S)-HETE receptor triggers also a second signal, or even more signals or additional signals are generated independently and simultaneously through a different receptor. The lymph endothelial cell would integrate these signals and responds in one way or another. Based on the observation that 1) Ca²⁺ signaling caused endothelial wall disintegration and that 2) the induction of intracellular Ca²⁺ release was very long lasting, we hypothesized that this may deplete intracellular Ca²⁺ stores when replenishment is inhibited. Therefore, we tested whether FDA-approved drugs, which reportedly interfere with extracellular calcium supply, reduce constitutive as well as 12(S)-HETE-induced intracellular Ca²⁺ levels. In fact, lidoflazine, ketotifen, epiandrosterone, and cyclosporine inhibited 12(S)-HETE-induced Ca²⁺ increase (thereby supporting the hypothesis). Ser19-MLC2 phosphorylation and CCID formation. All four drugs inhibited the NF- κ B luciferase reporter in HEK293 cells and the extent of upstream Ca²⁺ decrease roughly corresponded to downstream NF- κ B inhibition, which was consistent with the second role of Ca²⁺ as an inducer of PKC and NF- κ B [47,48]. However, the extent of Ca²⁺ decrease did not correlate with the extent of CCID formation among the four tested drugs with lidoflazine being the most active CCID inhibitor but least active reducer of Ca²⁺ level. Thus, also other signaling cascades that regulate LEC migration become affected by these drugs in one way or another, and finally the net-outcome of the signals defines the migratory response of LECs and CCID formation. In addition to intracellular Ca²⁺ release, 12(S)-HETE reportedly activates the mTOR/S6 axis causing blood endothelial cell migration [49]. Furthermore, mTOR regulates gap junctions and

F-actin organization [50] such as 12(S)-HETE. Rapamycin, the inhibitor of mTOR, modulates extracellular calcium influx and muscle cell function [51]. Therefore, rapamycin and rapalogs, which are used as anticancer agents [52,53], warrant testing regarding their role in cell junction retraction and tumor intravasation.

In a screening approach it was demonstrated that the FDA approved drugs isosuprine, nifedipine, acetohexamide [54] and carbamazepine [25] attenuated CCID formation. In retrospect, these drugs have all in common that they inhibit calcium channels or Ca²⁺ signaling. Among the tested drugs were also anticancer agents such as methotrexate, paclitaxel, nocodazol, tamoxifen, camptotecin, doxorubicin and etoposide [54] (and unpublished data), all of which were inactive in the CCID assay (tested at 20 μ M for 4 h). This is not surprising as these compounds affect the cell cycle/replication machinery or repair mechanisms, but not cell migration and endoMT. Thus, drug combinations consisting of classical anti-cancer agents together with calcium blockers are most likely more active *in vivo* in combating neoplastic outgrowth and prolonging metastasis-free survival than each drug alone, because they affect entirely different mechanisms. However, cross reactivity may cause problems.

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Conflict of interest

None.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.06.022.

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formation of LEC. However, the treatment of LEC with the specific inhibitor KN62 did not inhibit CCID formation regardless of whether it was induced by MCF-7- or MDA-MB231 spheroids (Fig. 3E and F).

Taken together, the data evidence that 12(S)-HETE triggered a signaling cascade involving the expression of Ca^{2+} -calmodulin kinase MYLK (not however, the activity of CAMKII), which correlated with the phosphorylation of Ser19-MLC2 as an effector of cell movement, and with the disintegration of the LEC barrier.

PLC β / Ca^{2+} signaling of 12(S)-HETE is independent of 12-HETER

It was reported that signaling through the 12(S)-HETE high affinity receptor, 12-HETER, did not trigger the release of intracellular Ca^{2+} in prostate carcinoma cells [9]. Therefore, we tested whether 12-HETER contributed to 12(S)-HETE triggered Ca^{2+} increase and to CCID formation in LEC. Consistently, the inhibition of 12-HETER (by siRNA; si12-HETER) did not reduce 12(S)-HETE-triggered Ca^{2+} induction (Fig. 4A; the proper downregulation of 12-HETER by si12-HETER transfection was monitored by Western blotting, see Supplementary Fig. S3). Nevertheless, the knock-down of 12-HETER expression attenuated the formation of CCIDs. Furthermore, simultaneous inhibition of 12-HETER expression (by si12-HETER) together with the inhibition of Ca^{2+} signaling (by BAPTA-AM), or together with the inhibition of IP_3 (by U73122), attenuated CCID formation additively (Fig. 4B). This indicated that two independent signal transduction pathways, which are both triggered by 12(S)-HETE, caused the destabilization of LEC barriers. Recently, the signal transduction of 12(S)-HETE in LEC through 12-HETER-RHO/ROCK to induce CCID formation was reported [28].

Inhibition of CCID formation by clinical modulators of calcium uptake

The increase in intracellular Ca^{2+} upon treatment with 12(S)-HETE lasted over 35–45 min (Fig. 1A and B) and even thereafter the Ca^{2+} level seemed to remain above normal. It was not unlikely that this depleted intracellular stores and that their re-filling was required to maintain elevated intracellular Ca^{2+} levels. To attenuate potential replenishment, we tested FDA (Food and Drug

Administration of the United States) approved clinical drugs that reportedly affect extracellular calcium supply by antagonizing L-type Ca^{2+} channels, store-operated Ca^{2+} channels and calcium homeostasis such as lidoflazine, ketotifen, epiandrosterone and cyclosporine [17–20]. Here, we confirmed that these drugs reduced the constitutive and 12(S)-HETE-induced levels of free intracellular Ca^{2+} in LECs (Fig. 5A) as well as phosphorylation of Ser19-MLC2 (Supplementary Fig. S4). To challenge the hypothesis that Ca^{2+} signaling in LECs is relevant for tumor intravasation *in vitro*, these drugs were tested regarding their effect on CCID formation. In fact, lidoflazine, ketotifen, epiandrosterone and cyclosporine significantly attenuated CCID formation triggered by MDA-MB231- and MCF-7 spheroids (Fig. 5B and C). Compared to the other drugs, lidoflazine was the most potent inhibitor of CCIDs, although it was least potent in inhibiting constitutive Ca^{2+} level and equally potent in inhibiting 12(S)-HETE-triggered Ca^{2+} levels. As there was no direct correlation between reduction of free intracellular Ca^{2+} levels and attenuation of CCIDs, we investigated whether lidoflazine, ketotifen, epiandrosterone and cyclosporine affected also NF- κB , which is another signaling pathway relevant for CCID formation. All four drugs down-regulated NF- κB activation in HEK293 cells stably transfected with a NF- κB luciferase reporter (Fig. 5D) and the extent of NF- κB inhibition roughly corresponded to Ca^{2+} reduction. Therefore, Ca^{2+} increase and NF- κB might have been along the same signal transduction pathway and drug treatment affected still another CCID-forming pathway. Finally, the integrate net-outcome of the signals would define the migratory response of LECs and CCID formation.

Discussion

12(S)-HETE, produced by ALOX12/15, is secreted by neutrophils and breast cancer spheroids and triggers CCID formation in LEC monolayers enabling their transmigration into the lymphatic vasculature [3,6]. Besides, ALOX5 activity in neutrophils supports lung metastasis of breast cancer cells [32]. It was shown that 12(S)-HETE increases the intracellular Ca^{2+} level independent of extracellular calcium level [14,16,33] suggesting that Ca^{2+} contributes to the transduction of a 12(S)-HETE-triggered signal. This *in vitro* study aimed to elucidate whether the 12(S)-HETE-generated intracellular Ca^{2+} increase leads to the disintegration of LEC barriers.

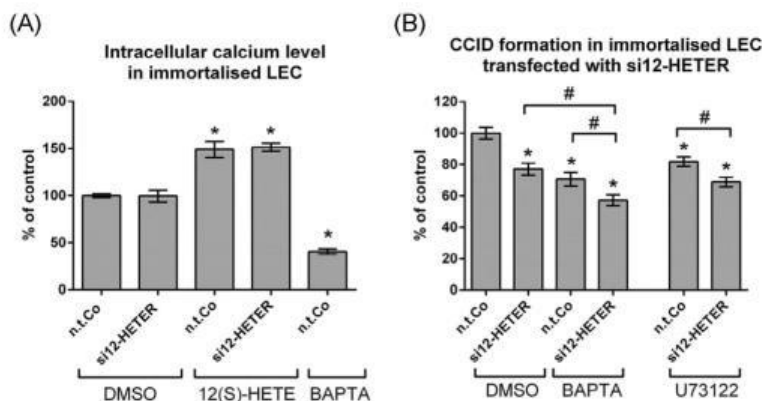


Fig. 4. 12(S)-HETE-triggered calcium increase is independent of 12-HETER expression. Immortalized LECs, which were transfected with either non-targeting control RNA (n.t.Co) or siRNA targeting 12(S)-HETE receptor (si12-HETER), were (A) stimulated with 1 μM 12(S)-HETE and then intracellular free calcium was measured. BAPTA-AM was used as positive control. Experiments were performed in sixuplicate. (B) Transfected LECs were pretreated with 1 μM BAPTA-AM or 1 μM U73122 and then, MDA-MB231 spheroids were placed on top of confluent LEC monolayers, and co-incubated for 4 h when CCID areas were measured. Error bars indicate means $\pm$ SEM, and asterisks and rhomboids significance ($p < 0.05$; t-test or ANOVA).

7. CONCLUSION

In this study, we investigated the role of identified two endothelium-specific transcription factors SOX18 and its transcriptional target, PROX1, during tumour intravasation by specifically inhibiting them with si-RNAs in triple negative breast cancer cell. MDA-MB231 breast cancer cells secrete the pro-metastatic arachidonic acid metabolite 12(S)-HETE as a trigger factor, which causes the retraction of lymph endothelial cells (LECs) and opens entry ports for tumour cell intravasation, This 12(S)HETE was transduced by GPR31 as high-affinity receptor and BLT2 as low-affinity receptor

Specific inhibition by both of SOX18- or PROX1 significantly attenuated in vitro intravasation of MDA-MB231 spheroids through the LEC barrier. SOX18 was upregulated RELA, NFKB1, and ICAM1. CCID formation was attenuate by Si-RNA-mediated inhibition of SOX18 and PROX1 as well as si-RNA-mediated inhibition of RELA, NFKB1 or NEMO for a crosstalk between SOX18 and NF- κ B, which is a show to contribute to MDA-MB231 or lymph endothelial intravasation.

Anti-metastatic property in vitro showed by several of approved clinical drugs and flavanoids Therefore authorized drugs may prevent metastatic outgrowth and should be tested in the animal in vivo model and eventually later on in human.