Dissertation zur Erlangung des Doktorgrades

der Fakultät für Chemie und Pharmazie

der Ludwig-Maximilians-Universität München

Anti-angiogenic effects of Cyclin dependent kinase inhibitors - Novel function of Cyclin dependent kinase 5 in endothelial cell migration

Johanna Liebl

aus Pfarrkirchen

2008

Erklärung:

Diese Dissertation wurde im Sinne von §13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn PD Dr. Stefan Zahler betreut am Lehrstuhl für Pharmazeutische Biologie von Frau Prof. Dr. Angelika M. Vollmar.

Ehrenwörtliche Versicherung:

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, 13. Oktober 2008

Johanna Liebl

Dissertation eingereicht am:	13. Oktober 2008
1. Gutachter:	PD Dr. Stefan Zahler
2. Gutachter:	Prof. Dr. Martin Biel
Mündliche Prüfung am:	21.November 2008

Für Mama und Papa

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2 INTRODUCTION

2.1 Background and aim of the study

Angiogenesis, the process of vascular outgrowth by sprouting from pre-existing vessels, is fundamental for the vascularization during embryonic development. During adultness, it is required for menstruation cycle and wound healing. However, angiogenesis is also involved in pathological processes, including diabetic retinopathy, arthritis, psoriasis, and tumor growth.[1] Consequently, the search for anti-angiogenic compounds and their molecular targets has been intensified during the recent years.[2, 3] Compounds that control the first step in the angiogenic cascade, the response of endothelial cells to growth factors have already been established. They include the monoclonal antibody Bevacizumab (Avastin®, Roche), and kinase inhibitors like Sunitinib (Sutent®, Pfizer) and Sorafenib (Nexavar®, Bayer). However, compounds targeting the following steps, e.g. endothelial cell migration or tube formation, respectively, are still missing.

Recently, anti-angiogenic agents have been shown to synergize with classical anti-proliferative drugs in cancer therapy.[4] Following this line of thought, it is surprising that the anti-angiogenic potential of a class of compounds, the inhibitors of cyclin dependent kinases (Cdks) – initially designed for the control of cell cycle progression – has never been studied in detail. Cdk inhibitors comprise such classical and well-characterized compounds as roscovitine, flavopiridol, and 7-hydroxystraurosporine. Roscovitine (Seliciclib, CYC202) is an orally available small molecule Cdk inhibitor that is currently evaluated in phase 2b clinical trials concerning cancer therapy ("Efficacy Study of Oral Seliciclib to Treat Non-Small Cell Lung Cancer").[5] A special feature of roscovitine is its grade of specificity: in contrast to many other small molecule kinase inhibitors, this compound only inhibits Cdk1, Cdk2, Cdk5, Cdk7, and Cdk9 to a relevant degree, other kinases are not targeted.[6, 7]

Cdk5 is an unusual member of the Cdk family. In contrast to the cell cycle-related Cdks (e.g. Cdk1, 2, 4 or 6) which regulate the main cell cycle transitions,[8] Cdk5 is not implicated in cell cycle control. Since it was discovered in the 1990s, it has emerged as a crucial regulator of neuronal migration in the developing nervous system.[9] In neurons, Cdk5 regulates cell migration, adhesion, and axon guidance by influencing the cytoskeleton, i.e. actin dynamics and microtubule organization and transport, via phosphorylation of various substrates.

Recently, the biological and molecular similarities between nervous and vascular systems have attracted a lot of attention, since repulsive cues first discovered in the neuronal system to inhibit axon migration were shown to inhibit angiogenesis as well, utilizing similar mechanisms.[10] Apparently, vascular and neuronal cells use similar signals and principles to

differentiate, grow and navigate.[11] Thus, various ligand/receptor pairs described to regulate neuronal guidance have been implicated in angiogenesis as well.[2, 10]

These parallels concerning the regulation of the growth of nerves and blood vessels led us to the hypothesis that Cdk5 might be involved in the regulation of endothelial cell migration and angiogenesis, and that, consequently, the Cdk inhibitor roscovitine and its derivatives have anti-angiogenic properties.

Thus, the aims of this study were

1. **to elucidate potential anti-angiogenic effects of the Cdk inhibitor roscovitine and its derivatives LGR561, LGR848, and LGR849, to compare their potency and**
2. **to characterize the role of Cdk5 in endothelial cell migration and angiogenesis.**

2.2 Cyclin dependent kinases

2.2.1 Functions

Cyclin dependent kinases (Cdks) are a family of small serine/threonine kinases that associate with cyclins. Cdk/cyclin complexes phosphorylate target proteins to induce cellular responses. Cdks were initially characterized about 30 years ago. Nurse *et al.* identified a gene in fission yeast that controlled entry into mitosis (cdc2 or Cdk1),[12] while Masui and Smith discovered a complex called maturation-promoting factor (MPF) in amphibian oocytes.[13, 14] Henceforward, Cdks have become generally known as master regulators of the main transitions of the eukaryotic cell cycle.[15] Cell cycle related Cdks include Cdk1, Cdk2, Cdk3, Cdk4, Cdk6, and Cdk7 as a component of the Cdk activating kinase (CAK), according to Figure 1.[8, 16]

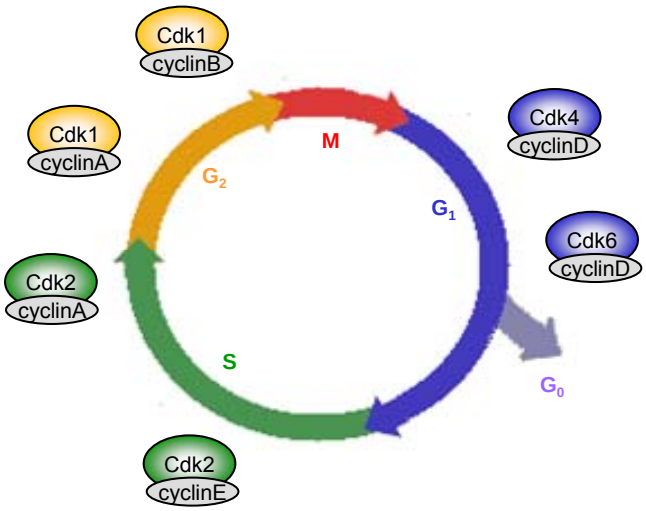


Figure 1 The cell cycle and its regulation by cell cycle related Cdks. Cdk4 and Cdk6 associate with cyclinD during G_1 phase. Cdk2 is activated by cyclinE during DNA synthesis and by cyclinA during S/G_2 transition. Cdk1 associates with cyclinA during G_2 phase and with cyclinB at G_2/M transition, respectively.

However, Cdk5, Cdk7, Cdk9, Cdk10, Cdk11, Cdk12, and Cdk13 are not directly implicated in the control of cell cycle transitions. Cdk5 is a crucial regulator of neuronal migration and development.[9] Cdk7 is a component of the CAK complex that activates other Cdks by phosphorylation.[17] Cdk7 also is involved in transcription control by phosphorylating the C-terminal domain of the large subunit of RNA-polymerase II, just as Cdk8, and Cdk9.[18] Cdks which regulate RNA splicing include Cdk10, Cdk11, Cdk12, and Cdk13.[16] In spite of their different biological functions, the family members share about 40% sequence identity.

2.2.2 Regulation

Cdks are regulated in different ways. For activation, the monomeric Cdk requires the association with a cyclin regulatory subunit. Originally, cyclins were defined as proteins whose levels oscillate during the cell cycle. Today, they are more accurately defined as members of a family of structurally related proteins that bind to and activate Cdks. Each Cdk interacts with a subset of cyclins, and mammalian cyclins can interact with several Cdks.[15]
In addition to their association with cyclins, Cdk activation requires phosphorylation of a conserved threonine by CAK (i.e. Thr160 for Cdk2, Thr161 for Cdk1).[19] Phosphorylation of a conserved threonine-tyrosine pair (Thr14 and Tyr15) mediated by Wee1 and Myt1 protein kinases inhibits Cdk-cyclin complexes. Cdc25 phosphatases reactivate Cdks by dephosphorylating Thr14 and Tyr15. Furthermore, Cdks are inhibited by binding to Cdk inhibitors (CKIs). Two classes of CKIs exist: the Cip/Kip-family is composed of the three members $p21^{waf1/kip1}$, $p27^{kip1}$, and $p57^{kip1}$ and the INK4 family includes the four members p15, p16, p18, and p19.[20]

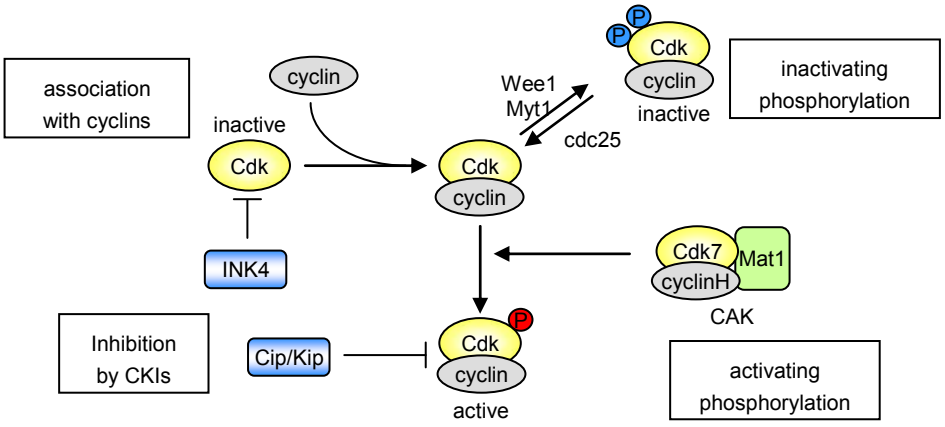


Figure 2 **Regulation of Cdks.** Activation of Cdks requires association with a cyclin regulatory subunit and phosphorylation by CAK. Cdks are inactivated by Wee1/Myt1-mediated phosphorylation and by association with Cip/Kip- or INK4-family members.

In summary, the different Cdk/cyclin complexes are required to catalyze the phosphorylation of distinct protein substrates which in turn regulate key cellular processes including cell cycle transitions, transcription, RNA splicing, apoptosis, and neuronal development.

2.3 Cyclin dependent kinase 5 (Cdk5)

Cdk5 belongs to the family of Cdks. Despite having 60% sequence identity with cell cycle related Cdks1 and 2, Cdk5 is not implicated in cell cycle control. Since it was discovered in the 1990s, it has emerged as a crucial regulator of neuronal migration in the developing nervous system. Despite its important role in the nervous system, Cdk5 is ubiquitously expressed, pointing to the question about its function in other tissues.

Cdk5 is a proline-directed kinase that phosphorylates serines and threonines immediately upstream of a proline residue. The consensus sequence for Cdk5 is (S/T)PX(K/H/R). S and T represent the phosphorylatable serine or threonine residues, X is any amino acid and P is the proline in the +1 position.[9]

2.3.1 Regulation of Cdk5

Although the regulation of Cdk5 shares common features with the mitotic Cdks, important differences exist. Like other Cdks, monomeric Cdk5 shows no enzymatic activity and requires association with a regulatory subunit for activation. Cdk5 associates with and gets activated by the two non-cyclin proteins p35 and p39. Both share about 57% amino acid identity and are themselves regulated by transcription and ubiquitin-mediated degradation. A myristoylation motif targets p35 and p39 to cell membranes and cell periphery, dictating the subcellular distribution of Cdk5.[9]

In contrast to mitotic Cdks which are inhibited by phosphorylation at Tyr15, phosphorylation of Cdk5 at Tyr15 by the tyrosine kinase c-Abelson (c-Abl) increases its activity. The role of phosphorylation of Cdk5 by CAK is not entirely clear. CAK was shown to activate Cdk5 by phosphorylation at Ser159.[21] However, phosphorylation of Cdk5 at Ser159 also was described as dispensable.[22, 23]

Moreover, Cdk5 is not inhibited by CKIs including $p21^{waf1/kip1}$ and $p27^{kip1}$. An overview over the regulation of Cdk5 is given in Figure 3.

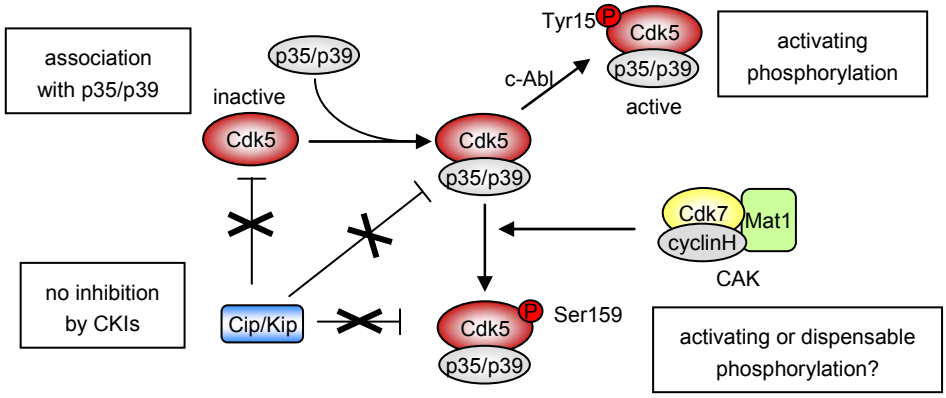


Figure 3 **Regulation of Cdk5.** Like the mitotic Cdks, activation of Cdk5 requires the association with a regulatory subunit. Cdk5 associates and is activated by the two non-cyclin proteins p35 and p39. In contrast to the mitotic Cdks, Cdk5 gets activated via phosphorylation at Tyr 15 by c-Abl. Furthermore, Cdk5 is not inhibited by CKIs.The role of phosphorylation at Ser159 by CAK is not entirely clear.

2.3.2 Function of Cdk5 in the nervous system

Cdk5 has been characterized as a signalling molecule with central functions in the nervous system. Mice that are deficient for Cdk5 show perinatal lethality with defects in neuronal layering of many brain structures, including the cerebral cortex, the hippocampus, the cerebellum, and the olfactory bulb, indicating an impact of Cdk5 on neuronal cell migration.[24-26] In the central nervous system, Cdk5 is essential for the regulation of the cytoarchitecture during brain development, neuronal migration, adhesion, axon guidance, membrane transport, neuronal survival, dopaminergic signalling, drug addiction, and synaptic function according to Figure 4.

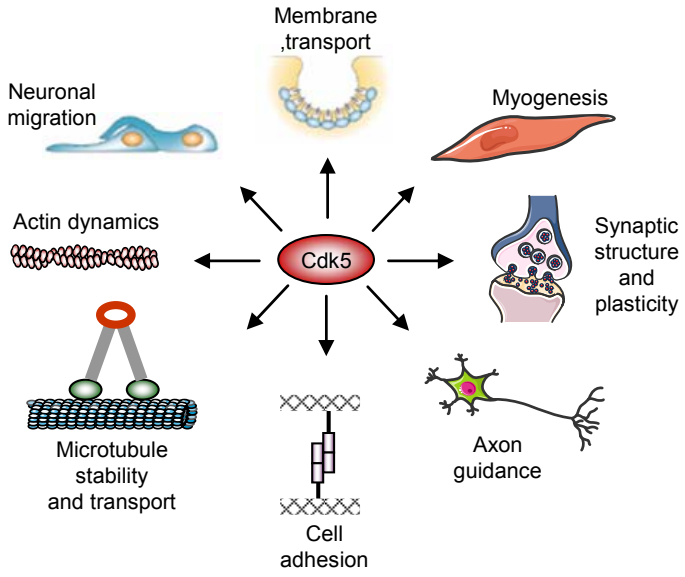


Figure 4 Cellular processes regulated by Cdk5.

Deregulation of Cdk5 leads to neurodegenerative diseases including Alzheimer's disease and amyotrophic lateral sclerosis (ALS) based on the hyperphosphorylation of the microtubule associated protein Tau by the p25-Cdk5 complex. Neurotoxic insults induce the cleavage of p35 into p25 that in turn is neurotoxic by mislocalizing Cdk5 to the cytoplasm, causing sustained activation. Thus, association of Cdk5 with p25 leads to hyperphosphorylation of Tau protein, cytoskeletal disruption, and neuronal death.[9]

The crucial role of Cdk5 in neuronal migration is based on its involvement in the regulation of the cytoskeleton. Cdk5 regulates actin dynamics as well as microtubule stability and transport via phosphorylation of various downstream substrates. Some of the substrates of Cdk5 that are linked to cell migration and motility are summarized in Table 1.

Table 1 Substrates of Cdk5 which are implicated in cellular motility.

Cdk5 substrate	Phosphorylation site(s)	Putative function of the phosphorylation	Reference
PAK1	Thr212	Inhibits PAK1 activity, regulation of actin dynamics, neuronal morphology	27, 28
Src	Ser75	Regulation of cell adhesion, actin dynamics, integrin signalling	29
WAVE1	Ser310, Ser397, Ser441	Regulation of actin polymerization and dendritic spine morphology	30
ephexin1	Thr41, Ser139	Increases ephexin1 activity towards RhoA, regulation of dendritic spine retraction, modulation of actin dynamics	31
Neurabin-I	Ser95	Regulation of the assembly of Neurabin-I with F-actin, neuronal morphology, and cortical migration	32
$p27^{kip}$	Ser10, Thr187	Stabilization of $p27^{kip}$, regulation of actin organization, neuronal migration	33
Disabled-1	Ser400, Ser491	Inhibits interaction with CIN85, regulation of actin dynamics	34
FAK	Ser732	Regulation of microtubule organization, nuclear movement, neuronal migration	35
Doublecortin	Ser297	Regulation of microtubule polymerization, neuronal migration	36
MAP1B	multiple	Regulation of microtubule stability	37, 38
Tau	multiple	Disorganization of microtubules	39, 40
Nudel	Ser198, Ser231, Thr219	Regulation of NUDEL distribution, axonal morphology, dynein behaviour, and microtubule organization	41
SIRT2	Ser331	Inhibits SIRT2 activity, regulation of microtubule stability, cell adhesion and motility, neurite outgrowth	42
Dynamin I	Ser774, Ser778	Synaptic vesicle endocytosis, axonal transport	43
β-catenin	Tyr654 (indirect) Ser191, Ser246	Regulation of cell adhesion	44-46
RasGRF1	Ser732	RasGRF1 degradation, regulation of neuronal nuclear organization	47
RasGRF2	Ser737	Downregulation of Rac activity and ERK1/2 activation, alters RasGRF2 and MAP1B distribution in neurons	48

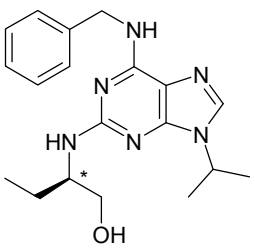
In summary, Cdk5 regulates neuronal cell migration, adhesion, and axon guidance by influencing microtubules and the actin cytoskeleton via phosphorylation of various substrates. To date, all these actions have only been examined in neuronal cells.

2.4 Cyclin dependent kinase inhibitors

Aberrations of cell cycle progression occur in the majority of human malignancies.[49] In cancer cells, there are fundamental alterations in the control of cell division, resulting in unrestricted cell proliferation.[50] Thus, to target cell cycle pathways has become an attractive approach in oncology.[51] Based on the crucial role of Cdks in regulating cell cycle transitions and, thus, cell proliferation, Cdk inhibitors have been designed as promising anti-proliferative agents for cancer therapy. During recent years, a great variety of chemical inhibitors of Cdks has been identified and characterized. Cdk inhibitors are small molecules which directly inhibit Cdks by competing with ATP for the binding at the catalytic site of the kinase. Although they all share the same principle of action, their selectivity towards the different Cdks varies greatly.[52] Some Cdk inhibitors currently are evaluated in clinical trials, including such classical and well-characterized compounds like roscovitine, flavopiridol, and 7-hydroxystraurosporine.[5, 50, 53]

2.4.1 Roscovitine (Seliciclib, CYC202)

Roscovitine (Seliciclib, CYC202) belongs to the family of 2, 6, 9-trisubstituted purines that encompasses some of the first Cdk inhibitors which have been described. In 1997, roscovitine was developed as a close analogue to the modestly potent Cdk inhibitor olomoucine and showed improved potency and selectivity in inhibiting Cdks.[6] Thus, a special feature of roscovitine is its relative specificity. In contrast to many other small molecule kinase inhibitors that also inhibit kinases not belonging to the Cdk family, roscovitine only inhibits Cdk1, Cdk2, Cdk5, Cdk7, and Cdk9 to a relevant degree.[6, 7] Structure/activity studies elucidated that the (R)-stereoisomer is about twice as potent in inhibiting Cdk1/cyclinB than the (S)-stereoisomer.[54] The chemical structure of (R)-roscovitine is displayed in Figure 5.



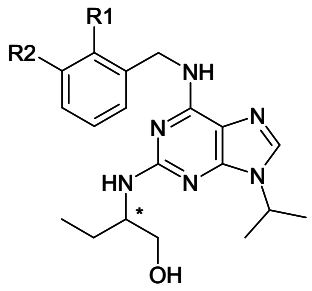
(R)-Roscovitine
(Seliciclib, Cyc202)

Figure 5 Chemical structure of roscovitine. Roscovitine competes with ATP for binding to the catalytic site of Cdks, due to its structural similarity. Thus, the purine portion binds to the adenine binding pocket of Cdks. The benzyl function enables the inhibitor to make contacts with the enzyme.

In recent years, the (R)-stereoisomer of roscovitine has developed to one of the prior used and most frequently studied Cdk inhibitors. Thus, roscovitine has been evaluated for its effects on a wide variety of cultured cancer cell lines. Two major effects have been described: roscovitine leads to an arrest in cell cycle progression and it induces cell death in most cancer cell lines. The anti-tumor activity of roscovitine has also been studied in various human tumor xenografts established on nude mice. Roscovitine was well tolerated when administerd orally, intravenously, as well as intraperitoneally and induced delay of tumor growth. In detail, roscovitine was most active in inhibiting the proliferation of colon, non-small cell lung, breast, and prostate cancer. Furthermore, roscovitine has been evaluated in several phase I clinical trials. Cyclacel is currently conducting a phase 2b clinical trial to test the efficacy and safety of roscovitine in cancer therapy. The study is evaluating roscovitine in patients with non-small cell lung cancer (NSCLC). ("Efficacy Study of Oral Seliciclib to Treat Non-Small Cell Lung Cancer").[5]

2.4.2 Derivatives of roscovitine: LGR561, LGR848, and LGR849

LGR561, LGR848, and LGR849 are close analogues of roscovitine which have been synthesized by Vladimir Krystof (Palacky University & Institute of Experimental Botany, Czech Republic). They have been developed to optimize the selectivity and potency of roscovitine in an EU project collaboration (PROKINASE No 503467). LGR561 is a (S)-stereoisomer with an additional *ortho*-hydroxyl-group in the benzyl function. LGR848 represents the (R)-stereoisomer and LGR849 the (S)-stereoisomer of a racemate with an additional *ortho*-hydroxyl-group and a *meta*-methoxy-group in the benzyl function (Figure 6).



Compound	R1	R2	stereoisomer
LGR561	-OH	-H	(S)
LGR848	-OH	$-OCH_3$	(R)
LGR849	-OH	$-OCH_3$	(S)

Figure 6 Chemical structures of LGR561, LGR848, and LGR849. All compounds are close analogues of the mother substance roscovitine. LGR561 only differs in residue R1 by the presence of an additional *ortho*-hydroxyl-group in the benzyl function. LGR848 and LGR849 differ in R1 and R2 by an additional *ortho*-hydroxyl-group and a *meta*-methoxy-group in the benzyl function.

2.5 Angiogenesis

Angiogenesis is the process of vascular growth by sprouting from pre-existing vessels. It is fundamental for the vascularization during embryonic development. During adulthood, most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and in the placenta during pregnancy. As endothelial cells retain their ability to divide rapidly in response to physiological stimuli (e.g. hypoxia), angiogenesis is reactivated during wound healing and repair. Physiologically, the onset of angiogenesis is regulated by a balance between pro-angiogenic and anti-angiogenic factors. In many pathological processes, including diabetic retinopathy, arthritis, psoriasis, and tumor growth, the pro-angiogenic stimuli become excessive and the balance between stimulators and inhibitors is disrupted, resulting in the angiogenic switch and leading to inappropriate vessel growth.[3] During tumor growth, angiogenesis is required for proper nourishment and removal of metabolic waste from tumor sites, and therefore accounts for tumor survival and proliferation.[1]

2.5.1 Angiogenic cascade

Angiogenesis involves a cascade of events with the migration of endothelial cells playing a central role.[55] Endothelial cells get activated by angiogenic factors such as VEGF-A, VEGF-C, bFGF, angiopoietins, interleukin-8, and PDGF, which are released by the diseased tissue (tumor) into the surrounding area. These factors activate resident endothelial cells by binding to specific receptors on their surface. Thus activated, endothelial cells produce molecules that locally degrade the basement membrane of the parent vessel and of the surrounding extracellular matrix (ECM). They begin to proliferate, show increased survival, change their adhesive properties, and vessel sprouting occurs.[56] Following appropriate guidance cues (growth factors like VEGF or bFGF), endothelial cells migrate towards the diseased tissue (tumor). Once the growing sprout of endothelial cells reaches its target (tumor), the tips of other sprouts, or existing capillaries, the formation of capillary tubes is induced. The primary sprout converts into a functional, blood carrying vessel. This process involves the adhesion of endothelial cells to the surrounding matrix, the formation of tight junctions, the deposition of a new basement membrane and the recruitment of other cells like pericytes or smooth muscle cells.[57] An overview over the different steps of the angiogenic cascade is displayed in Figure 7.

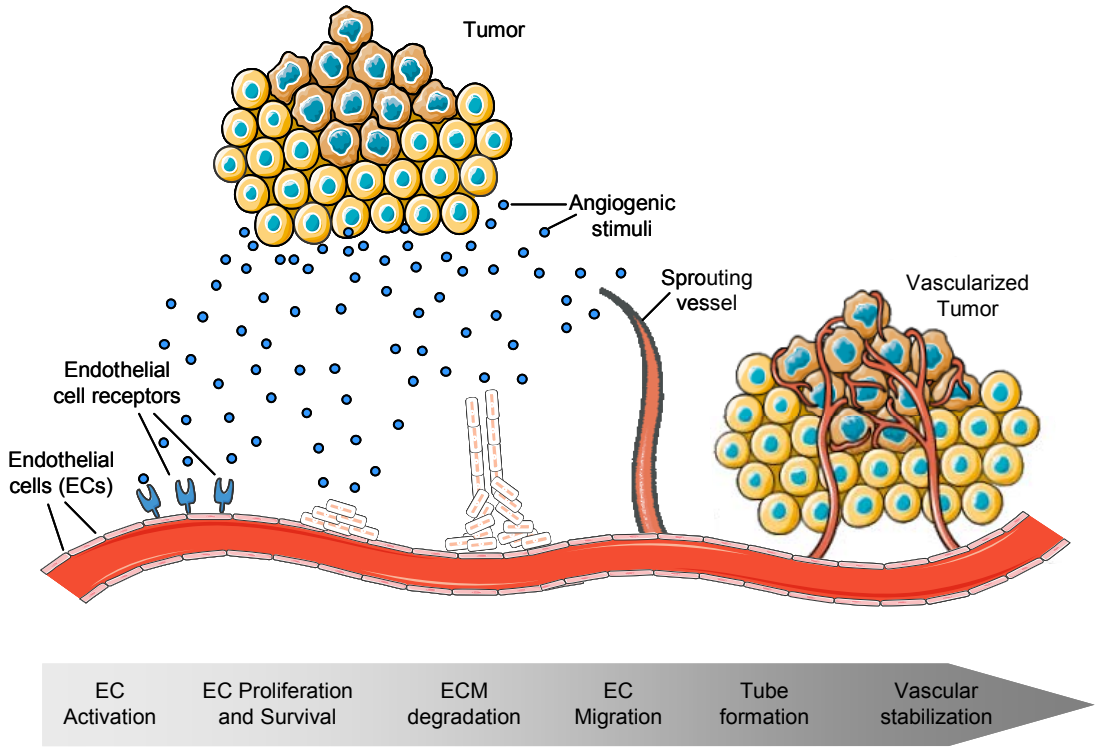


Figure 7 **Angiogenic cascade.** Endothelial cells get activated by angiogenic stimuli. They proliferate, show increased survival, degrade the extracellular matrix (ECM), and directly migrate towards the tumor. Finally, new tubes are formed and vessels are stabilized, leading to vascularization of the tumor.

The structure of tumor vasculature strongly differs from normal blood vessels. Tumor vessels follow tortuous paths and are highly disorganized, including an irregular shape with uneven diameter, and excessive branching and shunts. They are characterized by increased permeability (leakiness), abnormal blood flow, a discontinuous basement membrane and an unusual pericyte coat.[58]

Recently, the biological and molecular similarities between nervous and vascular systems have attracted a lot of attention, since repulsive cues first discovered in the neuronal system to inhibit axon migration, were also found to inhibit angiogenesis utilizing similar mechanisms.[10] Vascular and neuronal cells were shown to use similar signals and principles to differentiate, to grow, and to navigate.[11] Thus, various ligand/receptor pairs described to regulate neuronal guidance have been implicated in angiogenesis as well. They include semaphorin/neuropilin, ephrin/Eph, Slit/roundabouts (Robo), and Netrin/uncoordinated-5 (UNC5)[2, 10]. These parallels concerning the regulation of the growth of nerves and blood vessels not only link vascular biology and neuroscience, they furthermore promise to accelerate the finding of new mechanistic insights and therapeutic opportunities.

2.6 Endothelial cell migration

The migration of endothelial cells is essential to angiogenesis. It is a multistep process which involves changes in the cytoskeleton, i.e. remodelling of actin filaments and microtubules, cell-substrate adhesions, and the extracellular matrix. It is directionally regulated by chemotactic (VEGF, bFGF, angiopoietins, ephrins, TNFα), haptotactic (components of ECM and integrins), and mechanotactic (fluid shear stress) stimuli and requires the activation of several signalling pathways that converge on cytoskeletal remodelling. [55, 59]

2.6.1 Cell types

In endothelial cell migration, a distinction is drawn between two cell types. So called tip cells initiate the migration of endothelial cells and lead the endothelial cell sprout by following appropriate guidance cues. They are highly migratory and express numerous filopodia and lamellipodia that explore the surrounding tissue. In contrast, they show only low proliferative activity. Tip cells are followed by trailing cells, called trunk cells or stalk cells. Trunk cells maintain the connection to the parent vasculature. They have fewer and shorter filopodia and are highly proliferative.

Only few cells can be selected as tip cells, otherwise the patency of stable blood vessels would be lost, resulting in vascular leakage or haemorrhage. Thus, endothelial cells exposed to a similar degree of pro-angiogenic stimuli, have to exhibit distinct cellular behaviours. The decision whether a cell becomes a tip cell or a trunk cell, is regulated by the Notch/Dll-signalling pathway.[57, 60]

2.6.2 Regulation of endothelial cell migration

Endothelial cell migration can be devided into 5 sequential events. It is initiated by cell polarization and actin-dependent protrusion at the leading edge where lamellipodia and filopodia are formed. During cellular extension, under the leading edge, new adhesions to the substratum are generated. Next, the nucleus and the cell body are translocated forward through actomyosin-based contraction forces, mediated by stress fibres linked to focal adhesions. Then, retraction fibres pull the rear of the cell forward, adhesions at the rear of the cell disassemble, and the trailing edge retracts. The different steps of endothelial cell migration are displayed in Figure 8.

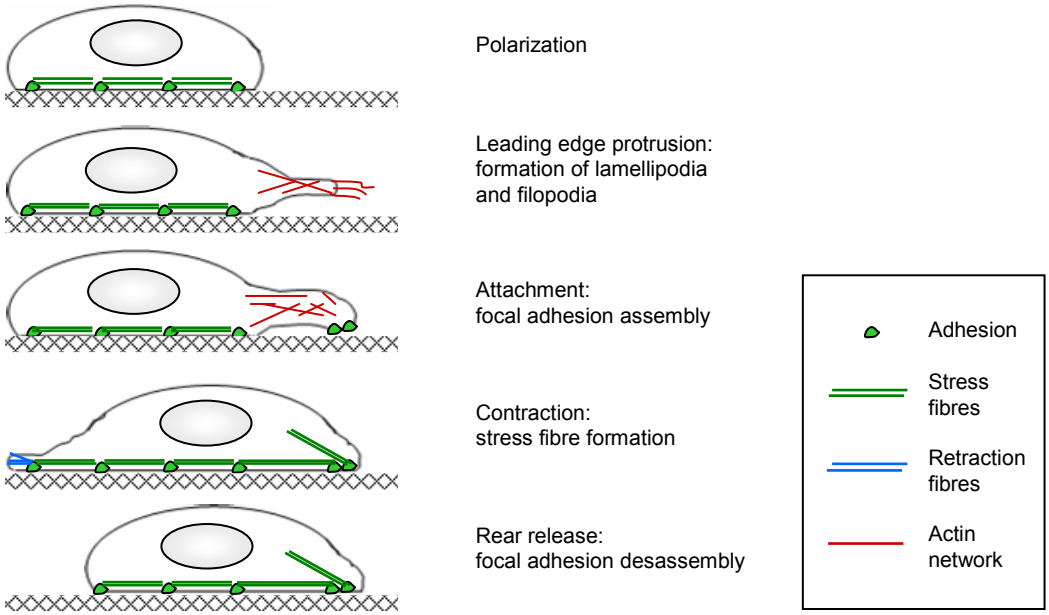


Figure 8 **Steps of endothelial cell migration.**

2.6.2.1 Cell polarization

Cell polarity, i.e. the asymmetric distribution of organelles, proteins, and/or surface projections, is substantial for proper cell migration. It is achieved by signalling molecules including the family of Rho GTPases, and phosphoinositide-3-kinases (PI3Ks). Cdc42, a member of the family of Rho GTPases, affects cell polarity along with the PAR-complex (polarity protein partitioning-defective-complex) including PAR6, PAR3, and aPKC. Cdc42 localizes the Golgi-apparatus and the microtubule organizing centre (MTOC) in front of the nucleus, oriented towards the cell front. Cdc42-induced MTOC orientation facilitates the polarized organization of microtubules which radiate from the MTOC with their plus-ends contacting the actin-rich leading edge as well as targeting focal adhesions in the rear of the cell.[61] The growth of microtubules stimulates Rac1 activity which mediates actin polymerization and lamellipodium formation. Shortening of microtubules stimulates RhoA, resulting in stress fibres formation and contraction. In this context, RhoA and Rac1 are mutually antagonistic. Thus, during cell migration, microtubules maintain cell polarity by proper positioning and stabilization of the leading edge and by contributing to rear retraction.[62] Cellular asymmetry is thereby regulated by APC proteins and by plus-end-tracking proteins (+TIPs), including cytoplasmic linker proteins (CLIPs) and CLIP-associating proteins (CLASPs), which localize and stabilize the ends of microtubules at the leading edge and promote microtubule growth.[63]

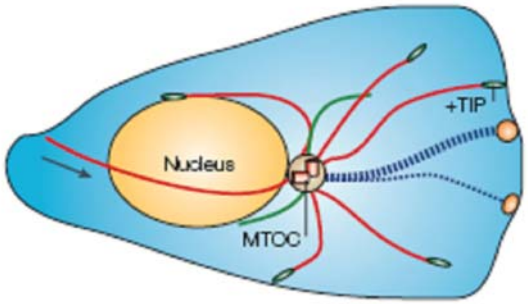


Figure 9 Microtubule dynamics during cell migration. Growing (red), shrinking (green), and stabilized (blue) microtubules radiate from the MTOC. Plus-end tracking proteins (+ tips) are localized to and direct growing microtubules. The image is adapted from Galjart.[63]

2.6.2.2 Extension of membrane protrusions

The extension of membrane protrusions in direction of migration is essential for a cell to migrate. These protrusions can be large, broad lamellipodia or spike-like filopodia. They are actin-based structures, driven by polymerization of actin filaments (F-actin) and stabilized by adhering to the extracellular matrix (ECM).[61] Thus, the constant remodelling of the actin cytoskeleton is essential for proper cell migration.[55]

Lamellipodia are highly dynamic, broad, sheet-like membrane protrusions formed at the leading edge of migrating and spreading cells.[64] Lamellipodia are characterized by a dendritic network of branched actin filaments which are formed by the actin-related-protein 2/3 (Arp2/3)-complex, dependent on Rac1. [65] The lamellipodium extends about several micrometers (1-3 µm) from the leading edge to the lamellum which connects to the cell body. At lamellipodia, spreading and migrating cells form new adhesions which connect the actin cytoskeleton to the ECM. This anchors the front of the cell and enables forward crawling. Thus, the extension of a lamellipodium is required for proper cell migration.[66]

Filopodia are thin finger-like protrusions that contain parallel bundles of actin filaments emanating from the lamellipodium or the lamellum. They function as sensors of the extracellular environment, either for soluble signals or for other cells.[64, 67] Cdc42 regulates the formation of filopodia by activation of the formin mDia2 which nucleates parallel actin bundles at the plasma membrane.[65, 68]

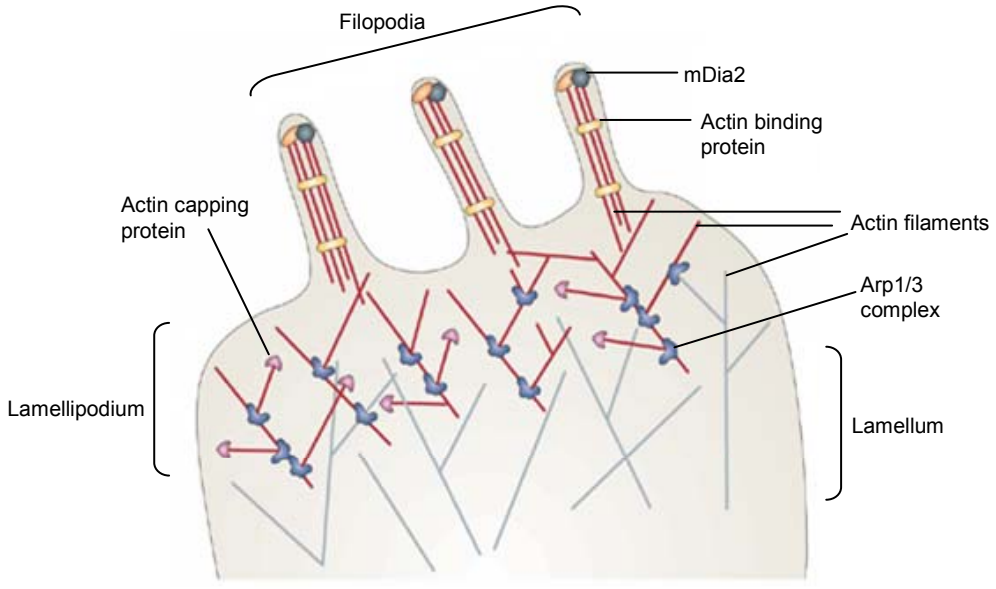


Figure 10 Membrane protrusions - Lamellipodia and filopodia. Lamellipodia are broad, sheet-like protrusions formed by highly branched actin filaments. Filopodia are thin, spike-like protrusions of bundled actin filaments. The image is adapted from Heasman and Ridley.[65]

2.6.2.3 Adhesion assembly and disassembly

During cellular extension, new adhesions are formed under the leading edge. These structures, named focal complexes and focal adhesions, control the integrin-mediated mechanical coupling between the actin cytoskeleton and the substrate.[69] Integrins are the major family of migration promoting receptors. They act as feet of a migrating cell by supporting adhesion to the ECM. Via adapters, integrins link the actin filaments of the cell to the ECM. Focal complexes are highly dynamic adhesions at the leading edge of migrating cells with high adhesion turnover. Depending on Rac1 and Cdc42, they stabilize the lamellipodium by attachment to the ECM. Focal adhesions are more stable structures with a slower turnover, located at the cell periphery and more centrally in less motile regions. They serve as traction sites for migration as the cell moves forward over them. The assembly of focal adhesions involves RhoA-stress fibres induced contractility.

Adhesion disassembly is observed both at the leading edge, where it accompanies the formation of new protrusions, and at the rear of the cell, where it allows the cell to detach and thus, promotes tail retraction and rear release. Adhesion turnover involves the interaction of Rho family GTPases and tyrosine kinases including Src kinases and the focal adhesion kinase (FAK), a key signalling component localized at focal adhesions.[70]

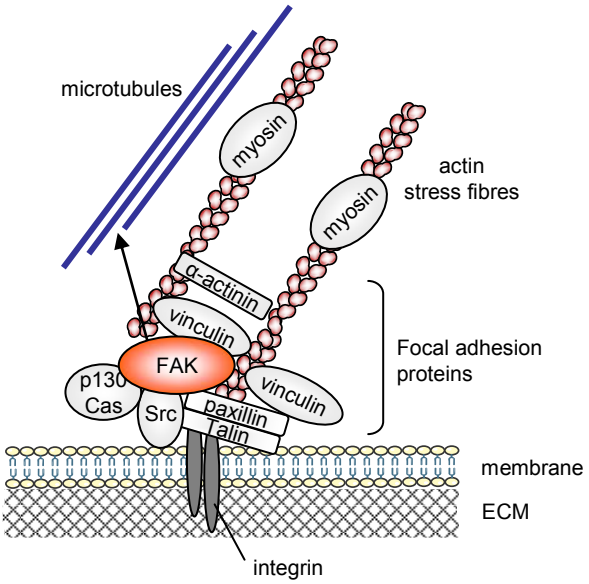


Figure 11 Focal adhesion: Interaction site of extracellular matrix, integrins, and the cytoskeleton. At focal adhesions, the extracellular matrix is connected to the cytoskeleton via integrins and various adapter proteins.[69] Activation of integrins results in a variety of intracellular responses including the recruitment of adapter proteins (vinculin, talin, α-actinin) and several cytoplasmic protein tyrosine kinases like members of the Src family kinases and the focal adhesion kinase (FAK).

2.6.2.4 Cell contraction

Cell contraction is mediated by the formation of stress fibres, i.e. contractile bundles of actin and myosin filaments connected with focal adhesions.[66] The force transmitted by stress fibres to focal adhesions mediates the translocation of the nucleus and the cell body towards the cell front and enables the retraction of the rear of the cell. Cell contraction is regulated by the RhoA-ROCK-MLC pathway via stress fibres formation.

2.6.3 Rho GTPases

The family of Rho GTPases is crucial for the regulation of the actin cytoskeleton in various cell types. They coordinate the cellular responses required for cell migration.[59, 71] 20 Rho family proteins have been identified in humans so far. RhoA, Rac1, and Cdc42 are the most prominent members and have been the most widely studied for their effects on cell migration.

2.6.3.1 Regulation

Rho GTPases cycle between a GTP-bound (active) and a GDP-bound (inactive) conformation. In the GTP-bound form, they interact with and activate downstream target proteins to induce cellular responses. The activity of Rho GTPases is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs stimulate the release of GDP and allow GTP to bind, thus activating the GTPase. GAPs catalyze GTP hydrolysis, converting the protein to the GDP-bound inactive conformation. Moreover, several Rho GTPases interact with guanine nucleotide dissociation inhibitors (GDIs), which prevent their association with the plasma membrane and downstream targets (Figure 12).[59, 67]

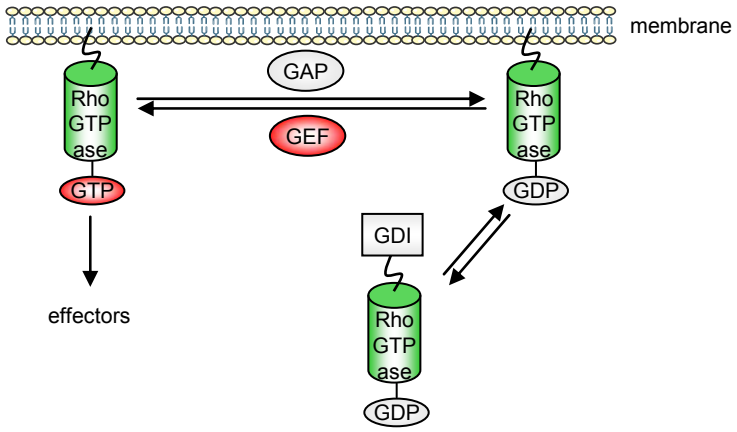


Figure 12 Regulation of Rho GTPases. Rho family proteins are active when bound to GTP and inactive when bound to GDP. They are activated in response to extracellular signals (i.e. cytokines, growth factors, extracellular matrix proteins). Activation is catalyzed by guanine nucleotide exchange factors (GEFs) and inactivation by GTPase activating proteins (GAPs). Furthermore, several Rho GTPases bind to guanine nucleotide dissociation inhibitors (GDIs) in the cytoplasm and are inactive in this complex. Bound to GTP, they interact with downstream target proteins and induce cellular responses.

2.6.3.2 Rac1

Rac1 is active at the cell front of migrating cells. It is a pivotal regulator of the extension of lamellipodia.[59] Rac1 mediates the polymerization of lamellipodial branched actin filaments via the Wiskott-Aldrich syndrome protein (WASP)-family verprolin-homologues protein (WAVE) complex. WAVE activates the Arp2/3-complex which nucleates new actin filaments from the side of an existing filament, resulting in a branched actin network, characteristic for lamellipodia. [64, 67] Rac1 also activates p21-activated kinases PAK1, PAK2, and PAK3. PAKs are serine/threonine kinases that phosphorylate and activate LIM kinases LIMK1 and LIMK2 which in turn phosphorylate and inactivate cofilin. Unphosphorylated cofilin stimulates severing and depolymerization of F-actin in lamellipodia.[67] Rac1 also is required for the targeting of cortactin to the cortical actin network. Cortactin is an Arp2/3 and F-actin binding protein and potentiates the nucleation of new actin filaments, playing a pivotal role in lamellipodia protrusion and integrity.[72] Tyrosine-phosphorylation of cortactin, required for efficient cell motility, is dependent on the targeting of cortactin to the cortical actin network and is regulated by the activation state of Rac1.[73] An overview over Rac1 signalling pathways is given in Figure 13.

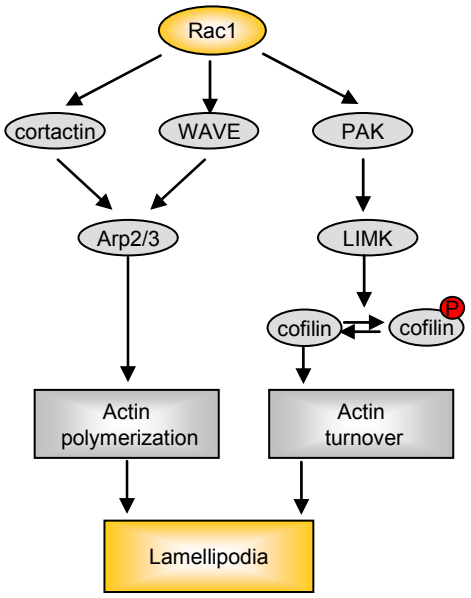


Figure 13 **Rac1 signalling.** Rac1 is active at the leading edge of a migrating cell, where it regulates the formation of lamellipodia.

2.6.3.3 Cdc42

Just as well as Rac1, Cdc42 also is active at the leading edge of migrating cells. Cdc42 regulates the formation of filopodia by activation of the formin mDia2.[67] Formins nucleate parallel actin bundles, generating filopodia.[68] Cdc42 also contributes to Rac1-mediated lamellipodium extension by organizing microtubules to the actin-rich leading edge, where microtubule growth stimulates Rac1 activation.[67] Furthermore, Cdc42 stimulates the formation of WASP and N-WASP proteins, which activate the Arp2/3 complex. Besides Rac1, it also activates the PAK-LIMK-cofilin pathway. Figure 14 displays Cdc42 signalling pathways.

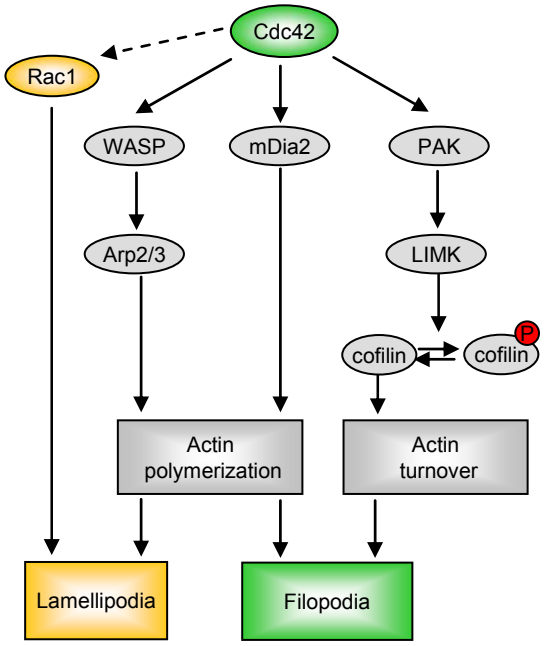


Figure 14 Cdc42 signalling. Cdc42 is also active at the cell front. It regulates formation of filopodia. Furthermore, it is implicated in formation of lamellipodia via influencing Rac1.

2.6.3.4 RhoA

In contrast to Rac1 and Cdc42, RhoA is active at the sides and the rear of the cell, associated with stress fibre formation and cell body contraction. RhoA acts via Rho-kinases (ROCKs) to affect the phosphorylation and the activation of myosin light chain (MLC), both by phosphorylating MLC and inhibiting MLC-phosphatase (MLCP). Phosphorylated myosin II assembles into fibres that associate with actin filaments to form contractile stress fibres. Furthermore, RhoA activates the assembly of parallel actin bundles (stress fibres) via mDia2. Like PAK, ROCK also activates LIMKs to inhibit cofilin-mediated actin-depolymerization, thereby promoting F-actin accumulation.[59, 67] RhoA signalling pathways are displayed in Figure 15.

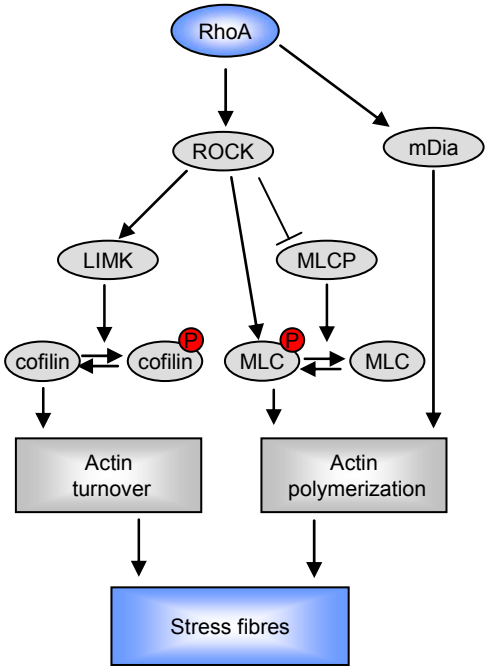


Figure 15 **RhoA signalling.** RhoA is active at the sides and the rear of the cell where it induces the formation of stress fibres, leading to cell contraction.

In summary, Rho GTPases are essential for the remodelling of the actin cytoskeleton and, thus, for cell migration. Rac1 and Cdc42 are active at the cell front, where they promote the extension of lamellipodia and filopodia, respectively. RhoA is active at the sides and the rear of the cell, associated with stress fibre formation and cell body contraction. RhoA and Rac1 are mutually antagonistic, each suppressing the others activity.[74]

2.6.4 Focal adhesion kinase (FAK)

FAK plays a crucial role in cell migration. It gets activated by numerous stimuli like integrins and growth factors and serves as a receptor-proximal regulator of cell motility. At focal adhesions, it functions as an adapter protein to recruit other adhesion proteins to their regulators, affecting the assembly and disassembly of focal adhesions. FAK activity and downstream-signalling affects cell-cell (cadherin-based) contacts and it is implicated in the regulation of microtubule structures and the actin cytoskeleton during cell migration. Thus, FAK can influence the activity of Rho GTPases via direct interaction with or phosphorylation of activators or inhibitors of Rho GTPases (Figure 16).

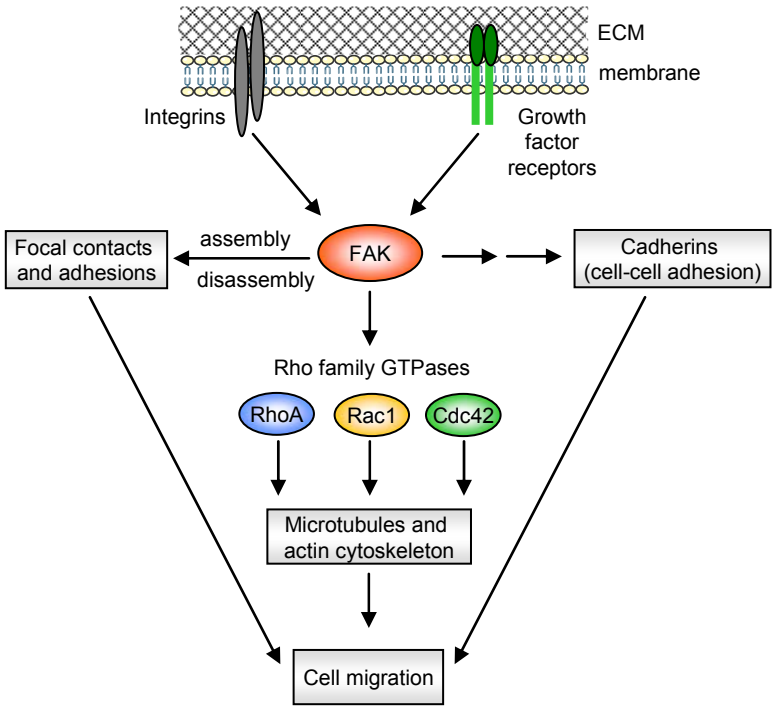


Figure 16 **The role of FAK in cell migration.** FAK gets activated by integrin clustering or growth factors, respectively. It influences cell migration by regulating focal adhesion turnover at the leading edge and the trailing edge of migrating cells. Furthermore, it regulates cell-cell adhesions, and it controls the organization of the cytoskeleton via Rho GTPases.

The activation of FAK is dependent on phosphorylation of tyrosine residues. FAK possesses six tyrosine residues that are phosphorylated by various agonists, transmitting different signals and effects. Tyr397 is an autophosphorylation site required for the catalytic activity of FAK.[75] Phosphorylation at Tyr397 recruites and activates Src homology-2 (SH2) domain-containing proteins, including members of the Src family kinases. These phosphorylate FAK at tyrosines Tyr576, Tyr577, and Tyr821 which is important for the maximal activation of

FAK and its signalling to downstream effectors.[75, 76] Phosphorylation of FAK at Tyr925 is important for the recruitment of other adapter proteins.

FAK also can get phosphorylated on serine residues, i.e. on Ser722, Ser732, Ser843, and Ser910.[75] The role of serine phosphorylation of FAK is poorly understood. In neurons, it has been shown that Cdk5 phosphorylates FAK at Ser732, which regulates microtubule organization, nuclear movement and neuronal migration, but not the catalytic acivity of FAK.[35] Another work illustrates that the phosphorylation of FAK at Ser732 is induced by RhoA-dependent kinase ROCK and is essential for the phosphorylation of FAK at Tyr407, promoting cell migration.[76] Phosphorylation sites of FAK are displayed in Figure 17.

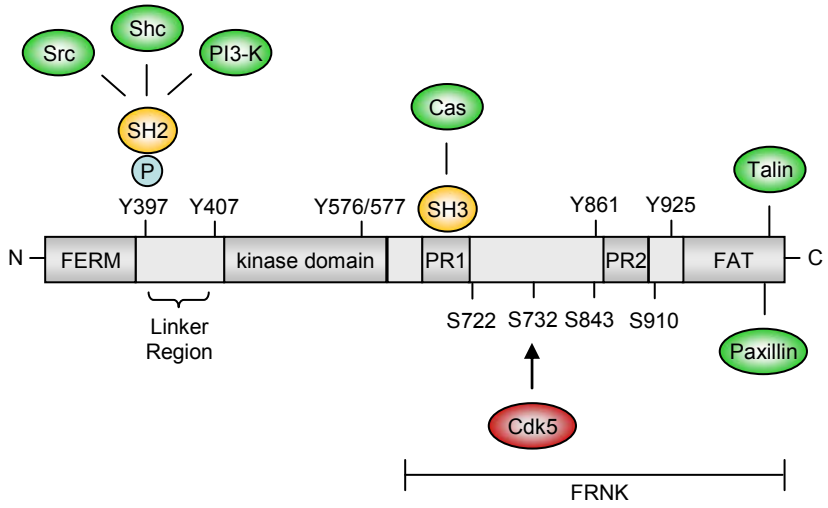


Figure 17 **Phosphorylation sites of FAK.** FAT: Focal adhesion targeting region, FERM: Protein four.1, ezrin, radixin, moesin domain (interacts with integrins and growth factor receptors), FRNK FAK-related-non-kinase domain (c-terminal non catalytic domain).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Roscovitine and its derivatives LGR561, LGR848, and LGR849

"Roscovitine" refers to (R)-roscovitine, as the (R)-stereoisomer was used. Roscovitine was from Sigma Aldrich, Munich, Germany. The LGR compounds were kindly provided by V. Krystof from Palacky University & Institute of Experimental Botany, Czech Republic.
All compounds were dissolved in DMSO at 100 mM. 10 µl aliquots were stored at -20°C for long time storage, and at 4°C for direct laboratory use.
For experiments, the compounds were freshly diluted in growth medium to 100 µM and further diluted to the indicated concentrations (10 µM and 30 µM, respectively).

3.1.2 Biochemicals and inhibitors

Biochemicals

bFGF	PeproTech, Rocky Hill, NY, USA
VEGF	PeproTech, Rocky Hill, NY, USA

Inhibitors

Complete™	Roche, Mannheim, Germany
Na_3VO_4	ICN Biomedicals, Aurora, Ohio, USA
NaF	Merck, Darmstadt, Germany
Phenylmethylsulfonylfluorid (PMSF)	Sigma Aldrich, Munich, Germany
Y27632	Sigma Aldrich, Munich, Germany

3.2 Cell Culture

3.2.1 Solutions and reagents

The following solutions and reagents were used for the isolation as well as for the cultivation of endothelial cells.

PBS (pH 7,4)

NaCl	123.2	mM
Na_2HPO_4	10.4	mM
KH_2PO_4	3.2	mM
H_2O		

PBS+Ca^{2+}/Mg^{2+} (pH 7,4)

NaCl	137	mM
KCl	2.68	mM
Na_2HPO_4	8.10	mM
KH_2PO_4	1.47	mM
$MgCl_2$	0.25	mM
$CaCl_2$	0.5	mM
H_2O		

Trypsin/EDTA (T/E)

Trypsin	0.05	%
EDTA	0.20	%
PBS		

Freezing medium

FCS	50	%
DMSO	8	%
ECGM		

Growth medium

ECGM	500	ml
Supplement	23.5	ml
FCS	50	ml
Antibiotics	3.5	ml

Stopping medium

M 199	500	ml
FCS	50	ml

Collagen A

Collagen A	10	%
PBS		

Collagen G

Collagen G	0.001	%
PBS		

Cell culture reagents

Collagen A	BIOCHROME AG, Berlin, Germany
Collagen G	BIOCHROME AG, Berlin, Germany
Collagenase A	Roche, Mannheim, Germany
Culture flasks, plates, dishes	TPP, Trasadingen, Switzerland
ECGM containing supplement and antibiotics	Provitro, Berlin, Germany
FCS	PAA, Pasching, Austria
ibidi slides	ibidi GmbH, Munich, Germany
M199	PAN Biotech, Aidenbach, Germany

Foetal Calf Serum (FCS)

FCS "Gold" was tested for mycoplasm and endotoxin. For heat inactivation, FCS was partially thawed for 30 min at room temperature. Subsequently, it was totally thawed at 37°C using a water bath. Finally, FCS was inactivated at 56°C for 30 min. Thereafter, 50 ml aliquots of heat inactivated FCS were stored at -20°C.

3.2.2 Endothelial cells

Endothelial cells (ECs) were cultured under constant humidity at 37°C and with 5% CO_2 in an incubator (Heraeus, Hanau, Germany). Cells were routinely tested for contamination with mycoplasm using the PCR detection kit VenorGeM (Minerva Biolabs, Berlin, Germany).

HMEC-1 – Human microvascular endothelial cells

The cell line CDC/EU.HMEC-1 was kindly provided by Centers for Desease Control and Prevention (Atlanta, GA, USA). The immortalized HMEC-1 cell line was created by transfection of human dermal microvascular endothelial cells with a plasmid coding for the transforming SV40 large T-antigen. HMEC-1 were shown to retain endothelial morphologic, phenotypic, and functional characteristics.[77, 78] HMECs were used for cell cycle analysis.

HUVEC – Human umbilical vein endothelial cells

Human umbilical cords were kindly provided by Klinikum München Pasing, Frauenklinik Dr. Wilhelm Krüsmann, and Rotkreuzklinikum München. After childbirth, umbilical cords were

placed in PBS+Ca^{2+}/Mg^{2+} containing Penicillin (100 U/ml) and Streptomycin (100 µg/ml), and stored at 4°C. Cells were isolated in the space of 1 week. The umbilical vein was washed with PBS+Ca^{2+}/Mg^{2+}, filled with 0.1 g/l collagenase A, and incubated for 45 min. at 37°C. To isolate endothelial cells, the vein was flushed with stopping medium and the eluate was centrifuged (1000 rpm, 5 min.). Afterwards, cells were resolved in growth medium and plated in a 25 cm^2 flask. After reaching confluency, cells were trypsinized and plated in a 75 cm^2 flask. Experiments were performed using cells at passage 3. HUVECs were used for all assays except cell cycle analysis.

3.2.3 Passaging

After reaching confluency, cells were either sub-cultured 1:3 in 75 cm^2 culture flasks or seeded either in multiwell-plates or dishes for experiments. For passaging, medium was removed and cells were washed twice with PBS before incubation with trypsin/ethylene diamine tetraacetic acid (EDTA) (T/E) for 1-2 min at 37°C. Thereafter, cells were gradually detached and the digestion was stopped using stopping medium. After centrifugation (1,000 rpm, 5 min, 20°C), the pellet was resuspended in growth medium and cells were plated.

3.2.4 Freezing and thawing

For freezing, confluent HMECs from a 75 cm² flask were trypsinized, centrifuged (1,000 rpm, 5 min, 20°C) and resuspended in 3 ml ice-cold freezing medium. 1.5 ml aliquots were frozen in cryovials. After storage at -80°C for 24 h, aliquots were moved to liquid nitrogen for long term storage.

For thawing, a cryovial was warmed to 37°C and the content was immediately dissolved in prewarmed growth medium. In order to remove DMSO, cells were centrifuged, resuspended in growth medium and transferred to a 75 cm² culture flask.

3.3 Angiogenesis assays

3.3.1 Proliferation assay (crystal violet staining assay)

The crystal violet staining assay was used to determine effects of the different compounds on endothelial cell proliferation.

1.5 x 10^3 cells in 100 µl growth medium were seeded into 96 well plates. After 24 h, cells in a reference plate were stained with crystal violet, serving as initial cell number. For stimulation, 100 µl growth medium containing the indicated compounds were added. After 72 h, the medium was removed and cells were stained with 100 µl of the crystal violet solution for 10 min. at room temperature. After washing five times with distilled water, the bound dye was solubilized by adding 100 µl of the dissolving buffer. The absorbance was measured at 540 nm in a plate-reading photometer (SPECTRAFluor Plus; Tecan, Crailsheim, Germany).

Crystal violet solution

Crystal violet	0.5	%
Methanol	20	%
H_2O		

Dissolving buffer

Sodium citrate 0.1 M	50	%
Ethanol	50	%

3.3.2 CellTiter-Blue® Cell Viability Assay

The CellTiter-Blue® Cell Viability Assay (CTB assay, Promega Corporation, Madison, WI, USA) is a method to determine the viability of cells. It uses the indicator dye resazurin to measure the metabolic capacity of cells which is considered as an indicator of viability (Figure 18).

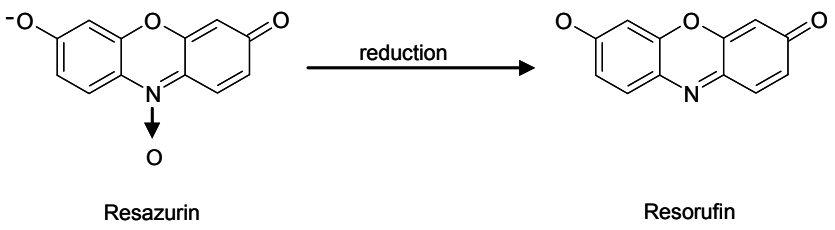


Figure 18 **CTB assay.** Reduction of resazurin to resarufin by metabolically active cells results in the generation of a fluorescent product.

The CTB assay was used to determine possible cytotoxic effects of Cdk5 siRNA.

Transfected cells were seeded into 96 Well plates (20,000 cells per well in 100 µl growth medium). After 24 or 48 h, 20 µl of the CellTiter-Blue Reagent® was added and cells were incubated for 1 h in an incubator. Afterwards, fluorescence was measured at 560 nm.

3.3.3 Scratch assay

Using the scratch assay, also called wound healing assay, the migration of cells was analyzed.

Experiments with the Cdk inhibitors:

HUVECs were seeded in a 24 well plate. After reaching confluency, cells were scratched using a pipette tip of a micropipette. The wounded monolayers were washed twice with PBS+Ca^{2+}/Mg^{2+} to remove floating cellular debris before adding growth medium containing roscovitine (10 µM and 30 µM) or LGR561, LGR848, or LGR849 (10 µM), respectively.

siRNA experiments:

Immediately after transfection with the respective siRNAs, HUVECs (500,000/well) were plated in a 24 well plate. 32 h after transfection, cells were scratched, washed twice with PBS+Ca^{2+}/Mg^{2+} and treated with growth medium.

After 16 h of migration, cells were washed with PBS+Ca^{2+}/Mg^{2+}, fixed with 3% formaldehyde and images were taken using the TILLvisON system (TILL Photonics GmbH, Gräfelfing, Germany) and a CCD-camera connected to an Axiovert 200 microscope (Zeiss, Oberkochen, Germany). Images were analyzed using specific software (S.CO LifeScience, Garching, Germany). Migration was quantified as the ratio of the number of pixels in the area covered with cells (yellow) and the number of pixels in the cell-free area (gray) (Figure 19).

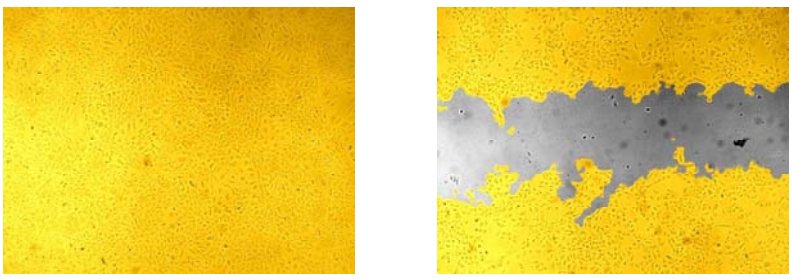


Figure 19 **Scratch assay**. Images represent cells after 16 h of migration treated with growth medium (left panel) or with serum-free medium (M199) (right picture).

3.3.4 Chemotaxis assay

The chemotaxis assay is a tool for observing chemotactical responses of adherent migrating cells over extended periods of time.

Seeding of cells and generation of the FCS gradient

6 µl of cell suspension containing $5x10^6$ HUVECs per ml was added into the observation channel of a special µ-slide for chemotaxis (ibitreat, chemotaxis, ibidi GmbH, Munich, Germany) and the slide was incubated until cells attached (4 h). To generate the FCS gradient, the chambers of the chemotaxis slide were completely filled with serum-free medium (M 199) Afterwards, growth medium containing 30 % FCS was added into one chamber. Accordingly to the manufacturers protocol, after a short time, the chemoattractant diffuses through the observation area and establishes a linear concentration profile from 0% FCS to 10% FCS over the cells. For stimulation, roscovitine (10 µM and 30 µM, respectively) was added to both media. The chemotaxis chamber is displayed in Figure 20.

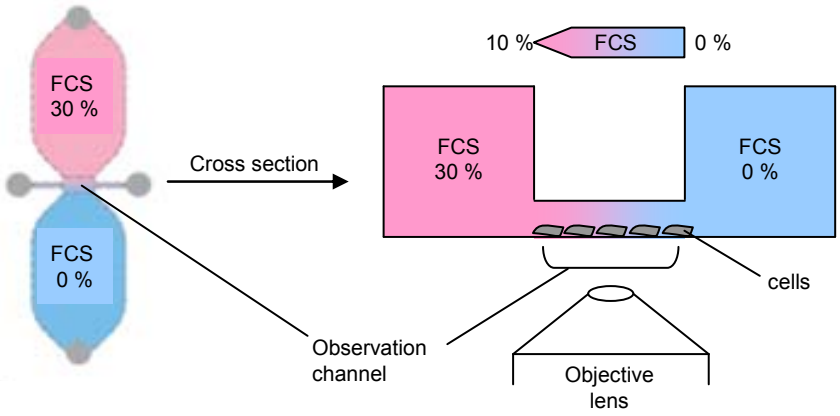


Figure 20 Chemotaxis chamber. Two large volume chambers are connected by a thin slit (observation channel). The reservoirs contain different chemoattractant (FCS) concentrations (blue: 0 % FCS; red: 30 % FCS). Inside the observation channel, a linear and stable concentration profile is generated by diffusion of the chemoattractant (0 % - 10 % FCS).

Life cell imaging:

Immediately after gradient formation, life cell imaging was performed using a Zeiss LSM 510 META confocal microscope equipped with a heating stage from EMBLem (Heidelberg, Germany). During observation, cells were incubated with constant humidity at 37°C and with 5% CO_2. Images of cells were obtained every 10 min. for 24h.

Cell tracking and evaluation:

Cells were tracked using the Manual Tracking plug-in by Fabrice P. Cordelieres (http://rsb.info.nih.gov/ij/plugins/manual-tracking.html). After tracking, movies were evaluated using the Chemotaxis and Migration Tool (Version 1.01) (ibidi GmbH, Munich, Germany) for ImageJ (http://rsb.info.nih.gov/ij/). For statistical analysis, Euclidean distance (direct cell path) and Cumulative distance (complete cell path) were calculated (Figure 21).

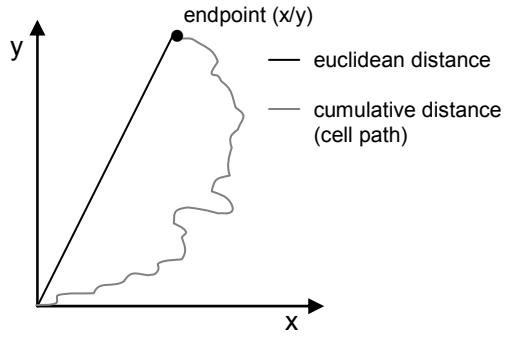


Figure 21 Euclidean and Cumulative distances. An intact Cumulative distance together with a reduced Euclidean distance refers to a loss of cell orientation. The shortening of both distances indicates a loss of cell motility.

3.3.5 Tube formation assay

Using tube formation assays, the ability of endothelial cells to form three-dimensional capillary-like structures was analyzed. BD Matrigel® Matrix Growth Factor Reduced (GFR) (BD Biosciences, Heidelberg, Germany) was used as extracellular matrix (ECM). Matrigel® Matrix is a solubilised basement membrane preparation extracted from EHS mouse sarcoma.
The Matrigel® Matrix was thawed at 4°C overnight and kept on ice until use. Ibidi angiogenesis-slides (18-well, ibidi GmbH, Munich, Germany) were coated with the Matrigel® Matrix (19 µl per well) using precooled pipette tips. For polymerization of the Matrigel® Matrix, the slides were incubated at 37°C for 30 min. Afterwards, the gels were overlaid with 30 µl growth medium containing $1x10^4$ HUVECs and the respective compounds in the indicated concentrations. Cells were incubated for 16 h and images were taken using the TILLvisON system. Evaluation of pictures was performed by S. CO LifeScience (Garching, Germany). For quantification, two parameters were analyzed according to Figure 22: tube length (red) and number of nodes (blue crosses in yellow areas).

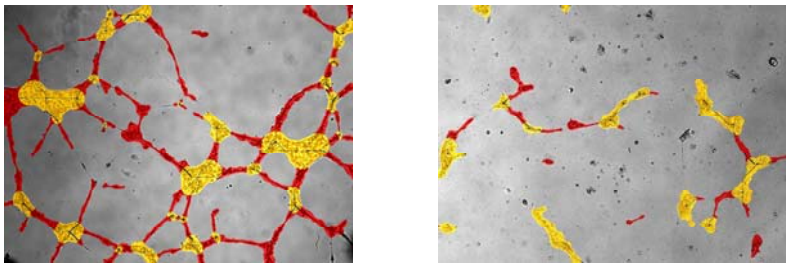


Figure 22 **Tube formation assay.** Images represent cells cultured onto Matrigel® for 16 h and treated with growth medium (left panel) or growth medium containing roscovitine (30 µM, right picture).

3.3.6 Mouse aortic ring assay

Mouse aortic rings were kindly prepared by PD Dr. Stefan Zahler and Bianca Hager (both Ludwig-Maximilians-University of Munich, Department of Pharmacy – Centre of Drug Research). After preparation, mouse aortic rings were embedded into BD Matrigel® Matrix Growth Factor Reduced (GFR) (BD Biosciences, Heidelberg, Germany), overlaid with growth medium and incubated with constant humidity at 37°C and with 5% CO_2. Once endothelial cell sprouting occurred (about 3 to 5 days), the growth medium was removed, and rings were either treated with growth medium or with growth medium containing the different compounds in the indicated concentrations. After further incubation for 72 h, images were taken using the TILLvisON system. The experimental procedure of the mouse aortic ring assay is displayed in Figure 23.

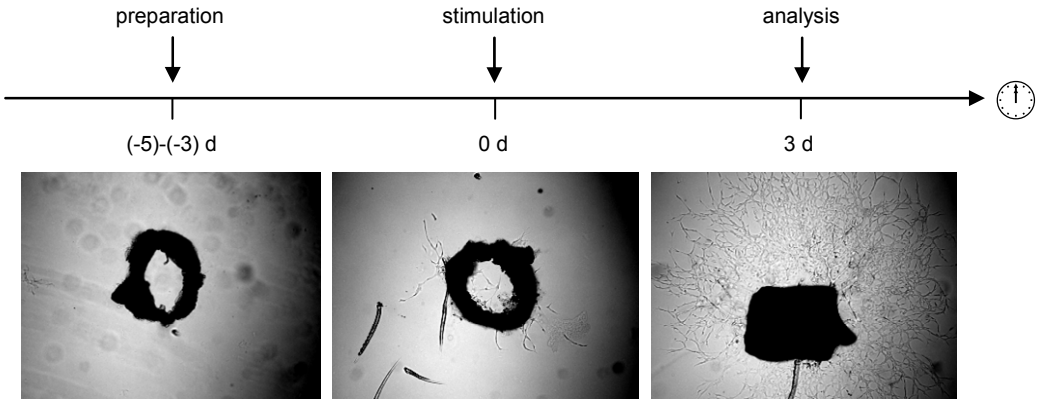


Figure 23 **Mouse aortic ring assay.** After preparation, mouse aortic rings were embedded into Matrigel® Matrix. Once endothelial cells started to sprout, rings were stimulated with the indicated compounds. After another 3 days, images were taken.

3.3.7 Chick chorioallantoic membrane (CAM) assay

The CAM assay currently is one of the most widely used *in vivo* models for the study of anti-angiogenic agents due to its technical simplicity and low cost.[79]

Preparation of cellulose disks:

After mixing the contents of the cellulose solution, the mixture was autoclaved, resulting in a homogenized, clear solution. For each disk, 200 µl of the warm solution were given into the preformed circles of the lid of a 96 well plate. For polymerization, the solution was dryed under a laminar air flow for 48 h. Afterwards, the cellulose disks were removed using tweezers.

Cellulose solution

HEC (Hydroxyethyl-Cellulose)	2.5	%
PVP 17 (Polyvinylpyrrolidone)	2	%
PEG 400 (Polyethylenglycole)	2	%
H_2O		

Preparation and stimulation of the eggs:

Fertilized White Leghorn chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) were incubated at 37°C for 72 h with constant humidity. Afterwards, the eggs were cleaned with 70% ethanol, opened, and each egg was transferred into a 100 mm culture dish (whole embryo culture). During the following incubation for 72 h the embryo grew and the CAM developed over the yolk sac. At that time, the eggs were stimulated using the cellulose disks. Four cellulose disks were placed onto each egg, localized on similar sites of the CAM. Subsequently, VEGF (1 ng per disk) or VEGF and the indicated compounds (45 µg of the compound per disk) were placed directly onto the cellulose disks. Cellulose disks without any stimulus were used to verify that the procedure itself causes no vascularization. The next day, the vascular structure in the CAM was visualized using a stereomicroscope and a CCD camera (Olympus, Munich, Germany). The experimental procedure of the CAM assay is displayed in Figure 24.

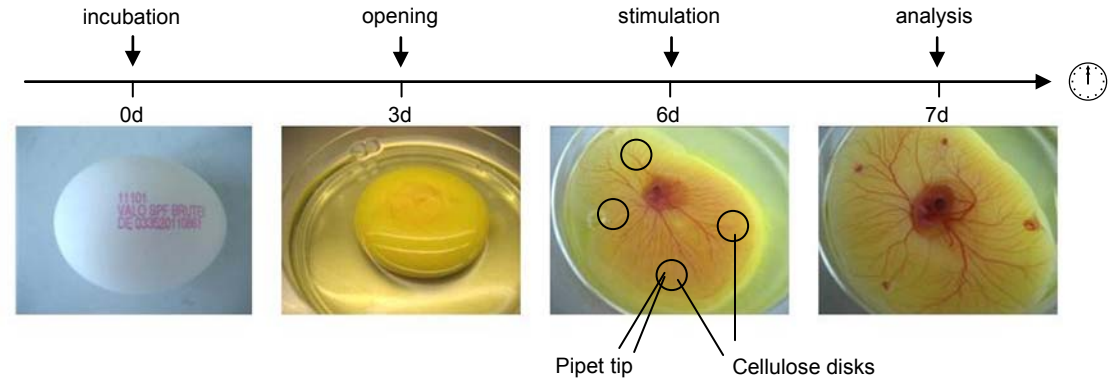


Figure 24 CAM-assay. Eggs were incubated for three days, opened, and stimulated after another three days with VEGF or with VEGF and the indicated compounds using the cellulose disks. The respective stimulus was directly given onto a cellulose disk. Four cellulose disks were given onto each egg. 24 h after stimulation, images were taken.

3.3.8 Mouse cornea micropocket angiogenesis assay

The mouse cornea micropocket angiogenesis assay uses the avascular cornea as a canvas to study angiogenesis *in vivo*. By the use of standardized slow-release pellets containing basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF), over the course of 5 days a predictable angiogenic response is generated and can be quantified.[80]

The mouse cornea micropocket assay was kindly performed by Prof. Dr. György Vereb (Medical and Health Science Center, Department of Biophysics and Cell Biology, University of Debrecen, Hungary) and by Dr. Lili Takács (Medical and Health Science Centre, Department of Ophthalmology, University of Debrecen, Hungary).
Both eyes of C57BL/6J 8-week old female mice (20-21 g body-weight) were implanted with pellets containing bFGF as described previously.[81, 82]

Pellet preparation:

A solution of 20 µg bFGF (R&D Systems) in 40 µl PBS was mixed with 10 mg sucralfate (Sucrose octasulfate-aluminium complex, Sigma Aldrich, Munich, Germany) and lyophilized. The lyophilisate was resuspended using 10 µl of 12% Hydron (Poly (2-hydroxyethyl-methacrylate), Sigma Aldrich, Munich, Germany) in 70% ethanol. This mixture was evenly spread into a 16x16-hole area of a Sefar PEEKtex open mesh precision fabric V17-300/36 (kindly provided by Dr. Thomas Wechsler, Sefar AG, Switzerland). Both sides of the mesh were covered with a solution of 12% Hydron in 90% ethanol. After complete drying (1 h), the fibres of the mesh were pulled apart and the size and shape of each pellet were examined.

Only pellets with proper height and square shape (0.3x0.3x0.2 mm) were used. The final bFGF content was 80 ng bFGF per pellet.

Pellet implantation:

Mice were anesthetized with 10 µl/g body weight pentobarbital. Polyvidon iodide eyedrop was used as local disinfectant, and tetracaine for local anesthesia of the cornea. Under a stereo microscope (Zeiss), a central vertical cut was made to mid-thickness of the cornea. Starting from this, a nasal pocket in the corneal tissue was generated using a modified von Graefe's knife, ending about 1.2 mm from the limbus. The pellet was slipped into the pocket and the edge of the wound was smoothed back. Tobramycine eye ointment was used to protect from immediate superinfection (Figure 25).

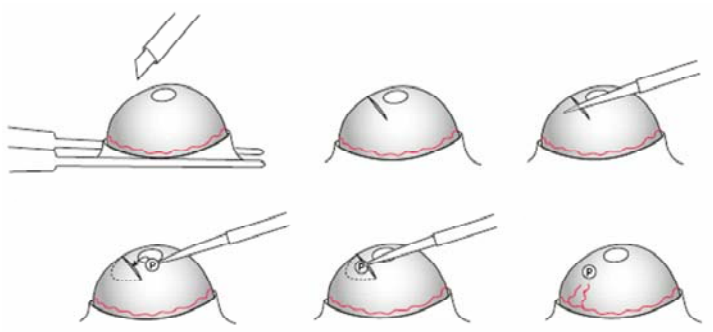


Figure 25 Creation of a corneal micropocket and pellet implantation. The image is adapted from Rogers *et al.*[80]

Drug administration:

Mice were daily injected with either DMSO (control) or with the indicated compounds for 5 days, starting at the time of operation. Roscovitine was dissolved in DMSO at 100 mg/ml and diluted with sterile PBS to 10 mg/ml. For complete dissolving, HCl had to be added to a final concentration of 20 mM. The LGRs were dissolved in DMSO at 70 mg/ml and diluted with sterile PBS to 3 mg/ml. For complete dissolving of LGR561, HCl had to be added to a final concentration of 10 mM. For each application, 200 µl of the solutions were injected intra-peritoneally. For each time, mice received 100mg/kg body weight of roscovitine or 30 mg/kg body weight of the LGR compounds, respectively.

Quantification of the neovascularization:

On postoperative day 6, vascularization was quantified under a stereo microcope, using a custom made calibrated ocular scale. Vessel length (VL) was defined as the length of the longest vessel emanating from the limbus towards the pellet. Clock hours (CH) were defined

as the fraction of limbal circumference giving rise to vessels in 1/12th units of the whole perimeter. Vascularized area was calculated as 1/2π x VL x 0.4CH, approximating the shape to a half ellipse, and taking the perimeter of the cornea as 9.6 mm. Eyes with inflammation, destroyed pellet (possibly owed to scratching by the animal), or operation error were omitted from the analysis. Pellets without bFGF but in every other respect identically created and implanted were used to verify that the procedure itself causes no vascularization.

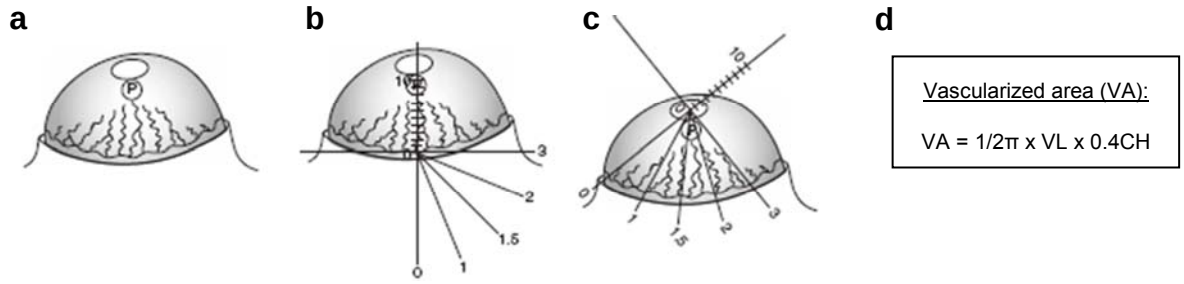


Figure 26 Assessment of corneal neovascularization. (a) The image displays corneal neovascularization. (b) Measurement of vessel length (VL). (c) Analysis of clock hours (CH, right picture) (d) Calculation of the Vascularized Area from VL and CH (the formula is displayed in the figure). The image is adapted from Rogers *et al*.[80]

3.4 Western Blot analysis

Western blot analysis is an extensively used technique to identify specific proteins in various protein mixtures, e.g. cell lysates or tissue homogenates. It includes the electrophoretic separation of proteins accordingly to their molecular weights, their transfer to a membrane ("blotting"), and their visualization by immunodetection.

3.4.1 Preparation of samples

Endothelial cells were treated as indicated, washed once with icecold PBS and subsequently lyzed in Ripa lysis buffer. Immediately, cells were frozen at -85°C. Afterwards, cells were scraped off and transferred to Eppendorf tubes (Peske, Aindling-Arnhofen, Germany) before centrifugation (14,000 rpm, 10 min, 4°C). Protein concentration was determined using the BCA or the Bradford assay, respectively. Afterwards, Laemmli sample buffer (3x) was added and samples were heated at 95°C for 5 min. The reducing agent β-Mercaptoethanol cleaves disulfide bonds and boiling leads to complete denaturation of the proteins. SDS is an anionic detergent that attaches to hydrophobic parts of the proteins, resulting in permanent negatively charged proteins. Samples were kept at -20°C until Western blot analysis.

Ripa lysis buffer

NaCl	150	mM
Tris	50	mM
Nonidet P-40	1	%
Deoxycholat	0.25	%
SDS	0.10	%
H_2O		
add before use:		
Complete™	4	mM
PMSF	1	mM
NaF	1	mM
activated Na_2VO_3	1	mM

Laemmli sample buffer (3x)

Tris-HCl	187.5	mM
SDS	6	%
Glycerol	30	%
Bromphenolblue	0.025	%
H_2O		
add before use:		
β-mercaptoethanol	12.5	%

3.4.2 SDS-PAGE electrophoresis

Proteins were separated by discontinuous SDS-polyacrylamid gel electrophoresis (SDS-PAGE) according to Laemmli.[83] Equal amounts of protein were loaded on gels and separated using the Mini-PROTEAN 3 electrophoresis module (Bio-Rad, Munich, Germany). Discontinuous polyacrylamide gels were used consisting of separation and stacking gel. The concentration of Rotiophorese™ Gel 30 (acrylamide) in the separating gel was adjusted for an optimal separation of the proteins depending on their molecular weights (Table 2). Electrophoresis was carried out at 100 V for 21 min for protein stacking and 200 V for 45 min for protein separation. The molecular weight of proteins was determined by comparison with the prestained protein ladder (PageRulerTM, Fermentas, St. Leon-Rot, Germany).

Table 2 Acrylamide concentration in the separation gel

Protein	**acrylamide concentration**	
Cdk2, Cdk5, ERK, $p27^{kip1}$, RhoA, Rac1	15	%
β-tubulin, AKT	12	%
FAK	10	%

Separation gel 10/12/15 %

Rotiphorese™ Gel 30	33.3/40/50	%
Tris (pH 8.8)	375	mM
SDS	0.1	%
TEMED	0.1	%
APS	0.05	%
H_2O		

Stacking gel

Rotiphorese™ Gel 30	40	%
Tris (pH 6.8)	125	mM
SDS	0.1	%
TEMED	0.2	%
APS	0.1	%
H_2O		

Electrophoresis buffer

Tris	4.9	mM
Glycine	38	mM
SDS	0.1	%
H_2O		

3.4.3 Electroblotting

After protein separation, proteins were transferred onto either PVDF or nitrocellulose membranes by electroblotting. Electroblotting, also denoted as Western blotting, is the most commonly used method to transfer proteins from a gel to a membrane.[84] The protein transfer can be achieved either by placing the gel-membrane sandwich between absorbent paper soaked in transfer buffer (semi-dry transfer) or by complete immersion of the gel-membrane sandwich in a buffer (wet transfer). In the present work semi-dry transfer has been used.

Semi-dry transfer:

Using a Transblot SD semidry transfer cell (Bio-Rad, Hercules, USA), the separated proteins were electrophoretically transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) or to a nitrocellulose membrane (Hybond ECl, Amersham Biosciences, NJ, USA). Prior to blotting, the membrane was incubated for at least 30 min in Anode buffer on a shaking platform. For semi-dry transfer, the gel-membrane sandwich is placed between carbon plate electrodes. Therefore, one sheet of thick blotting paper (Whatman Schleicher & Schüll, Dassel, Germany) was soaked with Anode buffer and rolled onto the anode. Subsequently, the membrane and the gels were added. Finally the stack was covered with

another sheet of thick blotting paper soaked with Cathode buffer. The transfer cell was closed and transfer was carried out at 15 V for 1 h.

Anode buffer		
Tris	12	mM
CAPS	8	mM
Methanol	15	%
H_2O		

Cathode buffer		
Tris	12	mM
CAPS	8	mM
SDS	0.1	%
H_2O		

3.4.4 Protein detection

3.4.4.1 Specific protein staining with antibodies

Prior to the immunological detection of the relevant proteins, unspecific protein binding sites were blocked. Therefore, the membrane was incubated in Blotto 5% or BSA 5% for 2 h at room temperature. Afterwards, detection of the proteins was performed by incubating the membrane with the respective primary antibody at 4°C overnight. After four washing steps (each 5 min.) with PBS containing 0.1% Tween (PBS-T), the membrane was incubated with the secondary antibody, followed by 4 additional washing steps. All steps regarding the incubation of the membrane were performed under gentle agitation.
In order to visualize the proteins, two different methods have been used depending on the labels of secondary antibodies.

Antibodies directly labeled with infrared (IR) fluorophores:

Secondary antibodies coupled to IRDye^{TM}800 and Alexa Fluor$^{®}$ 680 with emission at 800 and 700 nm, respectively, were used. Membranes were incubated for 1 h. Protein bands of interest were detected using the Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE). After scanning the membrane with two-color detection, bands could be quantified using Odyssey software.

Antibodies coupled to horseradish peroxidase (HRP)

Membranes were incubated for 2 h with HRP-conjugated secondary antibodies. For detection, luminol was used as a substrate. The membrane was incubated in a 1:0.025 mixture of ECL solution A and B for 1 minute (ECL Plus Western Blotting Detection Reagent RPN 2132, GE Healthcare, Munich, Germany). The enzyme HRP catalyzes the oxidation of luminol in the presence of H_2O_2 (Figure 27). The appearing luminescence was detected by exposure of the membrane to an X-ray film (Super RX, Fuji, Düsseldorf, Germany) and subsequently developed with a Curix 60 Developing system (Agfa-Gevaert AG, Cologne, Germany).

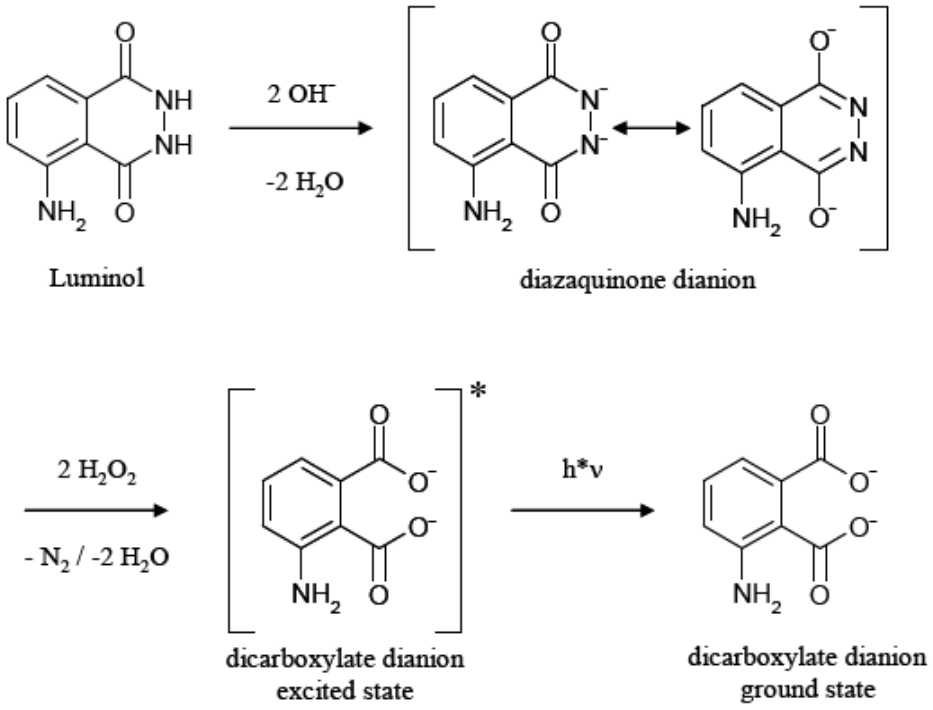


Figure 27 HRP-Luminol reaction

Primary antibodies used for protein detection are listed in Table 3; secondary antibodies are displayed in Table 4.

Table 3 Primary antibodies.

Antigen	Isotype	Dilution	in	Provider
Actin	mouse monoclonal	1:1,000	Blotto 1 %	Chemicon
AKT	rabbit polyclonal	1:1,000	Blotto 1 %	Cell Signaling
AKT phospho	mouse monoclonal	1:1,000	Blotto 1 %	Cell Signaling
β-tubulin	mouse monoclonal	1:500	Blotto 1 %	Santa Cruz
Cdk2	rabbit polyclonal	1:1,000	Blotto 1 %	Santa Cruz
Cdk5	mouse monoclonal	1:1,000	Blotto 1 %	Santa Cruz
ERK	rabbit polyclonal	1:1,000	Blotto 1 %	Cell Signaling
ERK phospho	mouse monoclonal	1:1,000	Blotto 1 %	Cell Signaling
FAK	mouse monoclonal	1:1,000	BSA 1 %	Santa Cruz
FAK phosphoSer732	rabbit polyclonal	1:1,000	BSA 1 %	Biosource
FAK phosphoTyr397	rabbit polyclonal	1:1,000	BSA 1 %	Santa Cruz
$p27^{kip1}$	mouse monoclonal	1:1,000	Blotto 1 %	Biosource
Rac1	mouse monoclonal	1:1,000	Blotto 3 %	Upstate
RhoA	mouse monoclonal	1:250	Blotto 5 %	Upstate

Table 4 Secondary antibodies.

Antibody	Dilution	in	Provider
Goat anti-mouse IgG1: HRP	1:1,000	Blotto 1%	Biozol
Goat anti mouse IgG2b: HRP	1:1,000	Blotto 1%	Southern Biotechnology
Goat anti-rabbit: HRP	1:1,000	Blotto 1%	Dianova
Alexa Fluor® 680 Goat anti-mouse IgG	1:10,000	Blotto 1%	Molecular Probes
Alexa Fluor® 680 Goat anti-rabbit IgG	1:10,000	Blotto 1%	Molecular Probes
IRDye™ 800 Goat anti-rabbit IgG	1:10,000	Blotto 1%	Rockland

3.4.4.2 Unspecific protein staining of gels and membranes

In order to ensure equal protein loading and blotting efficiency, gels as well as membranes were stained with Coomassie or Ponceau staining solution, respectively.

Staining of gels with Commassie staining solution:

After transfer, gels were incubated with Coomassie staining solution for 20 min. The dye penetrates the gel and stains all proteins without any specification. Afterwards, gels were extensively washed with Coomassie destaining solution for 60 min (6x, 10 min) until proteins appeared as blue bands.

Coomassie staining solution

Coomassie blue G	0.3	%
Glacial acetic acid	10	%
Ethanol	45	%
H_2O		

Coomassie destaining solution

Glacial acetic acid	10	%
Ethanol	33	%
H_2O		

Staining of membranes with Ponceau solution:

Membranes were incubated with Ponceau solution on a shaking platform for 5 min and were washed with water until the background disappeared.

Ponceau solution

Ponceau S	0.1	%
Glacial acetic acid	5	%
H_2O		

3.4.5 Membrane stripping and reprobing

In order to remove primary and secondary antibodies from the membrane (“stripping”), blots were incubated twice in stripping buffer for 15 min at room temperature. After extensive washing, stripping efficiency was confirmed by scanning the membrane to see if signals have been removed. Subsequently, the blot was re-blocked with Blotto 5% for 2 h and incubated with antibodies.

Stripping buffer (pH 2.0)

Glycine	25	mM
SDS	0.1	%
H_2O		

3.5 Protein quantification

In order to employ equal amounts of proteins in all samples for Western Blot analysis, protein concentrations were determined using either Bicinchoninic Protein Assay or Bradford Assay. After measurement, protein concentration was adjusted by adding Laemmli sample buffer (1x).

3.5.1 Bicinchoninic (BCA) Protein Assay

Bicinchoninic (BCA) Protein Assay (BC Assay reagents, Interdim, Montulocon, France) was performed as described previously.[85] 10 µl protein samples were incubated with 200 µl BC Assay reagent for 30 min at 37°C. Absorbance of the blue complex was measured photometrically at 550 nm (Tecan Sunrise Absorbance reader, TECAN, Crailsheim, Germany). Protein standards were obtained by diluting a stock solution of Bovine Serum Albumin (BSA, 2 mg/ml). Linear regression was used to determine the actual protein concentration of each sample.

3.5.2 Bradford Assay

Bradford Assay (Bradford solution, Bio-Rad, Munich, Germany) was performed as described previously.[86] It employes Coomassie Brillant Blue as a dye, which binds to proteins. 10 µl protein samples were incubated with 190 µl Bradford solution (1:5 dilution in water) for 5 min. Thereafter, absorbance was measured photometrically at 592 nm (Tecan Sunrise Absorbance reader, TECAN, Crailsheim, Germany). Protein standards were achieved as described above (BCA Assay).

3.6 Transfection of cells

Transfection refers to the introduction of genetic material into cultured mammalian cells. Genetic material (i.e. plasmid DNA or siRNA constructs) can be transfected using calcium phosphate, electroporation, lipofection or magnetofection.

For transient transfection with the indicated siRNAs or plasmids, respectively, HUVECs were electroporated using the Nucleofector® II device in combination with the HUVEC Nucleofector®Kit (both from Amaxa, Cologne, Germany).

Transfection with siRNA

In order to silence Cdk2 or Cdk5, respectively, HUVECs were transiently transfected with Cdk2 as well as Cdk5 siRNA. Two On-TARGETplus Individual Duplexes were used for each Cdk (Dharmacon, Lafayette, CO, USA). On-Targetplus siCONTROL Non-targeting siRNA was used as a control. SiRNAs were suspended in Dharmacon 1x siRNA buffer, aliquoted and stored at -80°C. The concentration of siRNA was verified using a NanoDrop (Wilmington, DE, USA).
For each transfection, $2x10^6$ HUVECs were suspended in 100 µl HUVEC Nucleofector Solution and added to 3 µg of the respective siRNA:

Cdk2 siRNA:

1.5 µg siRNA Duplex J-003236-11 (sequence: 5'-PUAUUAGGAUGGUUAAGCUCUU-3')
1.5 µg siRNA Duplex J-003236-12 (sequence: 5'-PUCUCCCGUCAACUUGUUUCUU-3')

Cdk5 siRNA:

1.5 µg siRNA Duplex J-003239-09 (sequence: 5'-PACAUCGGAUAGGGCUUAUAUU-3')
1.5 µg siRNA Duplex J-003239-10 (sequence: 5'-PGAUCUCAUGAGUCUCCCGGUU-3')

Non-targeting (nt) siRNA (transfection control):

3.0 µg ON-TARGETplus siCONTROL Non-targeting (nt) siRNA D-001810-01
(sequence: 5'-UGGUUUACAUGUCGACUAA-3')

The mixture of cells and siRNA was transferred to an amaxa certified cuvette and transfection was performed (program A-034). Immediately after electroporation, 900 µl of prewarmed culture medium was added to the cuvette. Afterwards, cells were seeded into 24-well

(500,000 cells per well) for scratch assays, in 6-well (500,000 cells per well) plates for Western Blot analysis, or into ibidi µ-slides (8-well ibiTreat, ibidi GmbH, Munich, Germany, 250,000 cells per well) for staining experiments. Scratch assays were performed 32 h after transfection for 16 h. Transfection efficiency was checked 24 and 48 h after transfection by Western Blot analysis. For staining experiments, cells were scratched 32 h after transfection and stained after migration for 16 h.

Transfection with DNA-constructs

To visualize the indicated proteins, HUVECs were transiently transfected with plasmids for eYFP-Rac1, eYFP-vinculin, eGFP-CLIP170, and eGFP-tubulin. eGFP CLIP-170 was kindly provided by N. Galjart (Rotterdam, Netherlands), eYFP-vinculin by A. Bershadsky (Rehovot, Israel), and eGFP-tubulin by Stefan Linder (Munich, Germany). eYFP-Rac1 was from ATCC/Promochem, Wesel, Germany.

For each transfection, $1x10^6$ HUVECs were suspended in 100 µl HUVEC Nucleofector Solution and added to 7 µg of the respective plasmid. Electroporation was performed analogical to the siRNA experiments. After transfection, cells were seeded into ibidi µ-slides (8-well ibiTreat, ibidi GmbH, Munich, Germany, 250,000 cells per well) or in 24 well plates (250,000 cells per well). Experiments were performed 48 h after transfection.

3.7 Confocal laser scanning microscopy

Confocal laser scanning microscopy is an extensively used method in life sciences due to its great advantages. The key feature of the confocal microscopy is that it only captures light reflected or emitted by a single plane of a specimen. Out of focus light is filtered out so that only light coming from in-focus objects can reach the detector. A laser beam scans the specimen pixel by pixel and line by line. Afterwards, data are assembled, resulting in an image that represents an optical section through the specimen. This results in high-quality images with high contrast and maximum resolution, gives information about colocalization of signals from different fluorochromes, and allows three-dimensional reconstructions of thick specimens.[87]

A Zeiss LSM 510 META confocal microscope (Figure 28) equipped with a heating stage from EMBLem (Heidelberg, Germany) was used for obtaining images of fixed cells as well as for life cell imaging experiments.

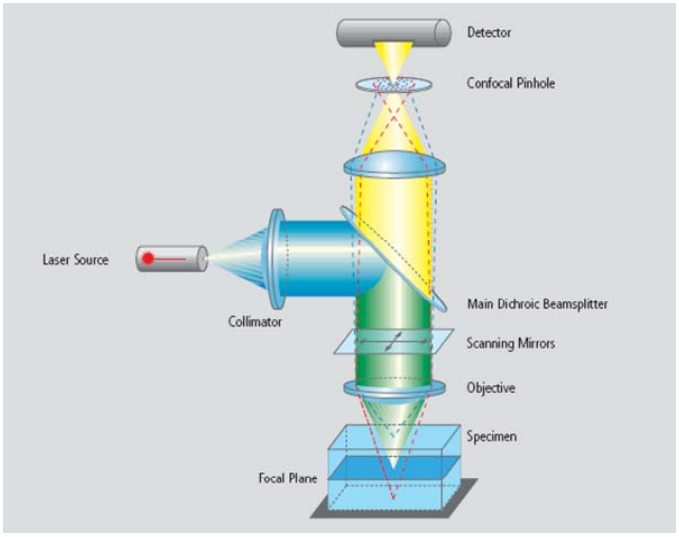


Figure 28 **Beam path in a confocal Laser Scanning Microscope** (adapted from Zeiss AG, [87]).

3.7.1 Microscopy with fixed cells

HUVECs were cultured in ibidi µ-slides (8-well ibiTreat, ibidi GmbH, Munich, Germany) until reaching confluency. Afterwards, cells were scratched, treated as indicated, and allowed to migrate. After 16 h, cells were washed with PBS and fixed with Cytoskelfix Cell fixative solution (Cytoskeleton, Denver, CO) containing 0.2% Triton X-100 (Sigma, Taufkirchen, Germany) at -20°C for 4 min. After washing with PBS for 10 min, unspecific binding was blocked by incubation with 0.2 % bovine serum albumin (BSA) solution for 30 min. Afterwards, cells were incubated with the respective primary antibody for 1 h at room temperature (Table 5).

Table 5 **Primary antibodies used for confocal microscopy.**

Antigen	Isotype	Dilution	in	Provider
Cortactin	rabbit polyclonal	1:100	BSA 0.2 %	Cell Signaling
Rac1	mouse monoclonal	1:100	BSA 0.2 %	Upstate

Following three washing steps with PBS, cells were incubated with the respective secondary antibodies or with rhodamin/phalloidin for staining of F-actin, respectively, for 30 min. at room temperature (Table 6).

Table 6 Secondary antibodies / dye used for confocal microscopy.

Antibody/Dye	Dilution	in	Provider
AlexaFluor488 goat-anti-mouse	1:400	BSA 0.2 %	Molecular Probes
AlexaFluor546 goat-anti-rabbit	1:400	BSA 0.2 %	Molecular Probes
Rhodamin/phalloidine	1:400	BSA 0.2 %	Molecular Probes

Finally, preparates were again washed three times with PBS (5 min) and embedded in FluorSave aqueous mounting medium (VWR, Darmstadt, Germany).

3.7.2 Life cell imaging

Life cell imaging was performed to visualize the dynamics of single cells during migration and spreading, as well.
HUVECs were transfected with 7 µg of the indicated plasmids as displayed in Table 7.

Table 7 Plasmids used for life cell imaging experiments.

Plasmid	visualization of	Provider
eGFP-CLIP170	microtubule +tips	N. Galjart, Rotterdam, Netherlands
eGFP-tubulin	microtubules	S. Linder, Munich, Germany
eYFP-Rac1	Rac1/lamellipodia	ATCC/Promochem, Wesel, Germany
eYFP-vinculin	focal adhesions	A. Bersadsky, Rehovot, Israel

Experiments with migrating cells:

After transfection with the plasmids as indicated, HUVECs were seeded into ibidi µ-slides (8-well ibiTreat, 250,000 cells per well). After 48 h, cells were scratched, treated as indicated and life cell imaging was performed for 60 min during migration.

Experiments with spreading cells:

After transfection with the plasmids as indicated, HUVECs were seeded into 24-well-plates (250,000 cells per well). After 48 h, cells were trypsinized, resuspended in growth medium containing the indicated stimuli, and plated into ibidi µ-slides (8-well ibiTreat). Life cell imaging was performed for 60 min during cell spreading.

3.7.3 Histological staining

Human umbilical cords were frozen at -20°C. Cryosections with a size of 20 µm were freshly prepared and applied onto glass objective slides. After drying (30 min), sections were reconstituted with PBS (10 min) and blocked with 0.2 % BSA in PBS (15 min). Afterwards, cells were incubated with the respective primary antibody for 1 h at room temperature (Table 8).

Table 8 Primary antibodies used for histological staining.

Antigen	Isotype	Dilution	in	Provider
Cdk5	mouse monoclonal	1:50	BSA 0.2 %	Santa Cruz
VE cadherin	rabbit polyclonal	1:400	BSA 0.2 %	Cell Signaling

Following three washing steps with PBS, cells were incubated with the respective secondary antibodies or with bisBenzimide H33342 trihydrochloride for staining of nuclei, respectively, for 30 min. at room temperature (Table 9).

Table 9 Secondary antibodies / dye used for confocal microscopy.

Antibody/Dye	Dilution	in	Provider
AlexaFluor488 goat-anti-mouse	1:400	BSA 0.2 %	Molecular Probes
AlexaFluor546 goat-anti-rabbit	1:400	BSA 0.2 %	Molecular Probes
bisBenzimide H33342 trihydrochloride	1:200	BSA 0.2 %	Sigma Aldrich

Finally, preparates were again washed three times with PBS (5 min), embedded in FluorSave aqueous mounting medium (VWR, Darmstadt, Germany), and covered with cover slips.

3.8 Flow Cytometry (FACS)

Flow cytometry allows counting, sorting, and analysis of various parameters of single cells or particles suspended in a fluid. Each cell passes a focused laser beam and scatters the illuminating light. If particles have previously been stained with a fluorescent dye, the same time fluorescence emission occurs and can be detected.
Flow cytometry has been used for the analysis of cell cycle and apoptosis. All measurements were performed on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Cells were illuminated by a blue argon laser (488 nm).

Cell cycle analysis and quantification of apoptosis rate

Cell cycle analysis and quantification of apoptosis rate was performed according to Nicoletti *et al.* [88]
Cells were seeded in 12-well plates and either left untreated or stimulated with roscovitine, LGR561, LGR848, or LGR849 in the indicated concentrations. After 48 h, cells were trypsinized, washed three-times with PBS, and centrifugated at 600 g and 4°C for 10 min. For permeabilization and staining, cells were incubated in hypotonic fluorochrome solution (HFS) buffer containing propidium iodide (PI). PI is a red fluorescent dye that intercalates with doublestranded DNA, detecting the DNA content of the cell. After incubation at 4°C overnight cells were analyzed by flow cytometry.

FACS buffer (pH 7.4)

NaCl	138.95	mM
K_2HPO_4	1.91	mM
NaH_2PO_4	16.55	mM
KCl	3.76	mM
LiCl	10.14	mM
NaN_3	3.08	mM
Na_2EDTA	0.97	mM
H_2O		

HFS buffer

Propidium iodide	75	nM
Sodium citrate	0.1	%
Triton X-100	0.1	%
PBS		

The respective fluorescence intensity gives information about the DNA content of a cell, and thus, about rate of apoptosis and cell cycle phases.

Apoptotic cells are characterized by DNA fragmentation, indicated by a low fluorescence. Thus, they appear "left" to the G_1/G_0 peak in the histogram plot ("sub-G1 peak") (Figure 29).

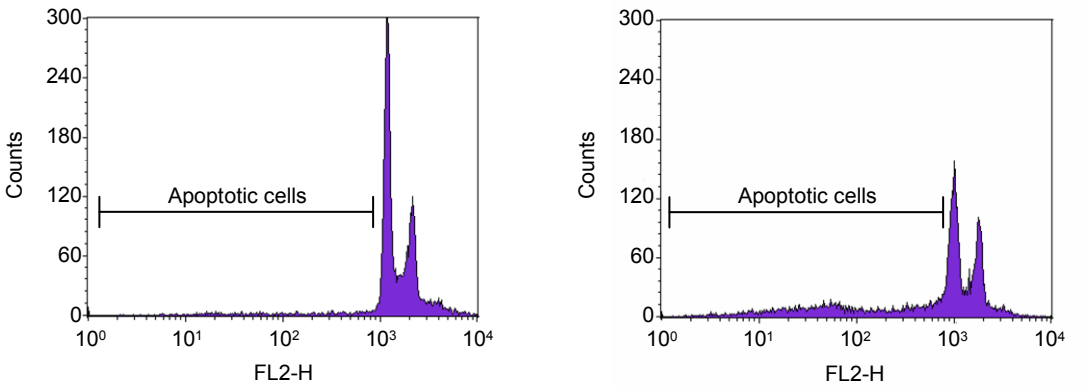


Figure 29 **Analysis of apoptotic cells.** In the left panel, untreated cells are displayed. The right panel refers to cells treated with LGR561 (100 µM) which show increased rate of apoptosis (sub-G_1 peak in the histogram plot).

The cell cycle consists of mitosis (M phase) and interphase. The interphase is subdivided into G_1/G_0-phase, S-phase, and G_2-phase, characterized by their DNA contents. Cells in G_1/G_0-phase exhibit haploid DNA contents ("normal" fluorescence), cells in G_2-phase diploid DNA contents ("high" fluorescence), and cells in S-phase DNA contents somewhere between. The respective fluorescence intensities of cell populations result in characteristic histogram plots (Figure 30).

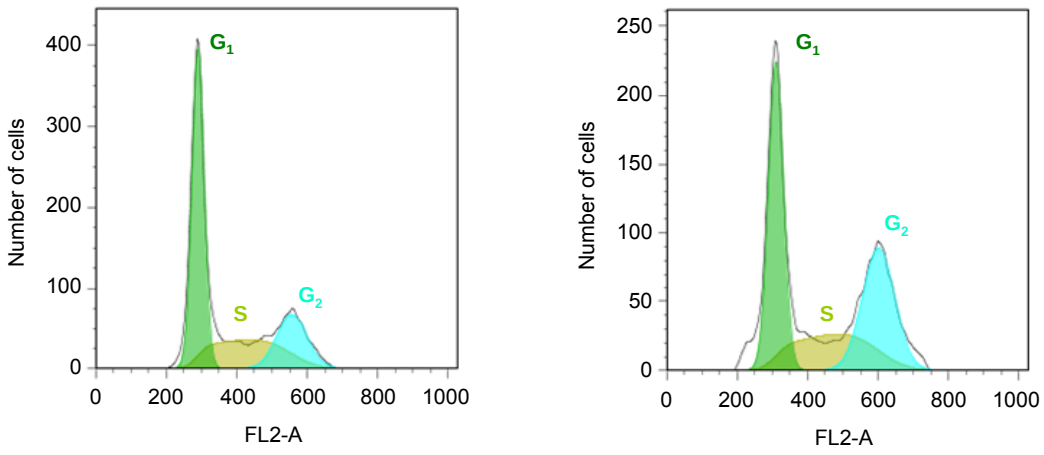


Figure 30 **Cell cycle analysis.** The Histogram plot in the left panel refers to untreated cells (normal division of cells in the respective phases). The Histogram plot in the right panel displays cells treated with LGR561 (100 µM). They are arrested in the G_2-phase of the cell cycle.

3.9 Tubulin fractionation

HUVECs were plated into 6 cm^2 dishes. After reaching confluency, cells were either left untreated (control) or treated with roscovitine (30 μM), taxol (1 μM), or vinblastine (10 nM), respectively. After 4 h, cells were lysed using PIPES lysis buffer. After incubation for 20 min. at room temperature, lysates were centrifuged for 45 min with 47,000 rpm (Optima™ TLX Ultracentrifuge, Beckman Coulter, CA, USA). Afterwards, the supernatant containing unpolymerized tubulin and the pellet containing polymerized tubulin were separated. The supernatant was mixed with Laemmli sample buffer (3x) containing β-mercaptoethanol (1:9) and boiled for 5 min. The pellet was incubated with 40 μl $CaCl_2$ buffer at 4°C for 60 min, mixed with Laemmli sample buffer (3x) containing β-mercaptoethanol (1:9) and boiled for 5 min as well. For the detection of β-tubulin, Western Blot analysis was performed.

PIPES lysis buffer (pH 6.9)

PIPES	100	mM
Glycerol	2	M
Triton X-100	0.5	%
$MgCl_2$	2	mM
EGTA	2	mM
H2O		
add before use:		
Taxol	5	μM
GTP	1	mM
PMSF	1	mM
Complete™	4	mM

$CaCl_2$ buffer (pH 6.8)

Tris/HCl	100	mM
$MgCl_2$	1	mM
$CaCl_2$	10	mM
H2O		

3.10 Pull-down assay

In a Pull-down assay, the activation state of GTPases can be analyzed. The assay is based on the principle that only the active form of the GTPase interacts with its specific downstream effectors. The GTPase-binding domains from these downstream effectors are expressed as recombinant glutathione S-transferase (GST) fusion proteins immobilized on glutathione resin and can be used for affinity precipitation (pull-down) of the active GTPase from cell lysates. The N-terminal regulatory region in p21-activated protein kinase 1 and 2 (Pak1 and 2) contains the p21-binding domain (PBD) for Rac and Cdc42.[9] The N-terminal region of Rhotekin contains the Rho-binding domain (RBD).[10] Pulled-down active GTPases are eluted from the resin and detected by immunoblotting with a specific antibody (Figure 31).

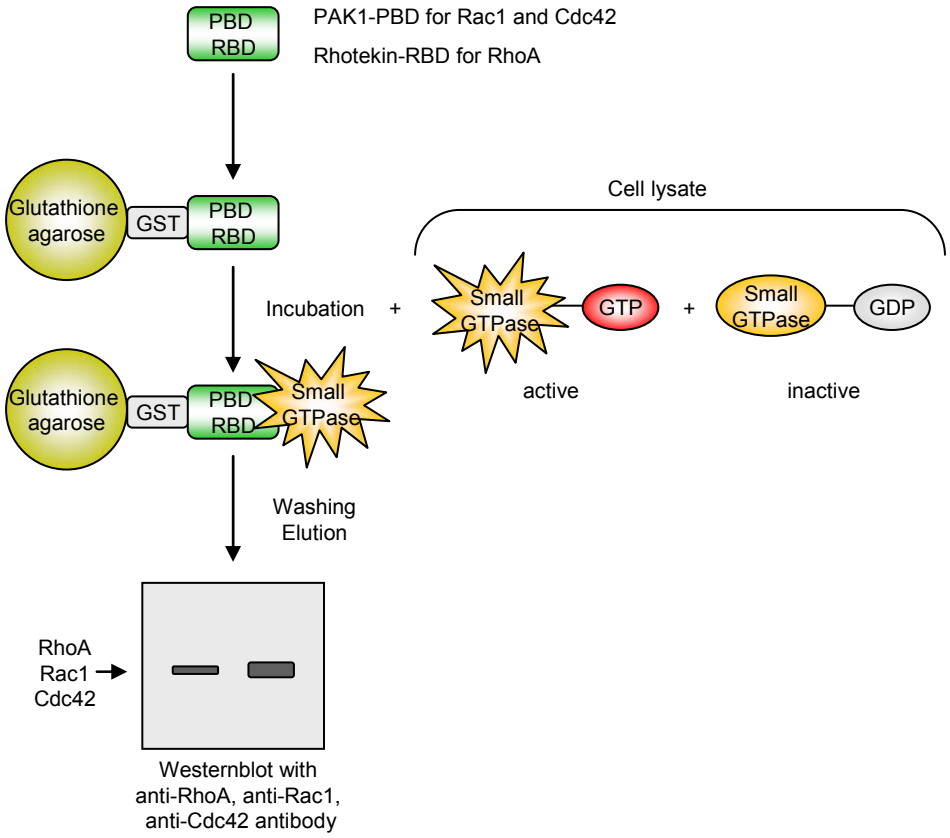


Figure 31 **Principle of a Pull-down assay**.

Pull-down assays were performed using the RhoA Activation Assay Kit 17-294 and Rac1/Cdc42 Activation Assay Kit 17-441 (both from Millipore, Billerica, MA, USA).

HUVECs were trypsinized, suspended in growth medium (control) or growth medium containing roscovitine (30 µM) and subsequently plated into two 100 cm^2 dishes (about 6 mio

cells per sample). After 30 min, cells were lyzed using Mg^{2+} lysis/wash buffer (MLB, 250 µl per dish), scraped off, and transferred to Eppendorf tubes before centrifugation (14,000 rpm, 10 min, 4°C). For adjustment of protein contents of the respective samples, protein concentration was determined using the Bradford-assay. Afterwards, 10 µg of the Rac1/Cdc42 assay reagent (PAK1 PBD agarose) or 20 µg of the Rho assay reagent (Rhotekin RBD agarose), respectively, were added to 1.0 ml of each lysate. The mixtures were incubated at 4°C for 1 h with gentle agitation. After separation of beads and supernatant, the supernatant was mixed with Laemmli sample buffer (2x) containing β-Mercaptothanol (1:9) and boiled for 5 min at 95°C. The beads were washed three times with 500 µl MLB and resuspended in Laemmli sample buffer (2x) containing β-Mercaptothanol (1:9) and boiled for 5 min at 95°C. For Western Blot analysis, 40 µl of the samples were loaded onto gels.

3.11 Adhesion assay

24-well plates were either left uncoated or coated with fibronectin (25 µg / ml), Collagen (0,001% in PBS) or Matrigel® (10% in serum free medium) for 2 h at 37°C. After washing with PBS, the plates were blocked with 0.2 % bovine serum albumin (BSA) solution for 1 h and again washed with PBS.

HUVECs were trypsinized, suspended in ECGM or ECGM containing roscovitine (30 µM), and $1x10^6$ cells per well were plated into 24 well plates which were prepared before. After 30 min., cells were stained with 300 µl crystal violet solution for 10 min. After washing five times with distilled water, the bound dye was solubilized by adding 300 µl of the dissolving buffer. The absorbance was measured at 540 nm in a plate-reading photometer (SPECTRAFluor Plus; Tecan, Crailsheim, Germany).

3.12 Statistical analysis

All experiments were performed at least three times unless otherwise indicated in the figure legend. Data are expressed as mean ± SEM. Statistical analysis was performed with SigmaStat software version 3.1 (Aspire Software International). Statistical tests are indicated in the figure legend. Statistical significance is assumed if $p \leq 0.05$.

4 RESULTS

4.1 Roscovitine, LGR561, LGR848, and LGR849 exert anti-angiogenic effects *in vitro* and *in vivo*

Angiogenesis involves a cascade of events with the endothelium playing an important role. Thus, to elucidate possible anti-angiogenic effects of the Cdk inhibitor roscovitine and its derivatives, we analyzed their influence on endothelial cell proliferation, migration and tube formation, which represent major steps in the angiogenic cascade.

4.1.1 Roscovitine and the LGRs inhibit endothelial cell proliferation

The influence of roscovitine and the LGRs on endothelial cell proliferation was analyzed by crystal violet staining. All compounds dose-dependently reduced HUVEC proliferation. The LGRs, especially LGR561 and LGR849, reduced HUVEC proliferation much more potently than roscovitine. The calculated EC50 for each compound is indicated in the respective diagram (Figure 32).

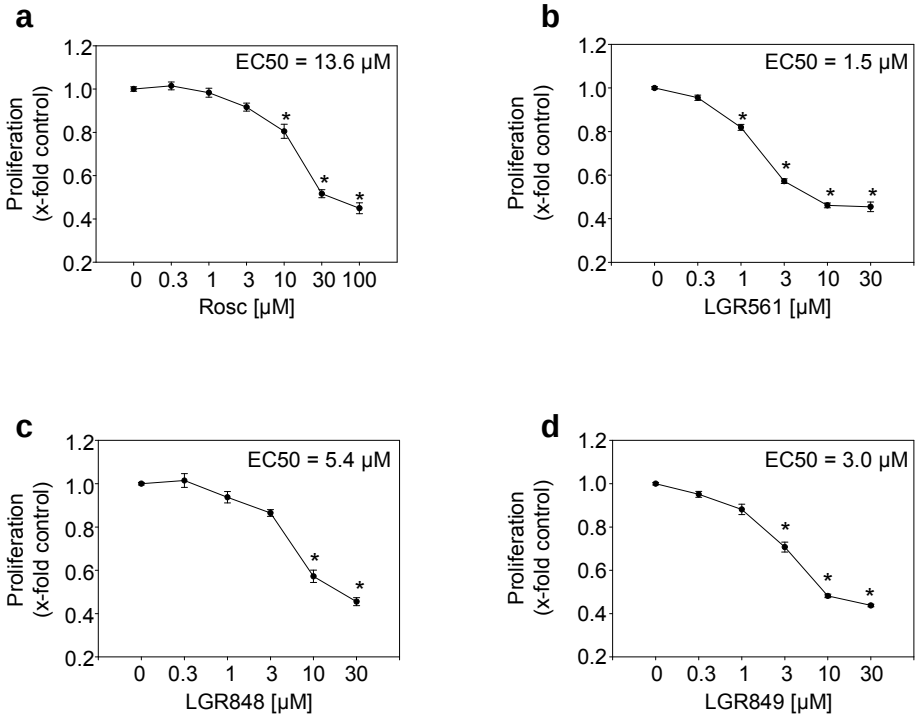


Figure 32 Roscovitine and the LGRs dose-dependently reduce endothelial cell proliferation. HUVECs were either left untreated or treated with the indicated compounds in increasing concentrations. After 72 h, cell proliferation was determined by staining of cells with crystal violet (Kruskal-Wallis One Way ANOVA on Ranks, * $p<0.05$). EC50 values were calculated using the SigmaStat software.

4.1.2 Effects of roscovitine and the LGRs on cell cycle and apoptosis

In order to understand the mechanism by which the compounds inhibit cell growth, we investigated their effects on cell cycle and apoptosis by flow cytometry analyses. The cell cycle comprises mitosis (M-phase) and interphase, subdivided into G_1/G_0-, S-, and G_2-phase, characterized by the DNA content of the cell. The G_1/G_0 -phase exhibits the haploid, the G_2-phase the diploid DNA content.

Treatment of proliferating endothelial cells with increasing concentrations of all compounds resulted in a cell cycle arrest in the G_2-phase. In detail, roscovitine caused a significant cell cycle arrest at a concentration of 100 µM. In comparison, only 10 µM of the two (S)-stereoisomers LGR561 and LGR849 significantly inhibited cell cycle progression. The (R)-stereoisomer LGR848 significantly inhibited cell cycle progression at a concentration of 100 µM (Figure 33).

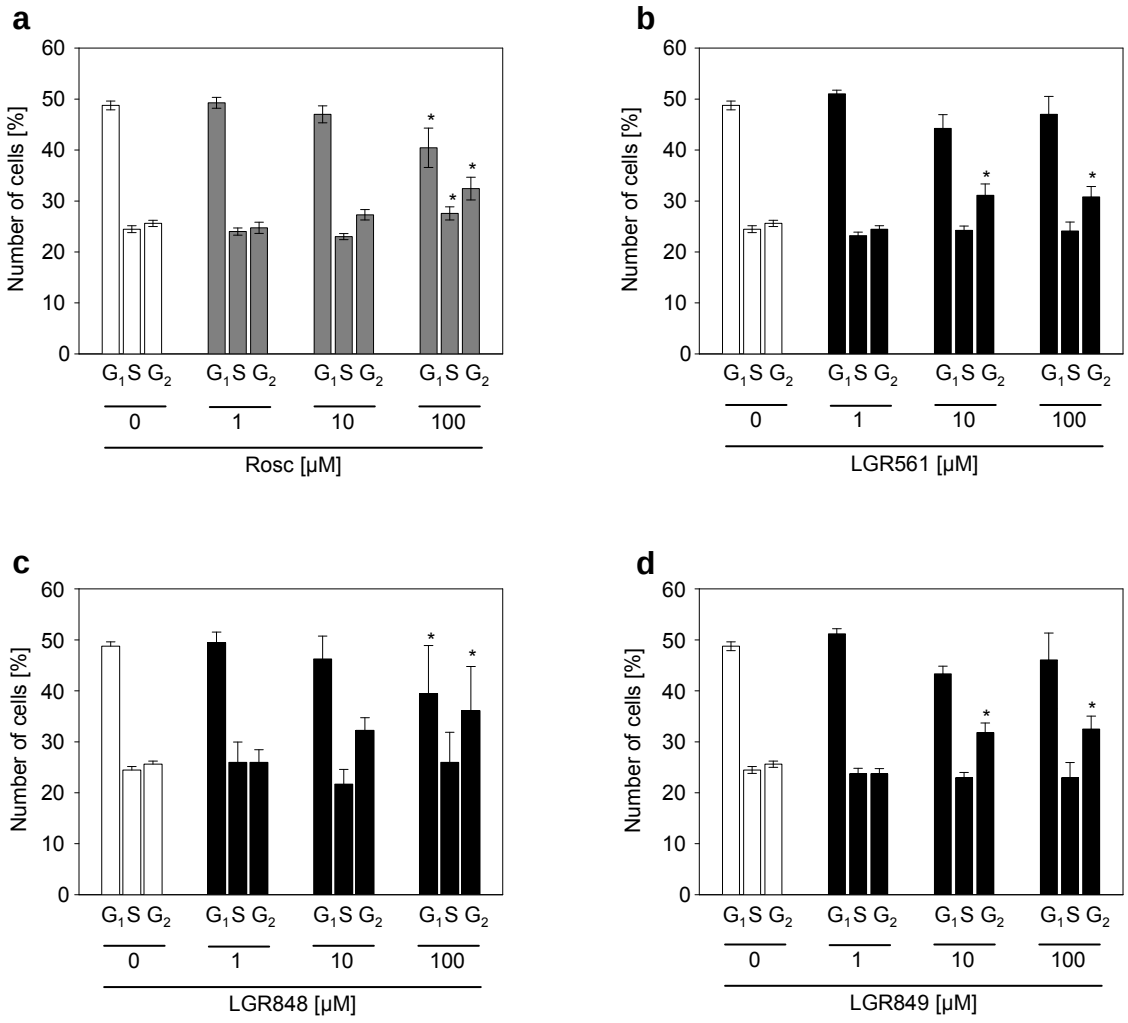


Figure 33 Roscovitine and the LGRs induce cell cycle arrest in G2-phase. Proliferating HUVECs were either left untreated or treated with increasing concentrations of roscovitine (Rosc), LGR561, LGR848, and LGR849. After 48 h, cells were stained with PI and the DNA content was determined by FACS analysis. (One Way ANOVA, Holm-Sidak * $p<0.05$).

Moreover, roscovitine and the LGRs dose-dependently increased apoptosis in proliferating endothelial cells (Figure 34). Roscovitine significantly increased apoptosis of HUVECs at a concentration of 100 µM, just as well as LGR848. The two S-isomers LGR561 and LGR849 again were more powerful: they significantly induced apoptosis at a concentration of 10 µM. (Figure 34)

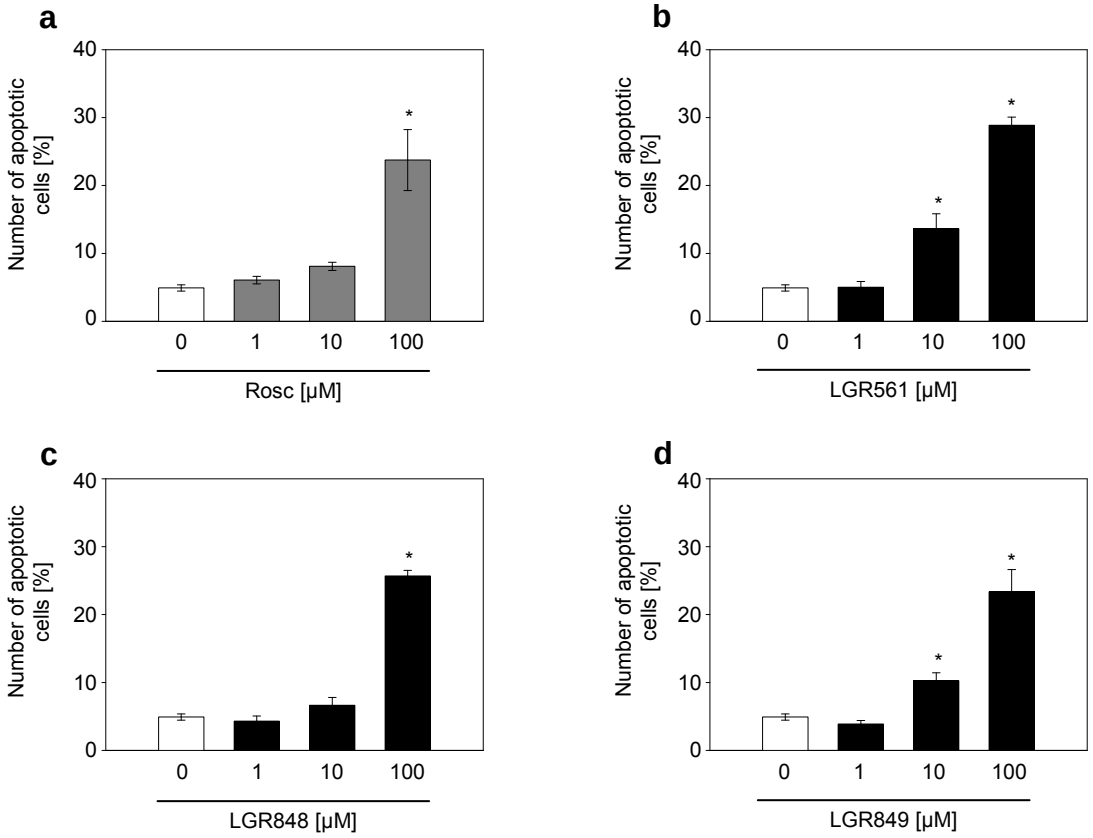
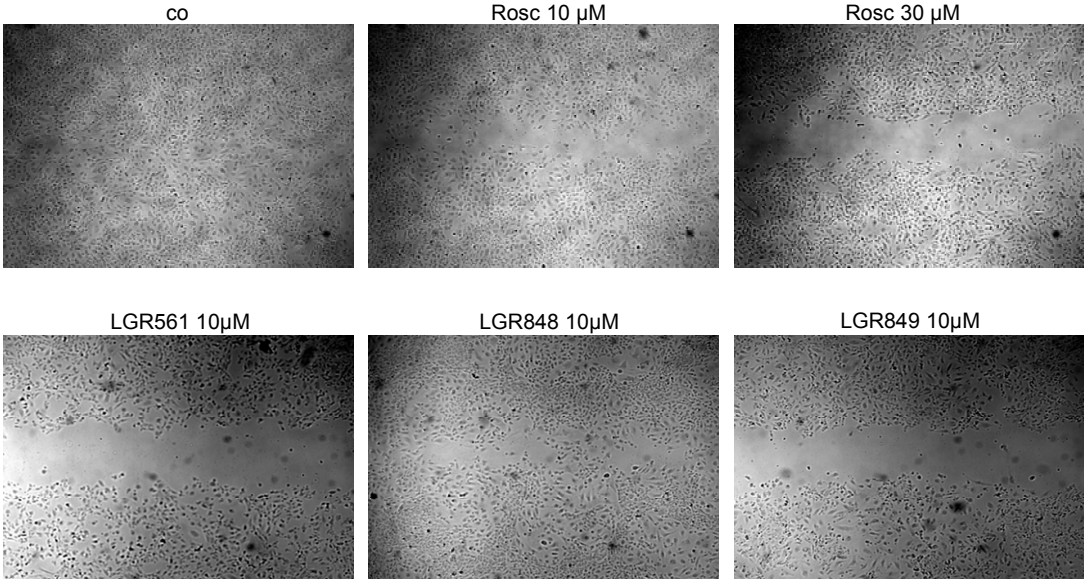


Figure 34 Roscovitine and the LGRs dose-dependently induce apoptosis in endothelial cells. Proliferating HUVECS were either left untreated or treated with increasing concentrations of roscovitine (Rosc), LGR561, LGR848, and LGR849 for 48 h. Apoptosis was determined accordingly to the Nicoletti method. (One Way ANOVA, Holm-Sidak * $p<0.05$).

4.1.3 Effects of roscovitine and the LGRs on endothelial cell migration

To analyze the influence of the compounds on the migration of endothelial cells, scratch assays were performed. Roscovitine significantly reduced HUVEC migration by 20 % (10 µM) or 67 % (30 µM), respectively. LGR561 decreased EC migration by 65 % (10 µM), LGR849 by 67 % (10 µM). Thus, both inhibited migration much more potently than their mother substance roscovitine. LGR848 reduced migration by 21 % at 10 µM, nearly to the same extent as roscovitine (Figure 35).



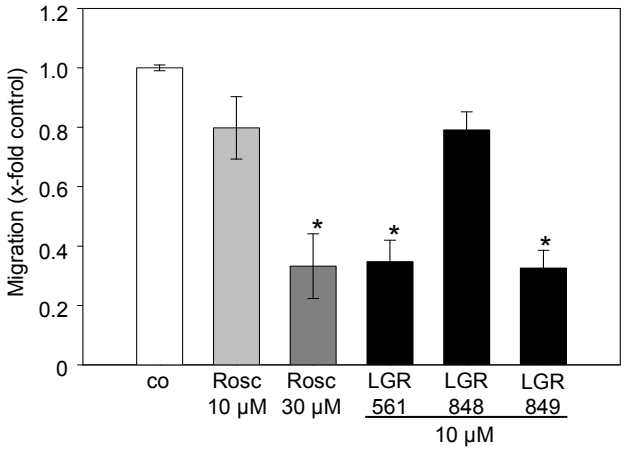


Figure 35 Roscovitine and its derivatives inhibit endothelial cell migration. HUVECs were scratched and either treated with growth medium (co) or growth medium containing roscovitine (Rosc), LGR561, LGR848, or LGR849 in the indicated concentrations for 16 h. Representative images out of three independent experiments are shown. The graph displays the ratio of pixels covered by cells and pixels in the wound area. (mean ± SEM, Kruskal-Wallis One Way ANOVA on Ranks, * $p<0.05$ vs control, n=3)

4.1.4 Effects of roscovitine on endothelial cell chemotaxis

To elucidate whether roscovitine abrogates cell migration via inhibiting cell motility per se (chemokinesis), or by causing a loss of orientation, migration experiments were performed in a chemotactic gradient (chemotaxis assay). Directional migration was induced by a gradient towards 10% fetal calf serum (FCS). Cumulative and euclidean distances were measured. Untreated cells moved along the FCS gradient (Figure 36 a, upper panel). Cells treated with 10 µM roscovitine still moved (intact cumulative distance), but did not follow the FCS gradient, suggesting a loss of orientation (reduced euclidean distance) (Figure 36 a, middle panel). Roscovitine at a concentration of 30 µM reduced both, cumulative and euclidean distance, which refers to a completely defective motility (Figure 36 a, lower panel).

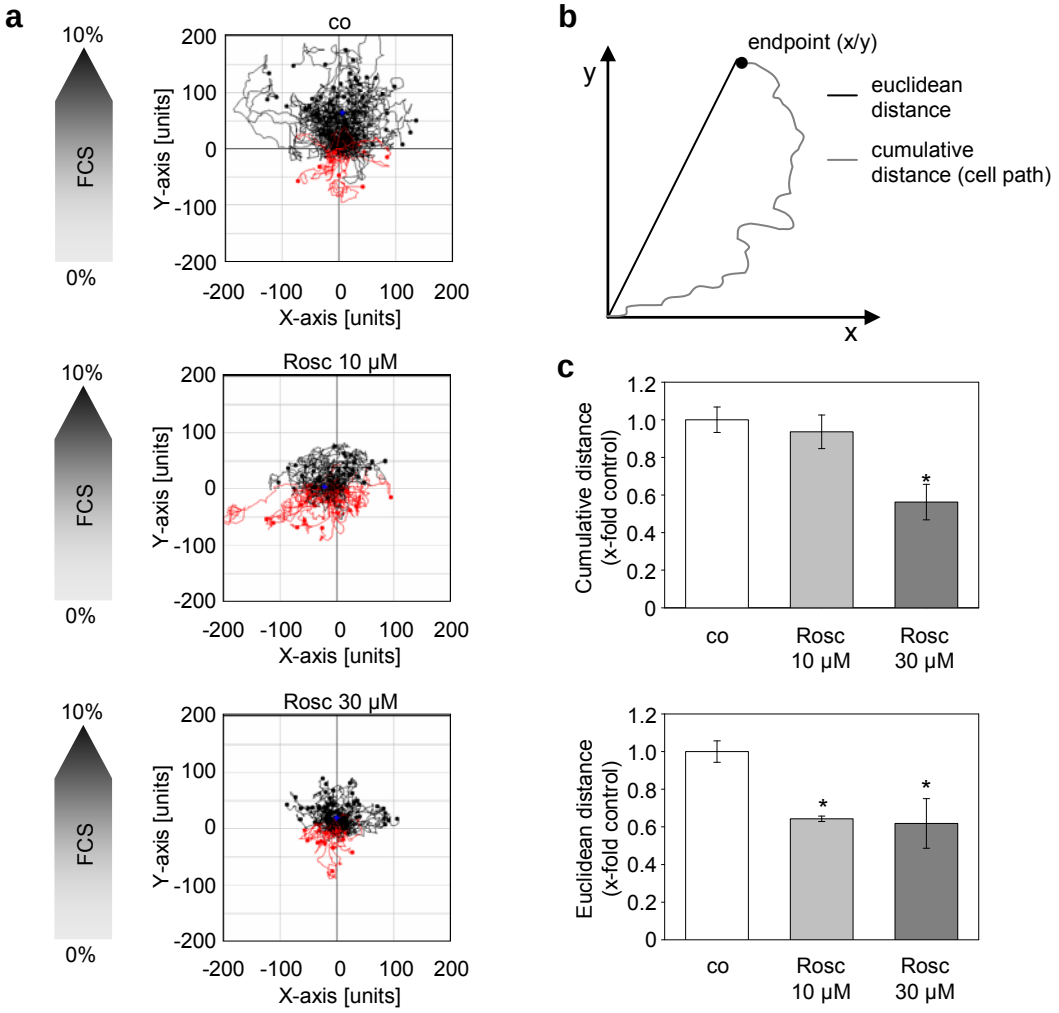


Figure 36 **Roscovitine inhibits chemotaxis in endothelial cells**. (a) Representative tracings of untreated cells (co, upper panel), cells treated with 10 µM roscovitine (Rosc, middle), and cells treated with 30 µM roscovitine (Rosc, lower panel) are displayed. (b) The diagram illustrates cumulative distance (complete cell path) and euclidean distance (directional cell path). (c) Quantitative evaluation of cumulative distance and euclidean distance is shown (One Way ANOVA/Dunnett, * $p<0.05$, n=3).

4.1.5 Roscovitine and the LGRs inhibit tube formation

In tube formation assay, the ability of cells to form three-dimensional structures can be measured. HUVECs were seeded onto Matrigel® in the absence or presence of roscovitine, LGR561, LGR848, and LGR849, respectively. In comparison to untreated cells, tube formation of cells treated with roscovitine was reduced dose-dependently (Figure 37). In comparison to roscovitine, LGR561, and LGR849 inhibited tube formation more powerfully, LGR848 showed a lower activity. For quantitative evaluation, tube length (displayed in red) and number of nodes (displayed as blue crosses in yellow areas) were analyzed.

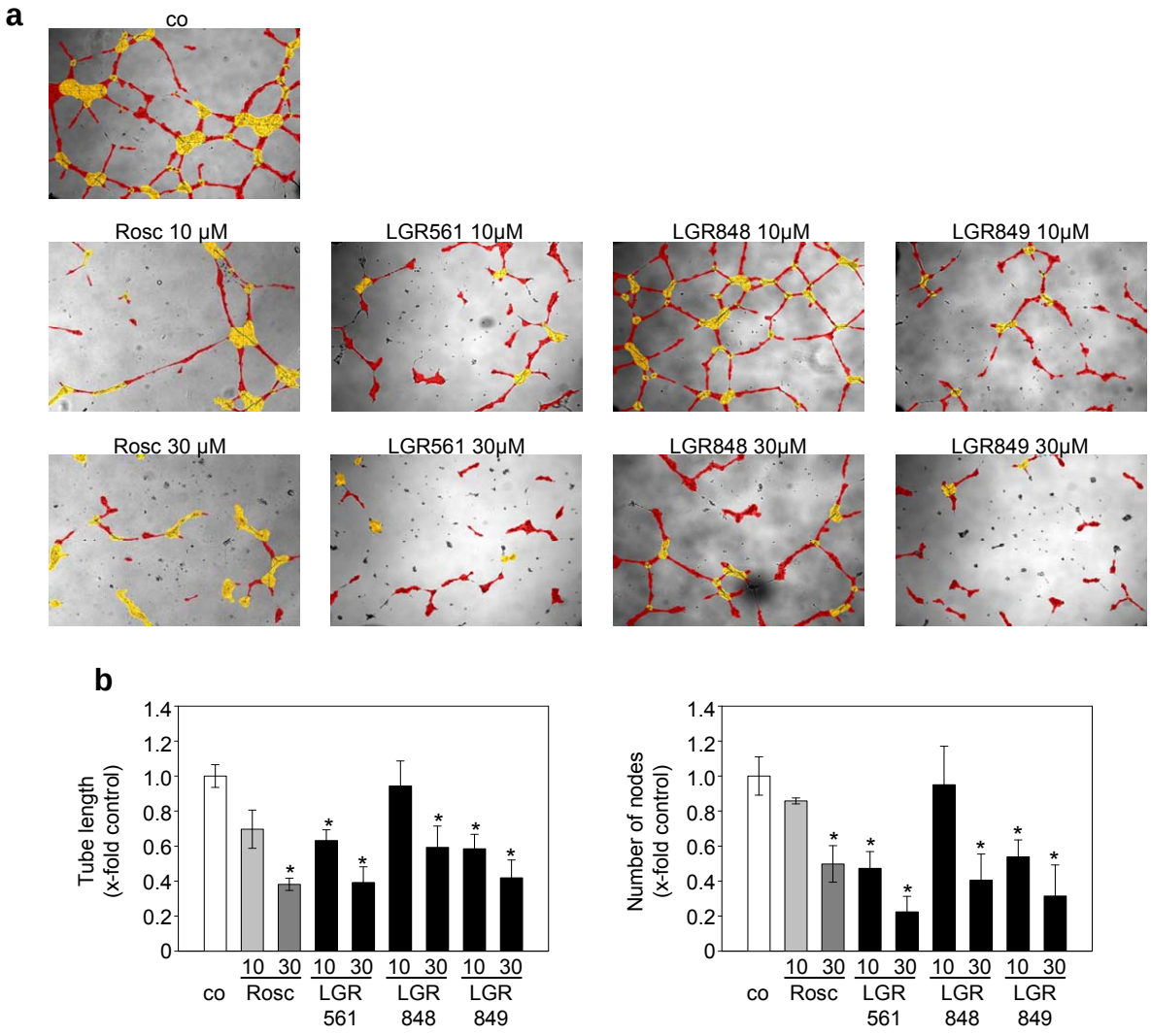


Figure 37 Roscovitine, LGR561, LGR848, and LGR849 dose-dependently inhibit tube formation. (a) Images show HUVECs seeded onto Matrigel® for 16 h in the absence or presence of the indicated compounds. Representative images out of three independent experiments are shown. Tube length is displayed in red, the number of nodes is indicated as blue crosses in yellow areas. (b) The graphs represent the quantitative evaluation of tube length (left panel, One Way ANOVA/Dunnett, * $p<0.05$, n=3) and of the number of nodes (right panel, One Way ANOVA/Dunn, * $p<0.05$, n=3).

4.1.6 Roscovitine and the LGRs reduce vessel sprouting out of mouse aortic rings

Besides endothelial cells, other cell types, such as pericytes or smooth muscle cells, are involved in angiogenesis as well.[3] Thus, we performed mouse aortic ring assays to test the anti-angiogenic properties of roscovitine and its derivatives LGR561, LGR848, and LGR849 in a more complex model *ex vivo*. Roscovitine (10 µM and 30 µM) dose-dependently reduced vascular sproutings out of aortic rings (Figure 38). LGR561, LGR848, and LGR849 decreased vessel sprouting at a concentration of 10 µM.

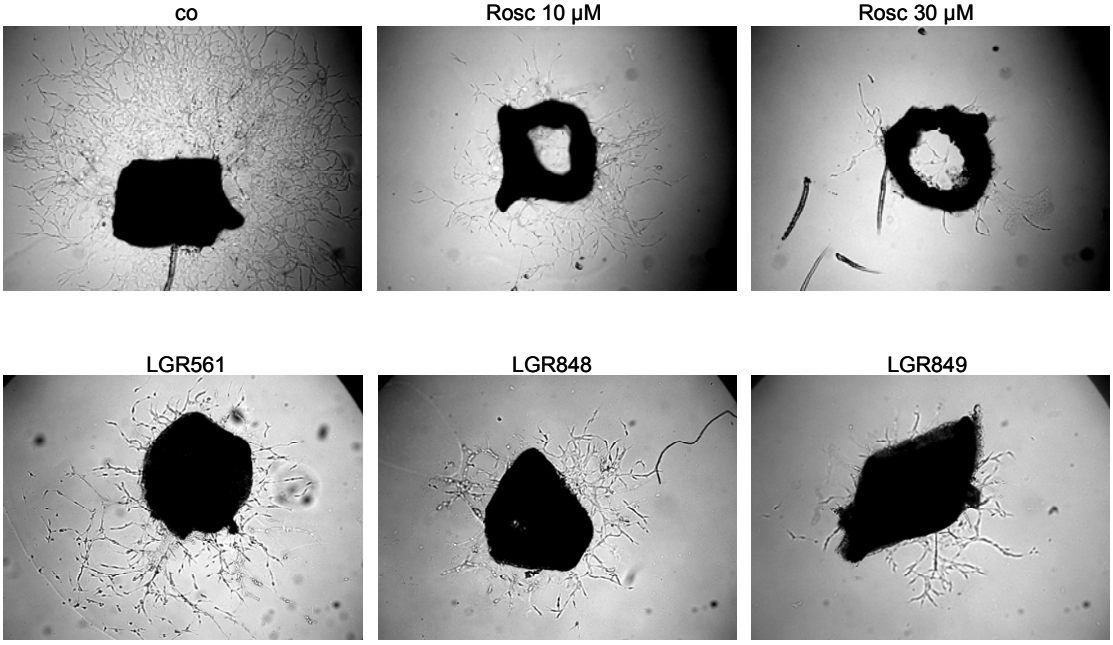


Figure 38 Roscovitine, LGR561, LGR848, and LGR849 reduce endothelial cell sprouting out of mouse aortic rings. Representative images out of three independent experiments display mouse aortic rings embedded into Matrigel® in the absence (co) or presence of roscovitine (Rosc, 10 µM and 30 µM) or its derivatives LGR561, LGR848, LGR849 (10 µM), respectively.

4.1.7 Roscovitine and the LGRs inhibit vessel formation in the CAM-assay

Using the chick chorioallantoic membrane (CAM) assay, we analyzed the anti-angiogenic properties of the investigated compounds *in vivo*. We found a strong induction of vessel formation by vascular endothelial growth factor (VEGF), which was completely blocked by concurrent treatment with roscovitine. LGR561 and LGR849 also strongly reduced VEGF-induced vessel formation in the CAM. LGR848 exhibited only a slight anti-angiogenic effect (Figure 39).

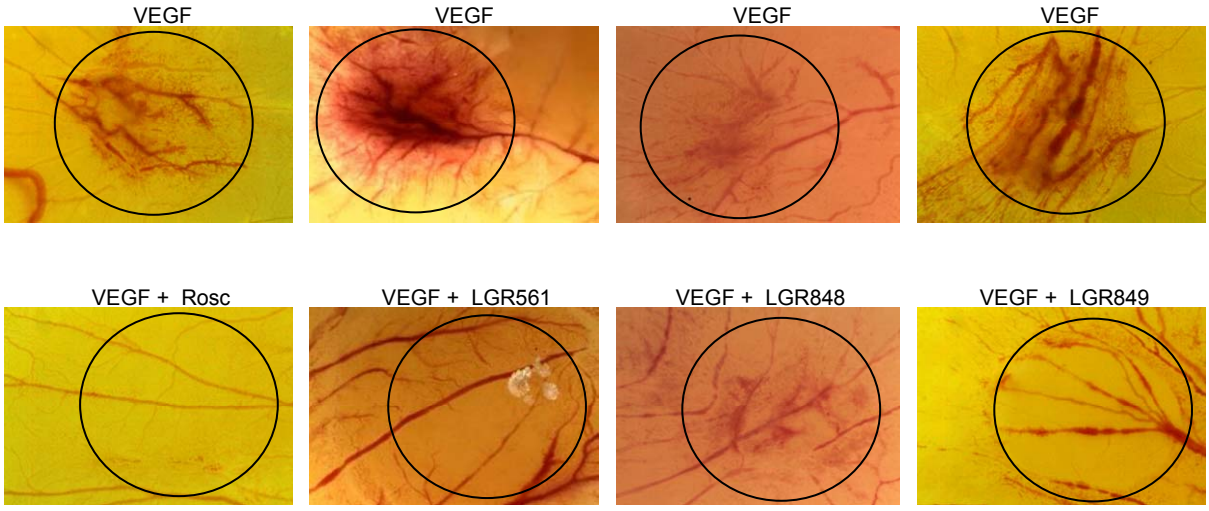


Figure 39 Roscovitine, LGR561, and LGR849 abolish VEGF-induced vessel formation in the chorioallantoic membrane assay, LGR848 shows only a slight effect. Circles represent localization of cellulose disks containing VEGF (1 ng/disk) or VEGF (1 ng/disk) combined with roscovitine (Rosc), LGR561, LGR848, or LGR849 (45 µg/disk), respectively. Representative images out of at least three independent experiments are shown.

4.1.8 Roscovitine and the LGRs reduce neovascularization in the mouse cornea micropocket assay

As an ultimate *in vivo* proof of principle, the mouse cornea micropocket assay was used to test the anti-angiogenic properties of the Cdk inhibitors.

Vascularization of the per se avascular cornea was induced by the implantation of slow-release pellets containing bFGF (80 ng per pellet). Intraperitoneally given roscovitine (100 mg/kg/day) as well as LGR561, LGR848, and LGR849 (30 mg/kg/day) strongly reduced bFGF-induced neovascularization *in vivo* (Figure 40).

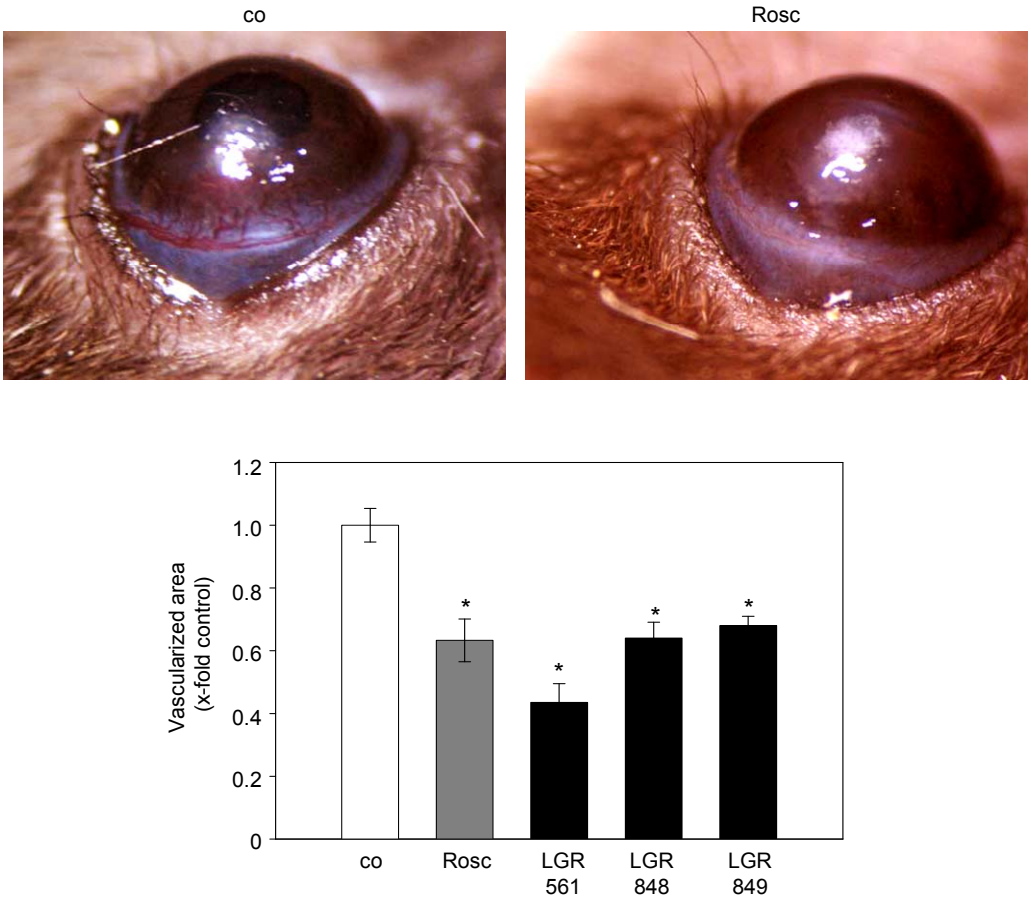


Figure 40 Roscovitine, LGR561, LGR848, and LGR849 significantly reduce bFGF-induced neovascularization in the mouse cornea micropocket assay. The ingrowth of blood vessels into the pellet containing bFGF of one eye of a mouse treated with solvent (DMSO) (co) is shown in the upper left panel. The right panel indicates one eye of a mouse injected intraperitoneally with roscovitine (Rosc, 100 mg/kg/day). The graph in the lower panel represents the quantitative evaluation of the vascularized area. (One Way ANOVA/Holm-Sidak, $p<0.05$, n=8 for control, n=15 for roscovitine, n=15 for LGR561, n=16 for LGR848, n=8 for LGR849).

4.1.9 Effects of roscovitine and the LGRs on the activity of different Cdks

To test and compare the selectivity and the potency of roscovitine and its derivatives LGR561, LGR848, and LGR849 towards different Cdks, in vitro activity assays for Cdk1, Cdk2, CDk4, Cdk5, Cdk7, and Cdk9 were performed. The Cdk5 activity assay was performed by Cerep (Paris, France). The activity assays for Cdk1, Cdk2, Cdk4, Cdk7, and Cdk9 were performed by ProQinase (Freiburg, Germany). Cdk2, Cdk5, Cdk7, and Cdk9 were strongly inhibited by roscovitine. In comparison to roscovitine, its derivatives LGR561, LGR848, and LGR849 much more potently decreased the activities of the respective kinases (Table 10). LGR561, which has been the most powerful anti-angiogenic agent, decreased the activities of the different Cdks most potently in comparison to the mother substance roscovitine and the other derivatives LGR848 and LGR 849. This confirms the data concerning the anti-angiogenic properties of the compounds.

Table 10 In vitro Cdk activity assays. The EC50 values of the different compounds towards the indicated Cdks are displayed.

Cdk	Roscovitine EC50 [µM]	LGR561 EC50 [µM]	LGR848 EC50 [µM]	LGR849 EC50 [µM]
Cdk1	4,472	0,422	2,973	2,002
Cdk2	0,441	0,054	0,265	0,127
Cdk4	9,173	4,944	18,008	6,263
Cdk5	1,4	0,4	1,5	1,3
Cdk7	0,916	0,23	0,647	0,387
Cdk9	1,431	0,145	0,421	0,268

4.1.10 Effects of roscovitine and the LGRs on AKT and ERK

To elucidate whether the compounds direct kinases beyond the Cdk family, we analyzed their influences on two central pathways during cell survival and proliferation: the AKT/PKB signalling pathway and the mitogen-activated protein kinases (MAPK) extracellular-signal-regulated kinases (ERK1/2) pathway.

AKT, also referred to as protein kinase B (PKB), is essential for various cellular processes including apoptosis, survival, cell growth, and proliferation.[89] The phosphorylation of AKT within the C-terminus at Ser473 is required for its full activation.[90] Thus, the impact of roscovitine and the LGRs on the phosphorylation of AKT at Ser473 was analyzed. We observed a strong induction of the phosphorylation of AKT by FCS (10%) which could not be reduced by preincubation with the tested compounds. This suggests that neither roscovitine nor its derivatives LGR561, LGR848, and LGR849 inhibit angiogenesis via effecting the activation of AKT (Figure 41).

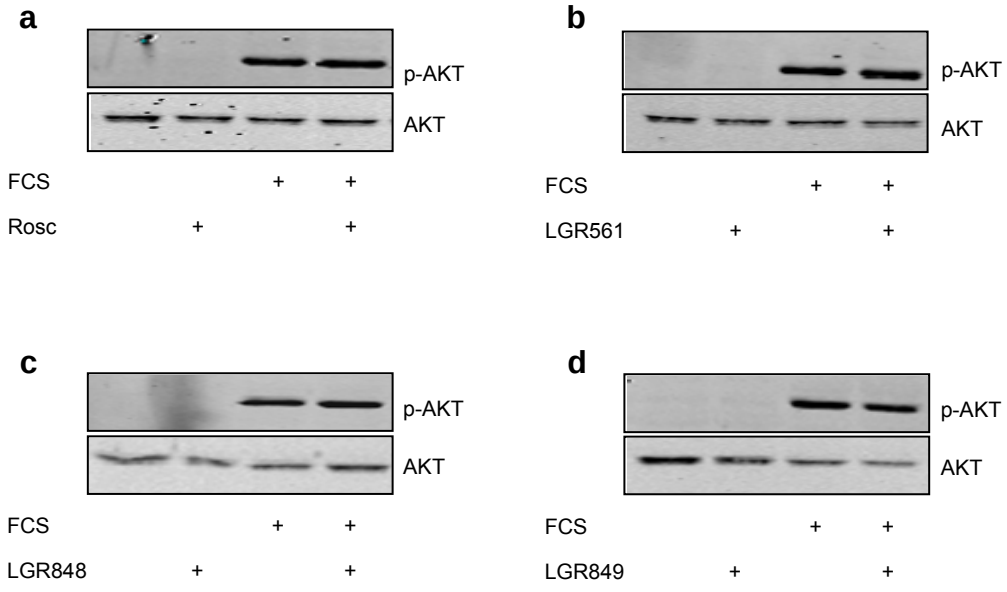


Figure 41 Roscovitine and its derivatives have no influence on the phosphorylation of AKT. Preincubation with roscovitine (Rosc, 30 µM) (a), LGR561 (10 µM) (b), LGR848 (10 µM) (c), and LGR849 (10 µM) (d), respectively, did not abolish the FCS (10%) induced phosphorylation of AKT. Levels of phospho-AKT (p-AKT, Ser473) and AKT were determined by Western Blot analysis. Representative data out of three independent experiments are shown.

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are the most prominent members of the family of mitogen-activated protein kinases (MAPK). MAPKs influence cell proliferation, differentiation, survival, apoptosis and development.[91] ERK1 and 2 are activated by dual-specific kinases MEK1/2 via phosphorylation at threonine and tyrosine residues in the ERK activation loop.[92] The phosphorylation of ERK1/2 was induced by bFGF. Preincubation with roscovitine or the LGRs, respectively, did not reduce FGF-induced phosphorylation of ERK, suggesting that the anti-angiogenic effects of the compounds are not due to an inhibition of ERK activity (Figure 42).

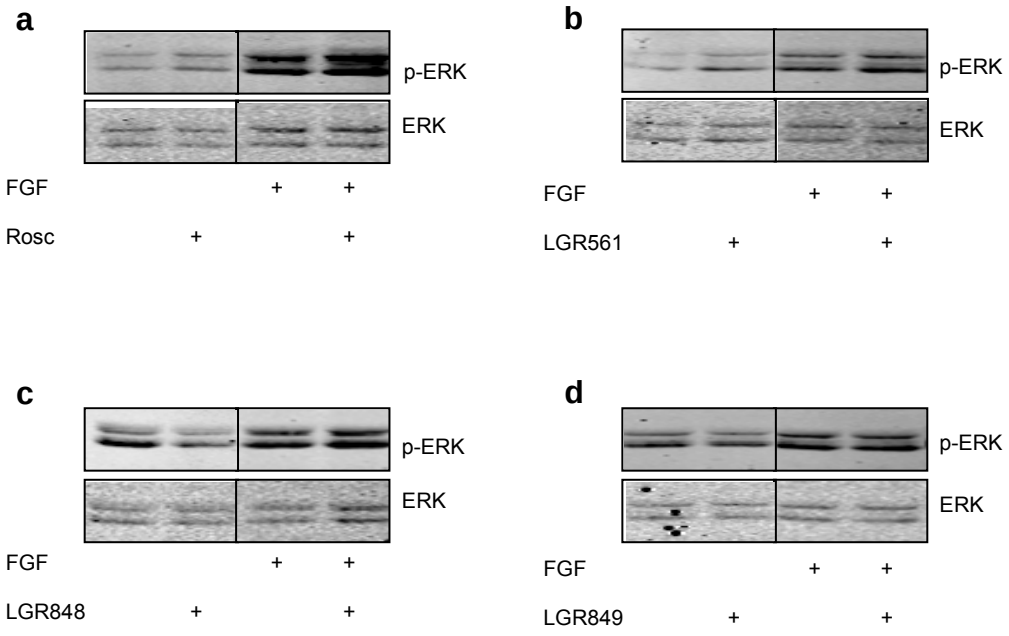


Figure 42 Roscovitine and the LGRs do not abolish the phosphorylation of ERK1/2. Preincubation with roscovitine (Rosc, 30 µM) (a), LGR561 (10 µM) (b), LGR848 (10 µM) (c), and LGR849 (10 µM) (d), respectively, did not abolish the FGF (5 ng/ml) induced phosphorylation of ERK1/2. Levels of phospho-ERK (p-ERK, Thr202 and Tyr204) and ERK were determined by Western Blot analysis. Representative data out of three independent experiments are shown.

4.2 Cdk5 is involved in the regulation of endothelial cell migration

4.2.1 Silencing of Cdk5 reduces endothelial cell migration

In order to elucidate the mechanisms causing the anti-angiogenic effect of the Cdk inhibitors, we analyzed the impact of Cdk2 and Cdk5 on the migration of HUVECs, as endothelial cell migration is a key process during angiogenesis.[55] Cdk2 and Cdk5 both are prominent targets of roscovitine. Cdk2 is an ubiquituously expressed Cdk which regulates cell cycle transitions. In contrast, Cdk5 is not implicated in cell cycle control. Since it was discovered in the 1990s, it has emerged as a crucial regulator of neuronal migration in the developing nervous system.[9]

We first checked the expression of Cdk5 in endothelial cells by Western Blot analysis. As shown in Figure 43, Cdk5 is expressed in HUVECs. Moreover, proliferating and confluent cells display the same amount of Cdk5 protein.

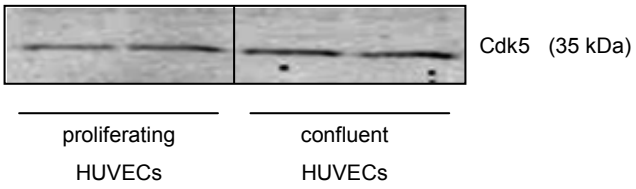


Figure 43 Cdk5 is expressed in endothelial cells. Cdk5 was detected by Western Blot analysis. No difference of Cdk5 protein level between proliferating and confluent HUVECs was determined.

To selectively inhibit Cdk2 and Cdk5, respectively, we silenced both Cdks using siRNA. Successful downregulation of the proteins was determined 24 and 48 h after transfection by Western Blot analysis. No cross-reaction was observed (Figure 44 a). In comparison to the treatment with non-targeting (nt) siRNA, silencing of Cdk5 led to an inhibition of HUVEC migration by 40%. In contrast, the treatment with Cdk2 siRNA showed no significant effect (Figure 44 b). To exclude a possible cytotoxic effect of the Cdk5 siRNA, we examined the viability of transfected cells by a CellTiter-Blue® assay. The viability of Cdk5 siRNA-treated cells was not changed in comparison to nt siRNA-treated cells after 24h or 48h (Figure 44 c).

Taken together, these data suggest an involvement of Cdk5 in the regulation of endothelial cell migration.

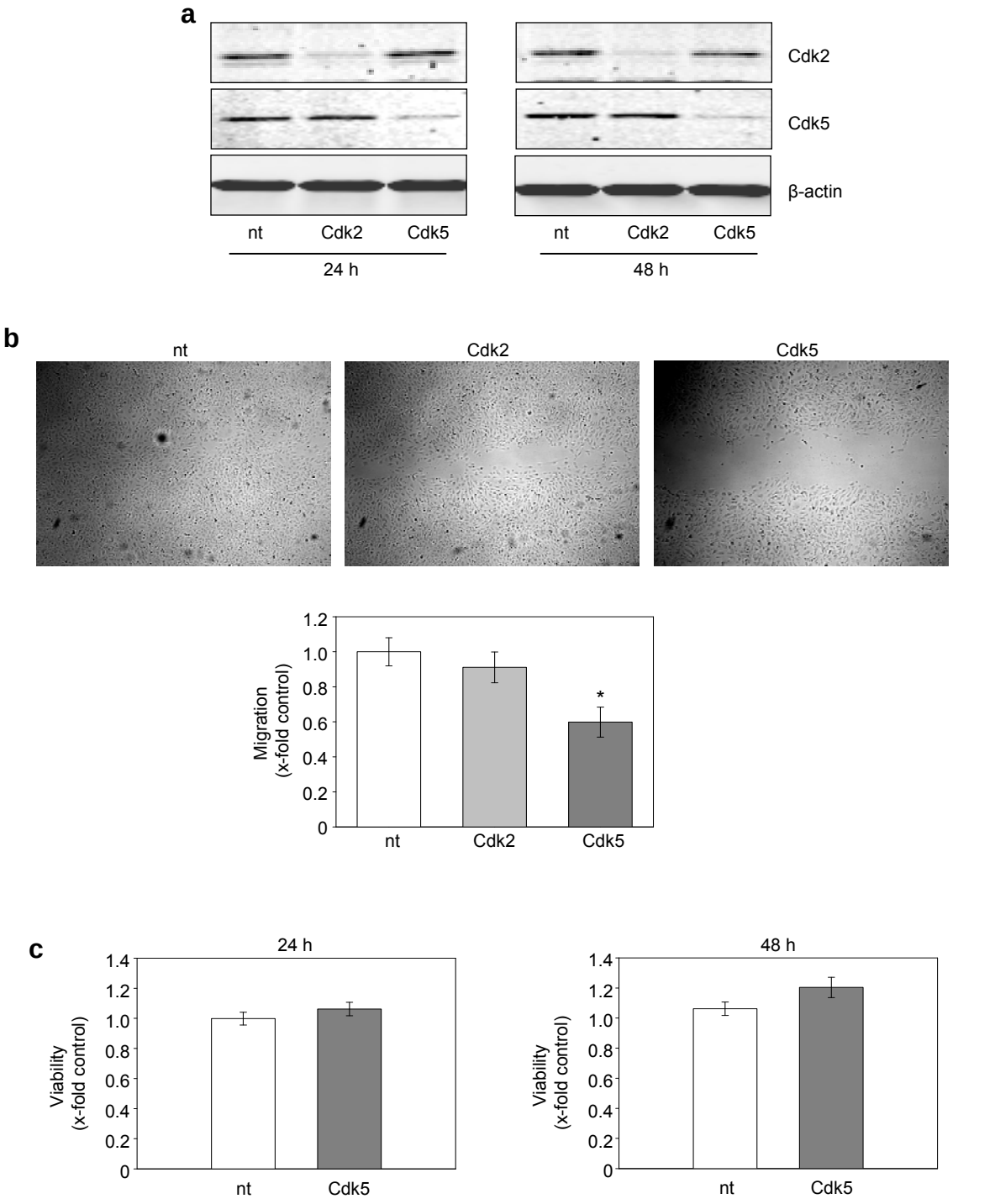


Figure 44 Silencing of Cdk5 reduces endothelial cell migration. (a) Successful downregulation by Cdk2 siRNA (Cdk2), and Cdk5 siRNA (Cdk5) is shown 24 h and 48 h after transfection by Western Blot analysis. Non-targeting siRNA (nt) served as control. β-actin served as a loading control (n=3). (b) Downregulation of Cdk5 by siRNA decreases endothelial cell migration. In scratch assays, transfection with Cdk5 siRNA (Cdk5) significantly decreased the migration of HUVECs, Cdk2 siRNA (Cdk2) had no influence. Non-targeting siRNA (nt) served as control. (One Way ANOVA/Dunnett, *$p<0.05$, n=5). (c) Silencing of Cdk5 does not influence cell viability. Viability of HUVECs transfected with Cdk5 siRNA is not decreased in comparison to cells transfected with nt siRNA (t-test, 24 h: p=0,974, 48 h: p=0,092, n=4).

In order to proof the expression of Cdk5 in the endothelium in vivo, histological staining of umbilical cords was performed. Cdk5 was colocalized with VE cadherin in the endothelium of the umbilical vein (Figure 45).

Thus, we clearly showed that Cdk5 is expressed in the endothelium *in vivo*.

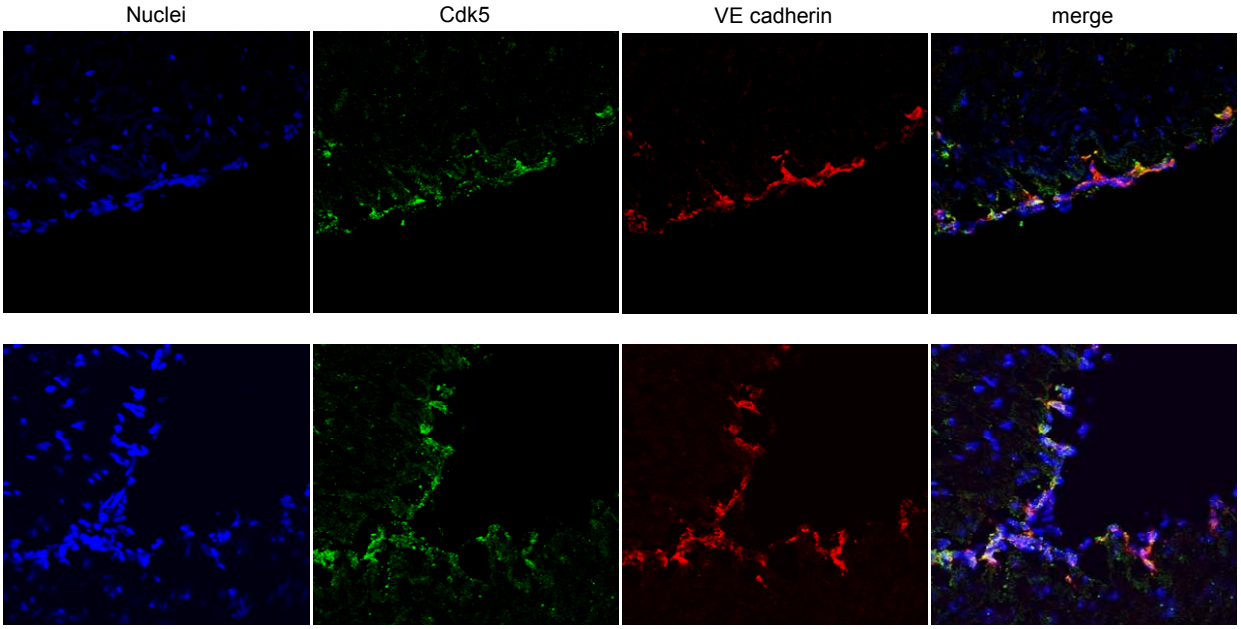


Figure 45 Cdk5 is expressed in the endothelium *in vivo*. Histological staining of umbilical cords was performed. Two representative stainings are shown. Representative images display the staining of nuclei (blue), of Cdk5 (green), and of VE cadherin (red). The overlay (merge) shows colocalization of Cdk5 with VE cadherin.

4.2.2 Inhibition of Cdk5 does not influence cell adhesion

To elucidate the mechanisms of Cdk5 to regulate endothelial cell migration, we first analyzed its impact on cell adhesion, one crucial element regulated during cell migration.[59] In neuronal cells, one reported target of Cdk5 is the Focal Adhesion Kinase (FAK).[35] FAK plays a central role in cell adhesion and migration. Thus, we investigated the effect of Cdk5 inhibition on the phosphorylation of FAK. Phosphorylation of FAK at Ser732, the site which is regulated by Cdk5 in neurons,[35] indeed was reduced after treatment with roscovitine as well as with Cdk5 siRNA. Interestingly, FAK autophosphorylation at tyrosine 397 was not affected (Figure 46).

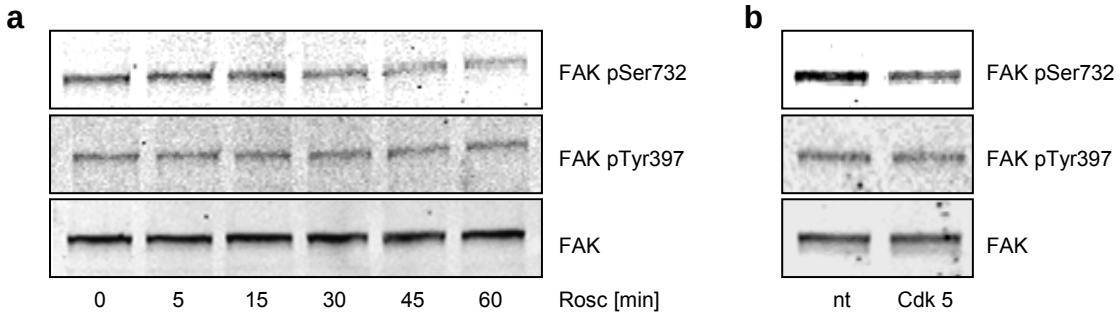


Figure 46 Inhibition of Cdk5 decreases the phosphorylation of FAK at Ser732. (a) Roscovitine (Rosc, 30 μM) time-dependently decreases the phosphorylation of FAK at Ser732. Phosphorylation at Tyr397 is not influenced. (b) Silencing of Cdk5 with siRNA (Cdk5) reduces the phosphorylation of FAK specifically at Ser732. Non-targeting siRNA (nt) served as control. (a, b) Phosphorylation of FAK was detected 48 h after treatment of HUVECs with Cdk5 siRNA by Western Blot analysis. Total FAK indicates equal loading (n=3)

In order to analyze the functional effects of the Cdk5 driven phosphorylation of FAK at Ser732, we analyzed the impact of roscovitine on focal adhesion dynamics and cell adhesion. Focal adhesions were visualized by overexpressing eYFP-vinculin. Vinculin is an adapter protein present at focal adhesions. Roscovitine caused no difference in focal adhesion structure (maturation) as well as in focal adhesion dynamics during cell migration and cell spreading. Furthermore, roscovitine did not functionally influence cell adhesion to various substrates.

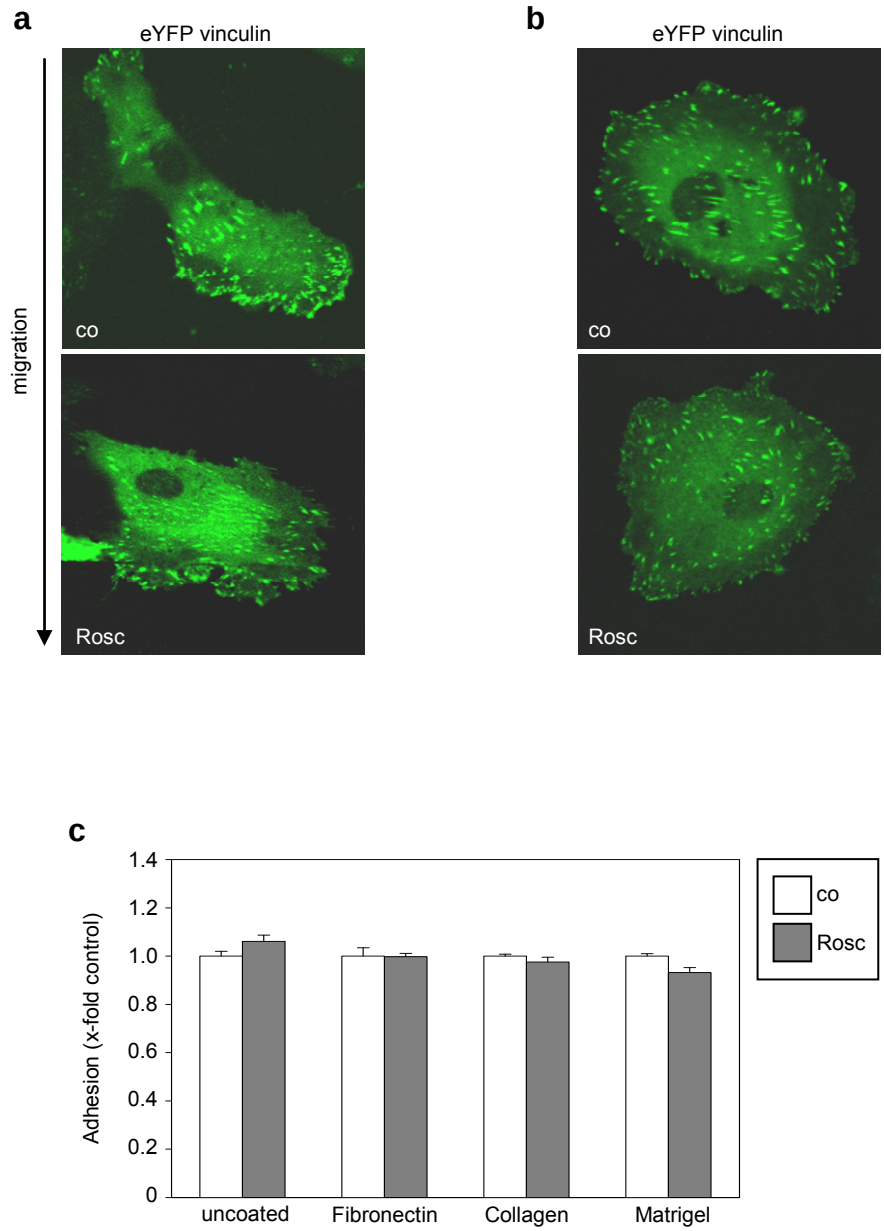
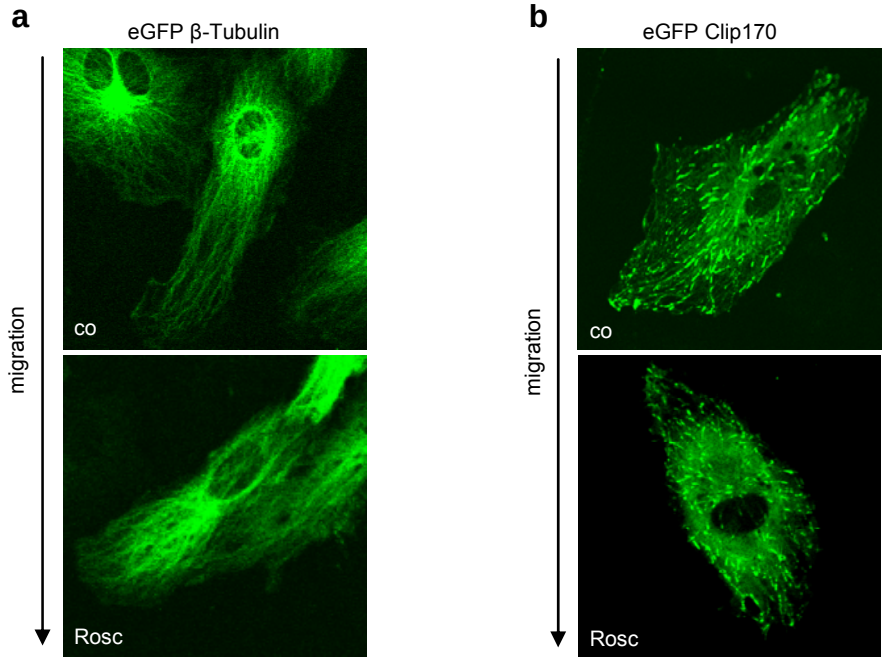


Figure 47 Cdk5 inhibition does not influence focal adhesions and cell adhesion. (a) Roscovitine neither influences the size, nor the localization, nor the dynamics of focal adhesions during cell migration. Images represent migrating HUVECs expressing eYFP-vinculin in the absence (co) or presence of roscovitine (Rosc, 30 µM) (n=2). (b) In spreading cells treated with roscovitine no change in focal adhesion localization, size, or turnover could be determined. Images represent HUVECs expressing eYFP-vinculin plated for 60 min in the absence (co) or presence of roscovitine (Rosc, 30 µM) (n=2). (c) Roscovitine (Rosc, 30 µM) exhibits no influence on cell adhesion. HUVECs were plated on differentially coated surfaces in the absence (co) or in the presence of roscovitine (Rosc, 30 µM). Adhesion was assessed by crystal violet staining of cells 30 min after plating (t-test, n=3).

4.2.3 Cdk5 inhibition exerts no effect on the microtubule cytoskeleton

The phosphorylation of FAK at Ser732 by Cdk5 is important for the organization of microtubules in neurons.[35] Thus, we analyzed the influence of roscovitine on the structure, dynamics, and polymerization of the tubulin cytoskeleton. To analyze microtubule structure, scratch assays with HUVECs overexpressing eGFP-tubulin in the absence or the presence of roscovitine were performed. Roscovitine had no effect on the structure of microtubules (Figure 48 a). To analyze microtubule dynamics, we overexpressed eGFP-CLIP170. CLIP170 is a microtubule plus-end-tracking protein (+tip) that contributes to cell polarization and directional migration by linking microtubule plus-ends to cortical regions.[63] Roscovitine did not influence microtubule dynamics (Figure 48 b). Furthermore, we performed tubulin fractionation assays to analyze possible effects of roscovitine on the polymerization of microtubules. In contrast to classical tubulin targeting compounds like taxol or vinblastine, roscovitine had no effect on the polymerization of tubulin (Figure 48 c). Altogether, we could not determine any effects of roscovitine on the tubulin cytoskeleton.



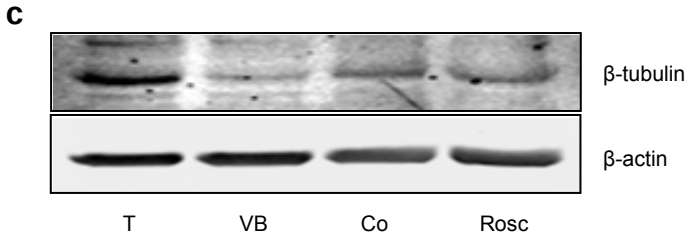


Figure 48 Inhibition of Cdk5 has no effect on the tubulin cytoskeleton. (a) Microtubule structure is not changed by roscovitine. Images represent HUVECs transfected with eGFP-tubulin after migration of 4 h in the absence (co) or presence of roscovitine (Rosc, 30 µM) (n=2). (b) Roscovitine has no influence on the dynamics of microtubules. Representative images show HUVECs expressing eGFP-CLIP170 during migration in the absence (co) or presence of roscovitine (Rosc, 30 µM) (n=2). (c) Roscovitine (Rosc, 30 µM) has no effect on the polymerization of tubulin. Taxol (T) and vinblastine (VB) showed the expected stabilization or fragmentation of microtubules, respectively. Fractions of polymerized tubulin are shown. β-actin indicates equal loading (n=3).

4.2.4 Cdk5 inhibition influences the actin cytoskeleton

Actin is a major component of the cytoskeleton. The constant remodeling of actin filaments during the formation of lamellipodia, filopodia and stress fibres is essential for cell migration.[55, 64] In order to investigate whether Cdk5 influences the actin cytoskeleton in endothelial cells we performed scratch assays and stained F-actin in migrating cells with rhodamine/phalloidin. Untreated cells as well as cells treated with nt siRNA formed lamellipodia with a densely packed actin seam at the leading edge, whereas cells treated with roscovitine or Cdk5 siRNA displayed a strong reduction of lamellipodia (Figure 49 a). Additionally, the inhibition of Cdk5 reduced the localization of cortactin, a protein regulating the protrusion and integrity of lamellipodia, to the leading edge (Figure 49 b).[72, 73]

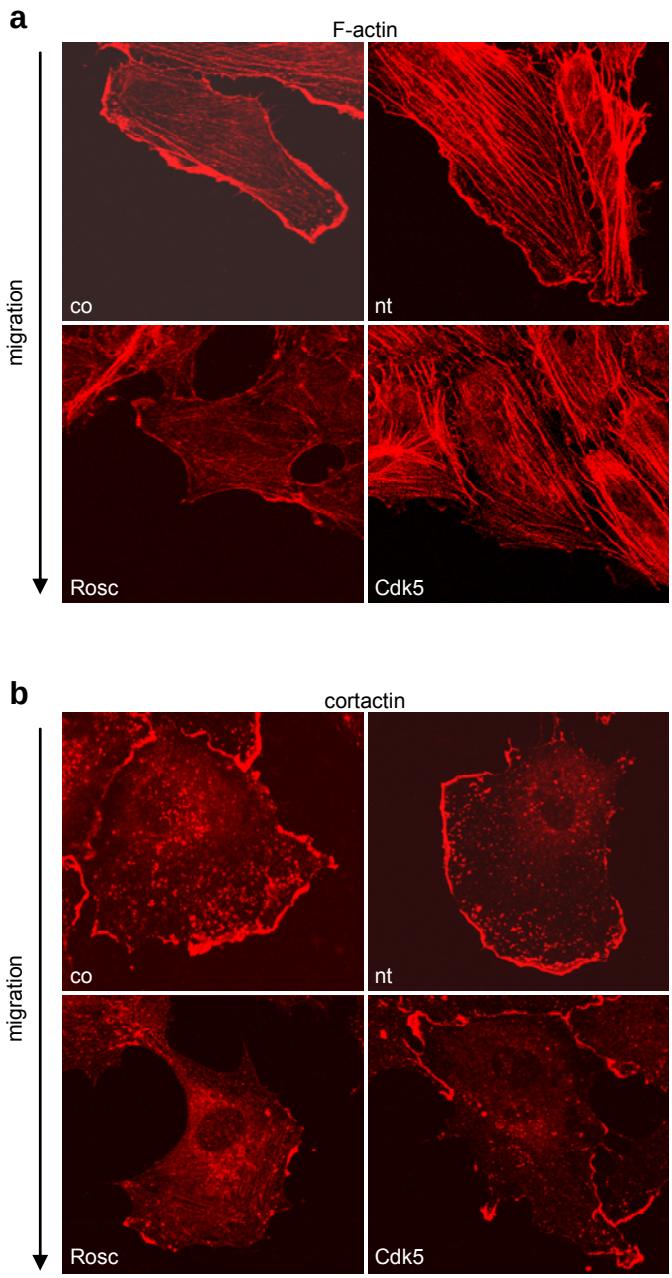


Figure 49 Cdk5 inhibition influences the actin cytoskeleton. (a, b) Inhibition of Cdk5 with roscovitine (Rosc, 30 µM) as well as silencing with Cdk5 siRNA (Cdk5) reduces the formation of lamellipodia during migration. Untreated cells are indicated as control (co), non-targeting siRNA as nt. (a) Images show migrating HUVECs stained for F-actin (n=3). (b) Images represent migrating cells stained with anti-cortactin antibodies (n=3).

4.2.5 Cdk5 inhibition regulates the small Rho GTPases RhoA and Rac1

In order to get insights into the signalling mechanisms underlying the effect of Cdk5 on remodeling of the actin cytoskeleton, we examined the impact of roscovitine on the activity of RhoA and Rac1, the most prominent regulators of the actin cytoskeleton during cell migration.[59]

Applying pull down assays, roscovitine highly increased levels of GTP-bound RhoA, suggesting an enhanced RhoA activity (Figure 50 a). This was accompanied by a decrease of $p27^{kip1}$ protein expression after Cdk5 inhibition with roscovitine (Figure 50 b) or Cdk5 siRNA (Figure 50 c), respectively. $p27^{kip1}$ is classically known as CKI, but it also modulates cell migration by preventing RhoA activation.[93] In neurons, $p27^{kip1}$ gets phosphorylated and stabilized by Cdk5, contributing to actin organization and neuronal migration.[33]

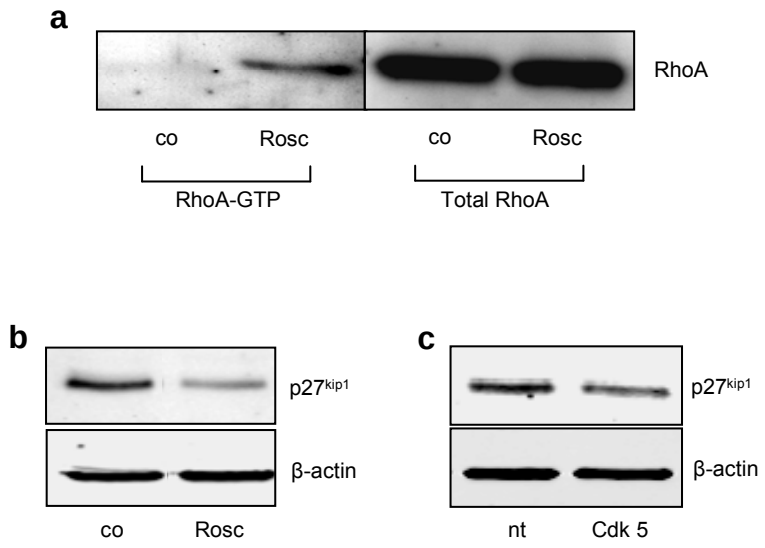


Figure 50 Roscovitine increases the activity of RhoA. (a) In pull-down assays, roscovitine (Rosc, 30 µM) increased the amount of RhoA-GTP. Untreated cells are indicated as control (co) (n=3). (b) Roscovitine (Rosc, 30 µM) as well as (c) Cdk5 siRNA (Cdk5) decreased $p27^{kip1}$ protein levels, in comparison to untreated cells (co) or cells transfected with nt siRNA (nt), respectively. Blotting for β-actin indicates equal loading. (n=3).

To determine whether the impaired endothelial cell migration upon Cdk5 inhibition is due to the increase in RhoA activity, we performed scratch assays with Y27632, an inhibitor of the RhoA downstream kinase ROCK I. Preincubation with the ROCK I-inhibitor Y27632 did not significantly abolish the inhibition of migration caused by roscovitine (Figure 51). This suggests that Cdk5 does not regulate endothelial cell migration via activation of the RhoA – ROCK I - pathway.

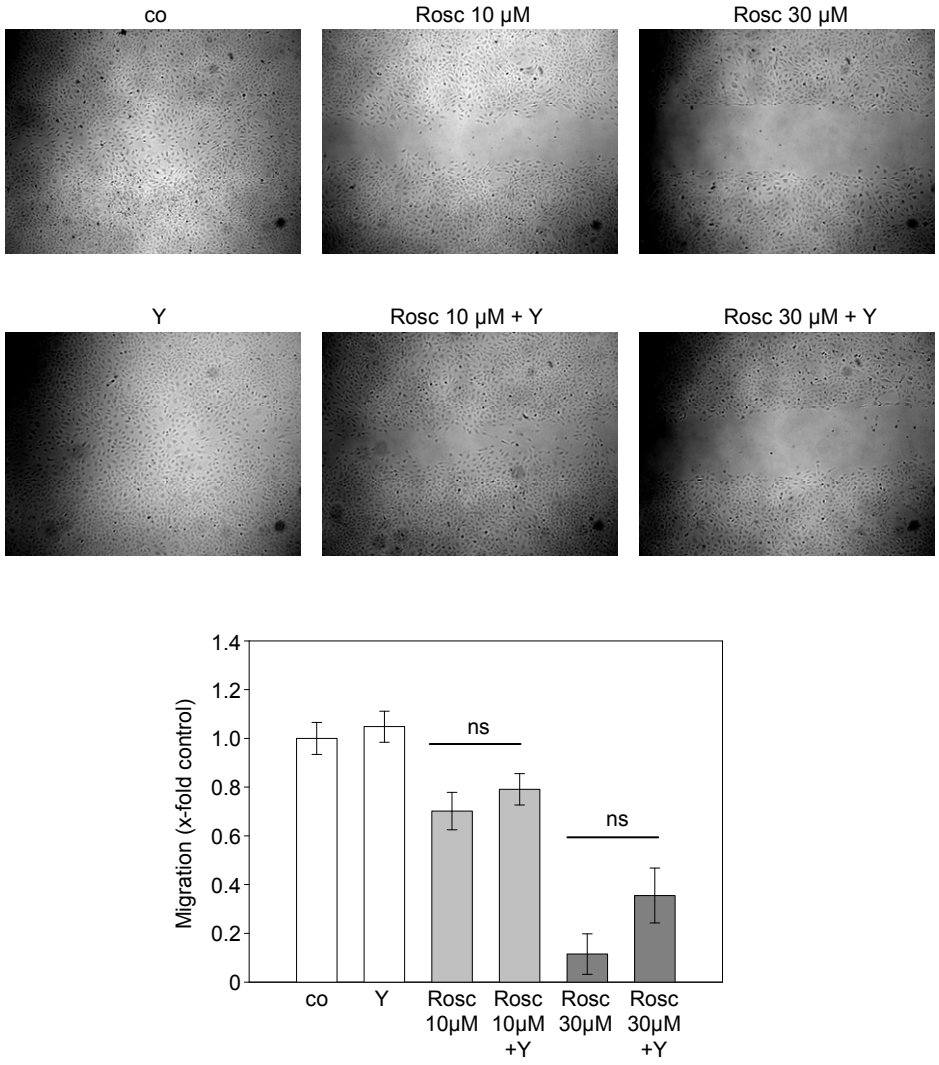


Figure 51 Inhibition of ROCK I does not rescue the effect of roscovitine on endothelial cell migration. Preincubation with Y27632 (10 µM) does not significantly abolish the effect of roscovitine (Rosc, 30 µM) on the migration of HUVECs. Representative images are shown. (not significant, ns), One Way ANOVA/Holm-Sidak, n=3).

Rac1 is a crucial regulator for lamellipodia formation and cell migration.[59] Applying pull-down assays, we detected a strong reduction of GTP-bound Rac1 upon treatment with roscovitine, suggesting a reduced Rac1 activity (Figure 52 a). In live cell imaging experiments with cells overexpressing eYFP-Rac1, we analyzed the distribution of Rac1 during cell spreading. Untreated cells displayed a regular cell shape with Rac1 localized to lamellipodia. In contrast, in cells treated with roscovitine, Rac1 was distributed over the whole cell which was accompanied by an irregular cell shape with large extensions of the cytoplasm and formation of ruffles (Figure 52 b). In contrast, the formation of filopodia seems not to be influenced upon inhibition of Cdk5 (Figure 52 c), suggesting that Cdc42 which regulates the formation of filopodia is not involved in the signalling of Cdk5 in endothelial cell migration.[59]

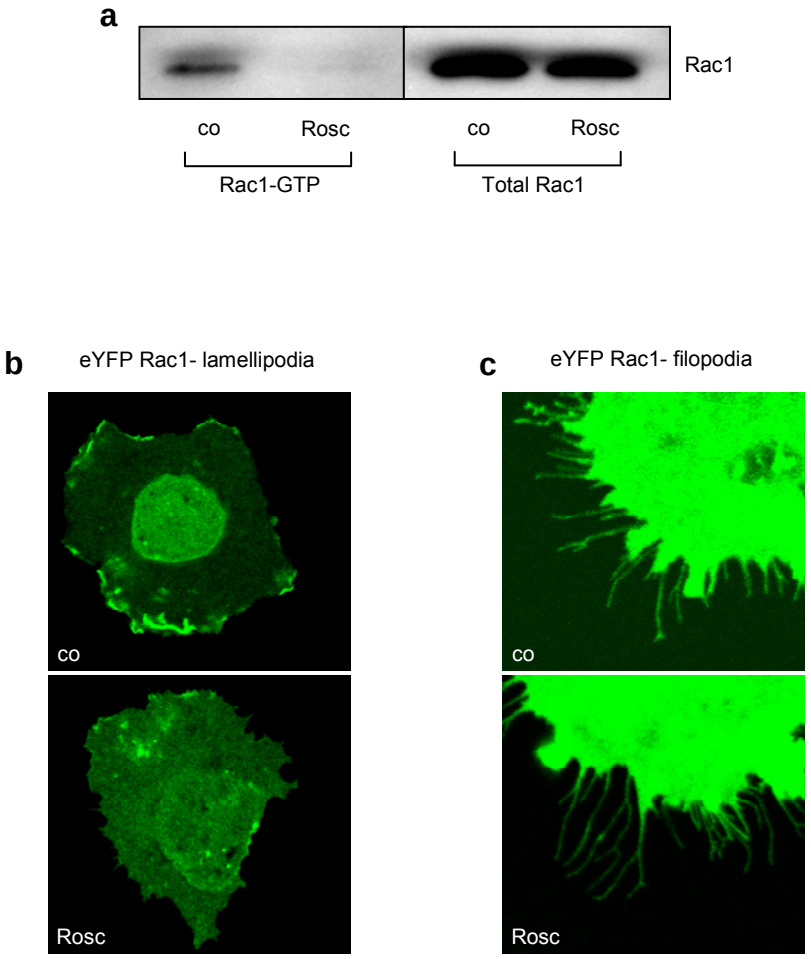


Figure 52 Inhibition of Cdk5 decreases Rac1 activity and inhibits its localization to lamellipodia.
(a) Roscovitine decreases the activity of Rac1. In pull-down assays, roscovitine (Rosc, 30 µM) reduced the amount of Rac1-GTP. Untreated cells are indicated as control (co) (n=3). (b) During cell spreading, roscovitine inhibits the localization of Rac1 to lamellipodia. Images show HUVECs expressing eYFP-Rac1 in the absence (co) or presence of roscovitine (Rosc, 30 µM) 60 min. after plating. Spreading cells treated with roscovitine display an irregular shape with Rac1 distributed over the cytoplasm (n=3). (c) Roscovitine seems not to influence the formation of filopodia. Filopodia could be visualized in HUVECs that extremely strong overexpress eYFP-Rac1. Images show HUVECs in the absence (co) or presence of roscovitine (Rosc, 30 µM) 60 min. after plating. Untreated cells (co) and roscovitine treated cells (Rosc) both form filopodia.

Furthermore, the inhibition of Cdk5 by treatment with roscovitine as well as with siRNA abolished the colocalization of Rac1 and cortactin, a protein that is crucial for the formation and integrity of lamellipodia, at the leading edge of migrating cells (Figure 53 a, b). These data suggest that Cdk5 regulates endothelial cell migration via influencing the actin cytoskeleton by activation of Rac1.

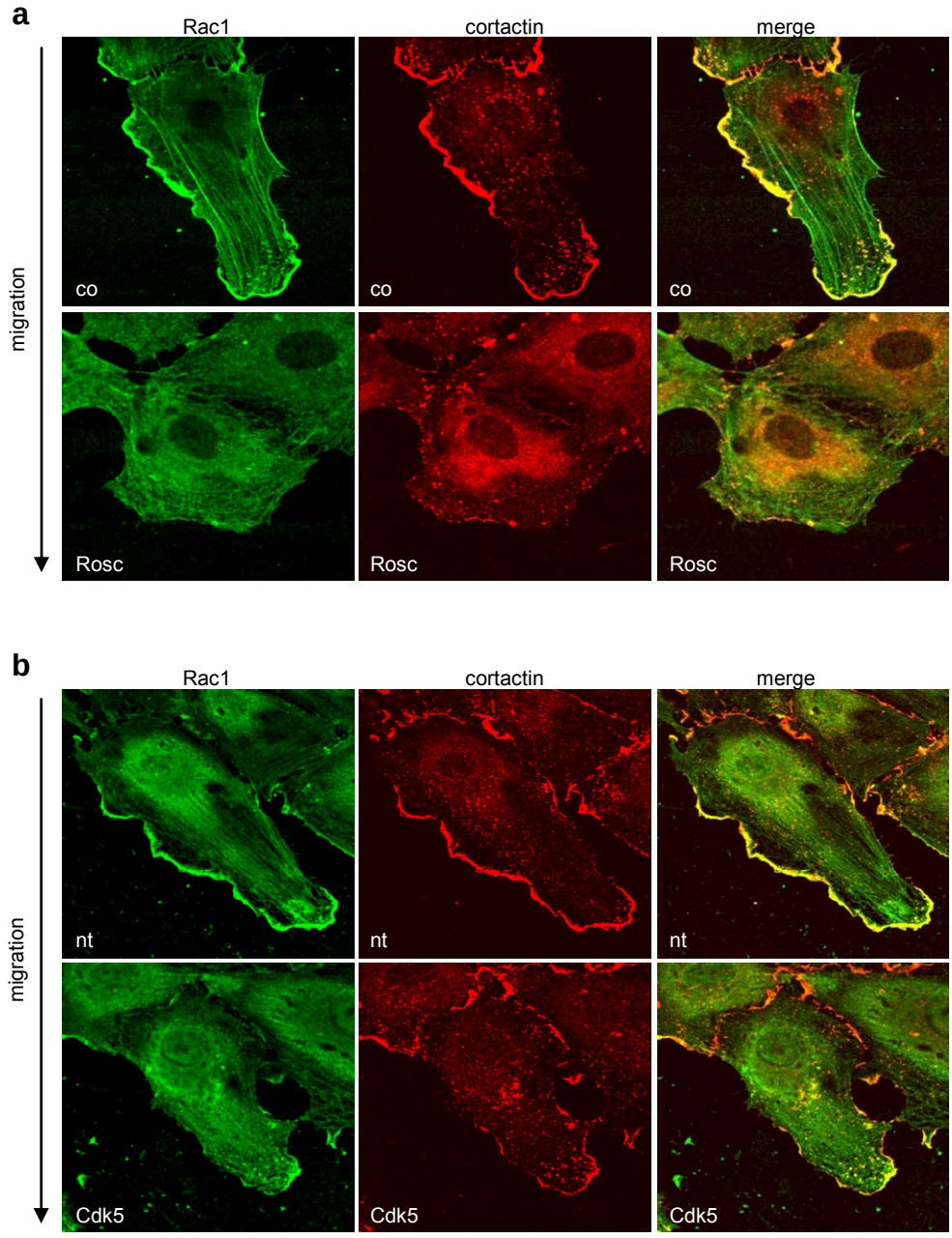


Figure 53 Inhibition of Cdk5 inhibits colocalization of Rac1 and cortactin at lamellipodia. Roscovitine (Rosc, 30 µM) (a) as well as Cdk5 siRNA (Cdk5) (b) inhibit colocalization of Rac1 and cortactin at lamellipodia during migration in comparison to untreated cells (co) as well as cells transfected with nt siRNA (nt). Images represent migrating cells stained for Rac1 (green) and cortactin (red) (n=3). The colocalization (yellow) is shown in the overlay (merge).

5 DISCUSSION

Angiogenesis, the formation of new blood vessels from existing ones, is a prerequisite for tumor growth and progression. Thus, in addition to the treatment of cancer cells with anti-proliferative compounds, anti-angiogenic therapy which is aimed to antagonize tumor derived blood vessel formation, represents a promising approach in oncology.[3] Many anti-angiogenic agents are currently tested in clinical trials and some have already been approved for cancer therapy. At present, inhibitors of the VEGF pathway are in the centre of clinical interest. They are very promising anti-angiogenic agents which stop the growth of tumor vessels, cause regression of existing vessels, normalize surviving vessels, and thus, provoke regression of the tumor itself. Consequently, they demonstrate therapeutic efficacy in an increasing number of human cancers.[2] However, in both preclinical and clinical settings, the benefits of existing anti-angiogenic agents are at best transitory and followed by a restoration of tumor growth and progression. Recently, two different modes of resistance to anti-angiogenic therapy were described. Adaptive resistance refers to the ability of the tumor, after an initial phase of response, to circumvent the specific angiogenic blockade, leading to renewed tumor growth and progression. Intrinsic non-responsiveness means the pre-existing indifference of the tumor towards anti-angiogenic therapy.[94] This resistance to anti-angiogenic therapy indicates a great need for new targets and treatment strategies to inhibit angiogenesis.

Cyclin dependent kinases (Cdks) are key regulators of cell proliferation. Since Cdk deregulation was found in most cancer cells, pharmacological inhibition of Cdks has become an attractive strategy in cancer therapy.[50] Over the last decade, a variety of agents has been developed to inhibit Cdks, and thus, to block cell cycle progression, to decrease proliferation, and to induce apoptosis in cancer cells. So far, some of these drugs have reached clinical evaluation for cancer therapies, including flavopiridol (Aventis), 7-hydroxystaurosporine (Kyowa Hakko Kogyo), and roscovitine (Cyclacel Pharmaceuticals). Surprisingly, very little is known about the effects of Cdk inhibitors on endothelial cells and angiogenesis. Only a few published results indicate potential anti-angiogenic properties of these agents. Flavopiridol, a non-specific Cdk inhibitor, was determined to induce apoptosis in HUVECs,[95] and to decrease blood vessel formation in an *in vivo* Matrigel® model.[96] Roscovitine was shown to inhibit proliferation and to induce apoptosis in bovine aortic endothelial cells (BAEC).[97] However, no in-depth-studies concerning the detailed mode of action, or identifying the respective Cdk and its substrates, have been performed to date.

5.1 Anti-angiogenic effects of the established Cdk inhibitor roscovitine and its new derivatives LGR561, LGR848, and LGR849

This work supports the idea of the application of Cdk-inhibitors as anti-angiogenic agents. For the first time we elucidate that the Cdk inhibitor roscovitine exerts anti-angiogenic effects *in vitro* and *in vivo*. Our work for the first time gives insights into the mechanism of roscovitine to disrupt the angiogenic cascade.

Moreover, the present study gives a first clue how the chemical structure of roscovitine could be optimized to increase its anti-angiogenic potency. In comparison to the mother substance roscovitine, its derivatives LGR561, LGR848, and LGR849 inhibited angiogenesis much more potently, especially LGR561 and LGR849. LGR561 was the most powerful anti-angiogenic agent. Thereby, the anti-angiogenic activity of roscovitine and its derivatives can be traced back to the fact that they inhibit Cdks. Neither the AKT/PKB signalling pathway nor the MAPK/ERK1/2 cascade, both central elements during cell survival and proliferation, was disrupted by the compounds.[89, 91] Furthermore, the anti-angiogenic activity of the respective compounds coincides with their potency to inhibit Cdks. In Cdk activity assay, LGR561 also was the most potent compound, followed by LGR849. LGR848 was only slightly more powerful than roscovitine. No preference of the LGRs to inhibit one certain Cdk could be distinguished: they inhibited Cdk1, Cdk2, Cdk5, Cdk7, and Cdk9, just as roscovitine. However, compared to the other compounds, the most effective anti-angiogenic agent LGR561 displayed a higher potency towards Cdk5. This suggests that the additional *ortho*-hydroxyl-group in the benzyl function of LGR561 optimizes the potency and the selectivity of roscovitine towards Cdk5. The *meta*-methoxy-group does not seem not be advantageous. The effectiveness of LGR561 to inhibit angiogenesis is reduced by the accessorily added *meta*-methoxy-group in LGR849 and LGR848.

The higher anti-angiogenic capacity of the (S)-stereoisomer LGR849 compared to the appropriate (R)-stereoisomer LGR848 is surprising. A comparison of the relative activities of the (R) and (S) stereoisomers of roscovitine towards Cdk1/cyclin B has indicated that the (R)-stereoisomer has about twice the inhibitory capacity than the (S)-stereoisomer, although this difference was barely detectable towards Cdk2/cyclinE.[54] Thus, both for *in vitro* and *in vivo* experiments and for clinical trials as well, investigators use and apply the (R)-stereoisomer whereas the (S)-stereoisomer never has drawn notable attention. Based on these data concerning the potencies of the stereoisomers of roscovitine to inhibit Cdks, we had estimated a higher anti-angiogenic capacity of the (R)-stereoisomer LGR848 in comparison to the (S)-

stereoisomer LGR849. A possible explanation for the higher potency of the (S)-stereoisomer might be a different type of binding of the LGR-compounds to the catalytic centre of Cdks, compared to roscovitine, due to the additional functional groups on the benzyl function. Furthermore, the isomers may partly form the racemate in solution.

Altogether, we elucidate for the first time that the Cdk inhibitor roscovitine exerts anti-angiogenic effects. This suggests a possible new application of Cdk inhibitors as anti-angiogenic agents. Moreover, we show that the new Cdk inhibitors LGR561, LGR848, and LGR849 inhibit the angiogenic cascade more powerfully, in comparison to the mother substance roscovitine. This gives a first clue to optimize the chemical structure of roscovitine to increase its anti-angiogenic potency.

5.2 Cdk5 is involved in the regulation of endothelial cell migration

To get insights into the mechanisms causing the anti-angiogenic effects of the Cdk inhibitors, we analyzed the influence of Cdk2 and Cdk5 on endothelial cell migration. The migration of endothelial cells plays a pivotal role during angiogenesis.[55] Cdk2 and Cdk5 both are prominent and well defined targets of roscovitine.[6, 7] They share about 60% sequence homology and partly have overlapping protein substrate specificity.[9] We focused on Cdk2 as a representative of the classical cell cycle related kinases which regulate cell cycle transitions and on Cdk5 based on its role in the developing nervous system, where it is essential for the regulation of neuronal cell migration.[9]

Roscovitine at concentration of 10 μM inhibits angiogenesis, but has no effect on the cell cycle. This suggests that a non cell cycle-related Cdk is involved in the regulation of endothelial cell migration. In fact, using siRNA to specifically downregulate Cdk2 and Cdk5, respectively, we elucidated an entirely new function of Cdk5 in the endothelium: we determined an involvement of Cdk5 in the regulation of endothelial cell migration. In contrast to Cdk5, Cdk2 was not involved in the regulation of cell migration. This suggests that the anti-angiogenic effects of roscovitine and its derivatives, the LGR-compounds, are most likely due to their inhibition of Cdk5 activity.

5.2.1 Influence of Cdk5 on cell adhesion, on microtubules and on the actin cytoskeleton

To reveal the mechanisms of Cdk5 to regulate endothelial cell migration, we analyzed its impact on the three superordinated elements regulated during migration: on cell adhesion, on the organization of microtubules and on the remodelling of the actin cytoskeleton. Cell migration is a complex process that results from coordinated changes in cell-substratum adhesions and the cytoskeleton. The microtubule network assumes a regulatory function by maintaining cell polarity. The actin cytoskeleton provides the driving force by remodelling into lamellipodia and filopodia at the leading edge and by the formation of stress fibres at the sides and the rear of the migrating cell. The formation of new adhesions to the ECM under the leading edge stabilizes the cell front whereas existing adhesions at the trailing edge are disrupted to allow cell detachment and forward crawling.[55, 61, 98]

In developing cell adhesions, FAK plays a pivotal role. It is a component of focal adhesions and focal complexes, serves as an adapter protein and is involved in integrin-mediated signal transduction. In response to growth factors or integrin clustering, FAK is regulated by phosphorylation at various tyrosine and serine residues.[75, 99] By analyzing the impact of the inhibition of Cdk5 on FAK, we determined a decrease in the phosphorylation of FAK at Ser732. In contrast, the autophosphorylation of FAK at Tyr397, which is required for the catalytic activity of FAK, was not affected.[75] Le Boeuf *et al*. found that a FAK mutant not phosphorylatable at Ser732 abrogates the recruitment of vinculin, an adapter protein present at focal adhesions, to the ventral focal adhesions.[76] In contrast, we could not observe a link between the reduced phosphorylation of FAK at Ser732 and a change in the formation of focal adhesions, both during cell migration and cell spreading. This lack of an effect was mirrored by the fact that roscovitine did not functionally influence cell adhesion on various substrates.

Several reports concerning the role of Cdk5 in neurons indicate an influence of Cdk5 on microtubules.[35, 36] Xie *et al*. showed that Cdk5-mediated phosphorylation of FAK at Ser732 is required for nuclear movement and neuronal migration via influencing microtubule organization.[35] Although Cdk5 inhibition led to a decreased phosphorylation of FAK at Ser732 in endothelial cells as well, neither microtubules structure, nor dynamics, stability, or polymerization was modified. The previously described fact that the phosphorylation of FAK at Ser732 does not influence its kinase activity,[35] might be a possible explanation. Taken together, we neither found an effect of Cdk5 on cell adhesion nor on the organization of microtubules.

Finally, we analyzed the influence of Cdk5 on the organization of the actin cytoskeleton in endothelial cells. The constant remodelling of the actin cytoskeleton represents the driving force during cell migration. Thus, an effect on its organization would result in a changed motility.[98] The fact that roscovitine inhibits cell motility per se in chemotaxis assay, displays a first hint for an effect of Cdk5 on the actin cytoskeleton. Indeed, our hypothesis could be confirmed: in HUVECs, the inhibition of Cdk5 caused a reduced formation of lamellipodia during migration. Lamellipodia are broad sheet-like membrane protrusions which are formed by the polymerization of actin into highly branched filaments. They are generated at the leading edge of migrating cells.[64] At the lamellipodium, new adhesions are formed connecting the actin cytoskeleton to the extracellular matrix which anchors the front of the cell and enables it to crawl forward. Thus, the formation of a lamellipodium is a prerequisite for proper cell migration.[66] Our work identifies Cdk5 to be required for the formation of lamellipodia in endothelial cells. This denotes that Cdk5 regulates endothelial cell migration via an involvement in the remodelling of the actin cytoskeleton.

5.2.2 Influence of Cdk5 on RhoA and Rac1

In order to investigate the mechanism of Cdk5 to regulate the actin cytoskeleton, we had a closer look at RhoA and Rac1, two members of the family of small Rho GTPases. Small Rho GTPases were proven as crucial regulators of the actin cytoskeleton in various cell types. During migration, Rac1 is active at the cell front, where it promotes the extension of lamellipodia. In contrast, RhoA is active at the sides and the rear of the cell, associated with stress fiber formation and cell body contraction.[59, 65, 67] Thus, RhoA and Rac1 are mutually antagonistic, thereby maintaining cell polarity and regulating cell migration.[74]

In neurons, Cdk5 is involved in the regulation of Rho GTPases. Cdk5 was shown to phosphorylate and stabilize $p27^{kip1}$ which suppresses RhoA activity. This leads to an increase in non-phosphorylated, activated cofilin, required for the organization of actin filaments during neuronal migration.[33] Furthermore, it was reported that Cdk5 associates with PAK1 in a Rac1-dependent manner, causing phosphorylation and down-regulation of PAK1 kinase activity, which promotes neuronal migration and neurite outgrowth and affects neuronal morphology.[27, 28]

In endothelial cells, RhoA and Rac1 both seem to be regulated by Cdk5. The inhibition of Cdk5 increased RhoA activity concomitant to a decrease of $p27^{kip1}$ protein levels. $p27^{kip1}$ is classically known as CKI but it also regulates cell migration by preventing RhoA activation.

However, Y27632, an inhibitor of ROCK-I, the most prominent downstream effector of RhoA, could not compensate for the inhibiting effect of roscovitine on endothelial cell migration. In contrast, Besson *et al*. showed that inhibition of ROCK-I rescued the migration defect of $p27^{kip1}$ –null cells.[93] Our data suggest that Cdk5 indeed regulates RhoA activity. Nevertheless, Cdk5 seems not to regulate endothelial cell migration exclusively via the $p27^{kip1}$ – RhoA – ROCK-I pathway. Our data point to an additional/another downstream target of Cdk5 that is involved in the regulation of endothelial cell migration.
In fact, the inhibition of Cdk5 strongly reduced Rac1 activity. Moreover, the colocalization of Rac1 and cortactin at the leading edge of migrating endothelial cells was impaired upon Cdk5 inhibition. Cortactin is an Arp2/3 complex and F-actin binding protein, that plays a pivotal role in lamellipodia protrusion and integrity.[72] Tyrosine-phosphorylation of cortactin, which is required for efficient cell motility, is dependent on the targeting of cortactin to the cortical actin network (lamellipodia) that in turn is regulated by the activation state of Rac1.[73] Our data clearly indicate that Cdk5 is required for the activation of Rac1 and, in turn, for the localization of Rac1 and cortactin to lamellipodia.

Altogether, our work for the first time elucidates a novel function of Cdk5 in endothelial cells. We found that Cdk5 is involved in the regulation of endothelial cell migration. Cdk5 is required for the formation of lamellipodia by influencing the remodelling of the actin cytoskeleton. It activates the small Rho GTPase Rac1 and regulates the localization of Rac1 and its downstream target cortactin to the leading edge of migrating cells.

5.3 Possible aspects of future research

Importantly, this work opens a new field of research with two central questions:

1. How does Cdk5 activate Rac1?

Is the Rac1 activation by Cdk5 due to the Cdk5 induced RhoA inhibition? RhoA and Rac1 can suppress each other's activity, acting antagonistic.[74] A recently recognized mediator of the antagonism of Rac1 by RhoA is FilGAP. FilGAP is a filamin A-binding RhoGTPase-activating protein that gets phosphorylated and activated by the RhoA downstream-effector ROCK I. This phosphorylation stimulates its GAP activity towards Rac1 and leads to Rac1 inactivation, suppressing leading edge protrusion and promoting cell retraction.[100]
However, we excluded the RhoA-ROCK I pathway to be the direct mediator of Cdk5 in the regulation of endothelial cell migration, suggesting that this theory is rather unlikely.

Does Cdk5 directly activate Rac1 e.g. by regulating a Rac1 specific GEF or GAP? Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) directly regulate the activation state of Rho GTPases. In neurons, Cdk5 was reported to regulate several GEFs. For example, Cdk5 phosphorylates Ras guanine nucleotide releasing factor-1 (Ras GRF1) and Ras guanine nucleotide releasing factor-2 (Ras GRF2), both GEFs responsible for the activation of Ras and Rac GTPases. Cdk5 mediated phosphorylation causes Ras GRF1 degradation and thus, reduces Ras activity. Phosphorylation of Ras GRF2 by Cdk5 leads to its inactivation, downregulating Ras GRF2-mediated Rac1 activity. Furthermore, Cdk5 was shown to activate ephexin1, a GEF that regulates the activation of RhoA.[31] These reports confirm the possibility that in endothelial cells, Cdk5 regulates Rac1 via (a) specific GEF(s) or GAP(s).

Does Cdk5 regulate Rac1 by influencing its spatial distribution? Cell motility critically relies on localized signalling. Studies on Rac1 suggested that the control of its intracellular traffic is important for determining the sites at wich Rac1 signalling occurs.[101] Recently, Palamidessi *et al*. showed that endocytic trafficking of Rac1 is required for the spatial restriction of signalling in cell migration. They reported, that the process of endocytosis controls actin dynamics via Rac1.[102] Furthermore, Cdk5 was determined to be an important regulator of synaptic vesicle endocytosis via phosphorylation of dynamin I, a GTPase whose activity is essential for synaptic vesicle fission.[103] The hypothesis that Cdk5 regulates Rac1 via its spatial distribution is strengthened by our data which show that the inhibition of Cdk5 impairs the localization of Rac1 to lamellipodia.

2. How is Cdk5 itself regulated in migrating endothelial cells?

Which relevance do p35 and p39, the neuronal activators of Cdk5, have in endothelial cells? By PCR experiments, we could show that p35 is expressed in endothelial cells (data not shown). This might suggest that p35 also regulates the activity of Cdk5 in endothelial cells.

Where is Cdk5 localized during migration? In cultured neurons, Cdk5 and p35 were shown to colocalize with F-actin and to concentrate at the leading edges of axonal growth cones, regulating neurite outgrowth.[104] Furthermore, p39 colocalizes with actin in cultured cells and coimmunoprecipitates with actin in brain lysates.[105] Thus, Cdk5 might be colocalized with F-actin at lamellipodia in migrating endothelial cells.

Is Cdk5 activity regulated by pro-angiogenic stimuli like growth factors? In neurons, Cdk5 is activated by phosphorylation at Tyr15. C-Abl, a nonreceptor tyrosine kinase related to the Src family, phosphorylates Cdk5 at Tyr15, increasing its kinase activity. Cables, a substrate of c-Abl, interacts with Cdk5 and enhances its phosphorylation.[106] The role of phosphorylation of Cdk5 by CAK is not entirely clear.[9] In neurons, Cdk5 kinase activity is also stimulated by Neuregulin, a growth factor that activates ErbB receptors which belong to the epidermal growth factor receptor related family of tyrosine kinases.[107] These reports suggest that in endothelial cells, Cdk5 might get activated by pro-angiogenic signals like VEGF or bFGF.

Is Cdk5 regulated by Cdk inhibitors (CKIs)? The CKIs $p21^{waf1/kip1}$and $p27^{kip1}$ which effectively inhibit Cdk2 have only minimal effect on p35/Cdk5.[9] Analysis of bovine brain extracts by gel-filtration chromatography yield an inactive p35/Cdk5 complex that is part of a macromolecular structure. An active p35/Cdk5 complex was obtained by subjecting the complex to gel-filtration chromatography in the presence of ethylene glycol, indicates that the macromolecular complex could contain a Cdk5 inhibitory factor.[108] However, no CKI for Cdk5 has been identified to date.

5.4 Conclusion

In conclusion, we identified the well-established Cdk inhibitor roscovitine as a new anti-angiogenic agent, suggesting a possible new application of Cdk inhibitors as anti-angiogenic agents.

The comparison of the anti-angiogenic potencies of roscovitine with its new derivatives LGR561, LGR848, and LGR849 gives a first clue for the optimization of the chemical structure of roscovitine to improve its anti-angiogenic properties. The addition of an *ortho*-hydroxyl-group in the benzyl function of roscovitine could contribute to develop even better new anti-angiogenic agents.

Interestingly, we elucidated for the first time an involvement of Cdk5 in endothelial cell migration. We found that Cdk5 regulates the organization of the actin cytoskeleton by influencing the formation of lamellipodia via Rac1.

Thus, our work highlights both the novel possibility of using Cdk inhibitors as anti-angiogenic agents, and Cdk5 as a new target for anti-angiogenic therapy. This novel role of Cdk inhibitors gives a first clue for their application in anti-angiogenic therapy to broaden the

spectrum and to overcome the drawbacks of already existing inhibitors of angiogenesis in oncology and other pathological processes involving excessive angiogenesis. Moreover, the dual effect of roscovitine on the cell cycle of tumor cells and tumor vascularization could be important in understanding its therapeutic efficacy under various clinical conditions.

6 SUMMARY

Anti-angiogenic agents have successfully been approved for the treatment of several disorders, including diabetic retinopathy, arthritis, psoriasis, and cancer. However, the benefits of existing anti-angiogenic agents are at best transitory and followed by a restoration of tumor growth and progression. This resistance to anti-angiogenic therapy indicates a great need for new targets and treatment strategies to inhibit angiogenesis.

Our work supports the idea of the application of Cdk-inhibitors as a novel class of anti-angiogenic compounds. We show for the first time that the clinically well-established Cdk inhibitor roscovitine exerts anti-angiogenic effects *in vitro* and *in vivo*.

Furthermore, our work gives a first clue for optimizing the chemical structure of roscovitine to improve its anti-angiogenic properties. In comparison to their mother substance roscovitine, the new Cdk inhibitors LGR561, LGR848, and LGR849 - especially the (S)-stereoisomer LGR561 - inhibited angiogenesis much more potently (Figure 54).

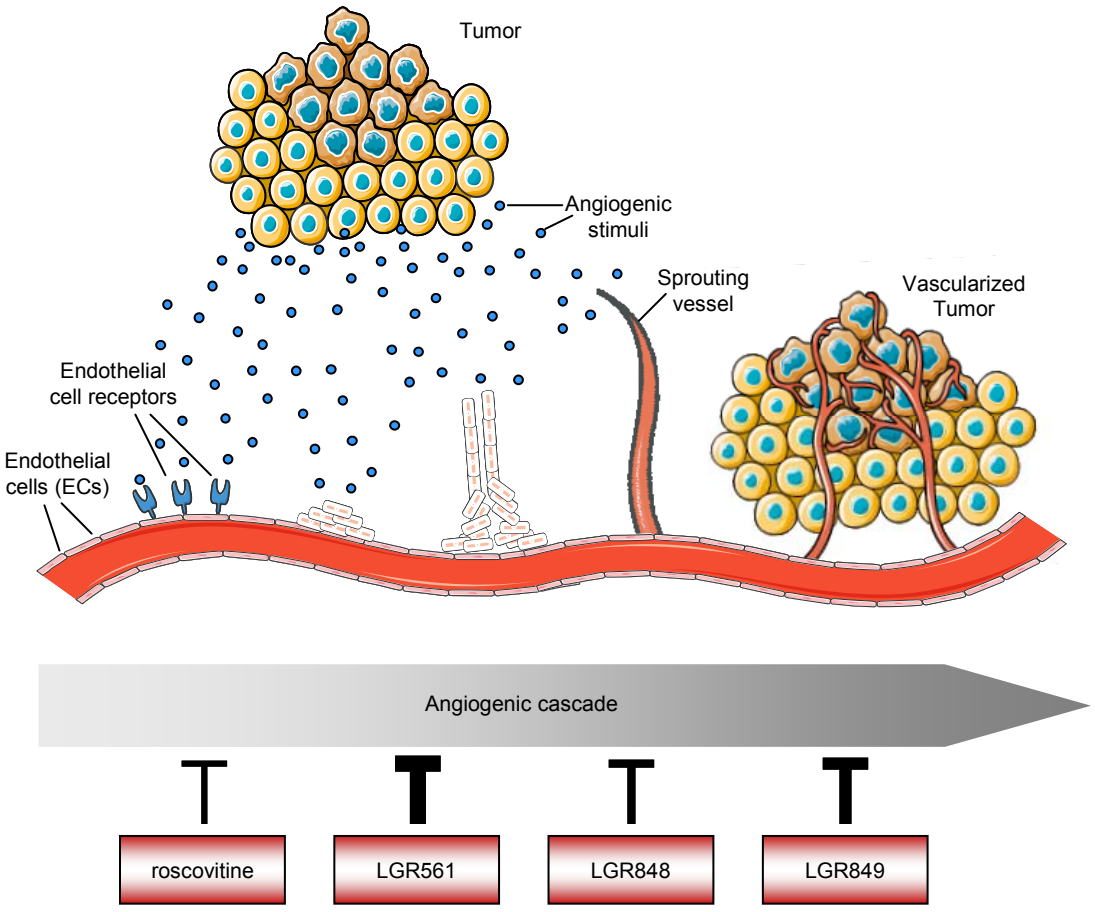


Figure 54 Roscovitine, LGR561, LGR848, and LGR849 inhibit angiogenesis *in vitro* and *in vivo*. In comparison to the mother substance roscovitine, the LGRs are more powerful. LGR561 most potently disrupts the angiogenic cascade.

By selectively analyzing the influence of Cdk2 and Cdk5 - both prominent and well defined targets of roscovitine[6, 7] - on endothelial cell migration, a key process during angiogenesis,[55] we elucidate an entirely new function of Cdk5 in the endothelium: an involvement of Cdk5 in the regulation of endothelial cell migration.

In migrating endothelial cells, Cdk5 influences the organization of the actin cytoskeleton by regulating the formation of lamellipodia via Rac1 and its downstream target cortactin.
Cdk5 also modulates the activity of RhoA via $p27^{kip1}$, but it does not regulate endothelial cell migration directly via the RhoA - ROCK I pathway. Furthermore, Cdk5 phosphorylates FAK at Ser732. However, its impact on endothelial cell migration is not clear. Finally, Cdk5 neither influences the microtubule network nor cell adhesion.

Figure 55 gives an overview over the signalling of Cdk5 in endothelial cell migration.

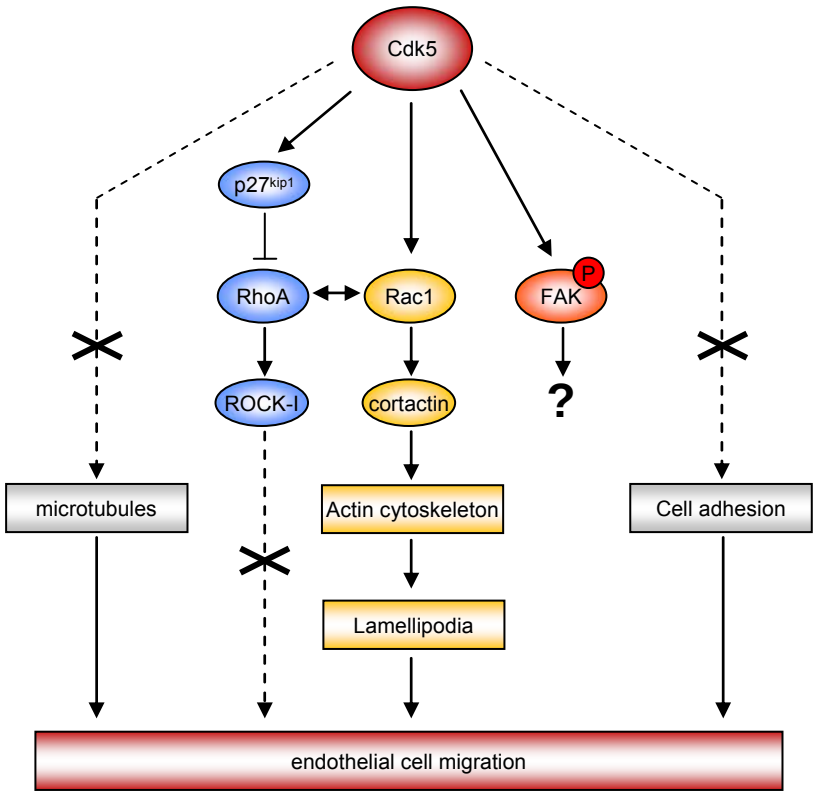


Figure 55 Signalling of Cdk5 in endothelial cell migration.

Thus, the anti-angiogenic effects of roscovitine and its derivatives LGR561, LGR848, and LGR849 are most likely due to their inhibition of Cdk5 activity.

In conclusion, our work

1. **highlights the potential new therapeutic indication of Cdk inhibitors as anti-angiogenic agents,**
2. **elucidates a novel role of Cdk5 in endothelial cell migration indicating Cdk5 as a new target for anti-angiogenic therapy, and**
3. **is the basis for a new field of research with the two central questions:**
   How are Cdk5 and Rac1 functionally connected?
   How is Cdk5 itself regulated in migrating endothelial cells?

This work was supported by the EU-project PROKINASE No 503467.

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8 APPENDIX

8.1 Abbreviations

ALS	amyotrophic lateral sclerosis
AKT	proteinkinase B
APS	ammonium persulfate
Arp2/3	actin-related protein-2/3
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
c-Abl	c-Abelson
CAM	chick chorioallantoic membrane
CAPS	cyclohexylamino-1-propane sulfonic acid
CCD	charge-coupled device
cdc	cell division cycle
CAK	Cdk-activating kinase
Cdk	cyclin dependent kinase
CKI	Cdk inhibitor
CLASP	CLIP-associating protein
CLIP	cytoplasmic linker protein
CLSM	confocal laser scanning microscopy
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECGM	Endothelial Cell Growth Medium
ECL	enhanced chemoluminescence
ECM	extracellular matrix
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FCS	foetal calf serum
FGF	fibroblast growth factor
FL2-H	fluorescent channel 2 height
FSC	forward scatter

GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GDP	guanosin-5'-diphosphate
GRF	guanine nucleotide releasing factor
GTP	guanosin-5'-triphosphate
h	hour
HFS	hypotonic fluorochrome solution
HMEC	human microvascular endothelial cell
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
LIMK	LIM kinase
MAP	microtubule associating protein
MAPK	mitogen-activated protein kinase
mDia	mammalian diaphanous protein
MEK	MAPK/ERK kinases
min	minute
MLC	myosin light chain
MLCP	MLC phosphatase
mRNA	messenger RNA
MTOC	microtubule orginizing centre
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PAK	p21 activated kinase
PAR-complex	polarity protein partitioning-defective-complex
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene fluoride

RNA	ribonucleic acid
ROCK	Rho associated kinase
Rosc	roscovitine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean value
Ser	serine
siRNA	small interfering RNA
TE	tris-EDTA buffer
TEMED	N, N, N', N' tetramethylethylene diamine
Thr	threonine
+Tip	plus-end tracking protein
TNF-α	tumor necrosis factor-α
Tris	trishydroxymethylaminomethane
Tyr	tyrosine
VEGF	vascular endothelial growth factor
WASP	Wiskott Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein complex

8.2 Publications

8.2.1 Original publications

Roscovitine inhibits angiogenesis – Novel function of Cyclin dependent kinase 5 in endothelial cell migration

Johanna Liebl[1], György Vereb[2], Robert Fürst[1], Angelika M. Vollmar[1], Stefan Zahler[1]

[1] Department of Pharmacy – Center for Drug Research, Pharmaceutical Biology, Ludwig Maximilians University, Munich, Germany

[2] Medical and Health Science Center, Department of Biophysics and Cell Biology, University of Debrecen, Hungary

Submitted

Ginkgo biloba extract EGb 761 exerts anti-angiogenic properties *via* activation of tyrosine phosphatases

Anja Koltermann[1], Johanna Liebl[1], Robert Fürst[1], Hermann Ammer[2], Angelika M. Vollmar[1], Stefan Zahler[1]

[1] Department of Pharmacy – Center for Drug Research, Pharmaceutical Biology, Ludwig Maximilians University, Munich, Germany

[2] Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Germany

In revision

Anti-angiogenic effects of new Cdk inhibitors LGR561, LGR848, and LGR849

Johanna Liebl[1], Vladimir Krystof[2], Paul Pechan[3], Angelika M. Vollmar[1], Stefan Zahler[1]

[1] Department of Pharmacy – Center for Drug Research, Pharmaceutical Biology, Ludwig Maximilians University, Munich, Germany

[2] Palacky University & Institute of Experimental Botany, Czech Republic.

[3] C3 Bio, Munich, Germany.

In preparation

8.2.2 Oral communication

Anti-angiogenic effects of new Cdk inhibitors LGR561, LGR848, and LGR849 on endothelial cells

Johanna Liebl[1], Vladimir Krystof[2], Paul Pechan[3], Angelika M. Vollmar[1], Stefan Zahler[1]

[1] Department of Pharmacy – Center for Drug Research, Pharmaceutical Biology, Ludwig Maximilians University, Munich, Germany

[2] Palacky University & Institute of Experimental Botany, Czech Republic.

[3] C3 Bio, Munich, Germany.

Frühjahrstagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie (DGPT); March 13-15th, 2007, Mainz, Germany.

8.2.3 Poster presentations

Antiproliferative effects of new Cdk inhibitors LGR 561 and LGR 849 on endothelial cells

J. Liebl[1], V. Krystof[2], P. Pechan[3], A. Vollmar[1], S. Zahler[1]

[1] Department of Pharmacy – Center for Drug Research, Pharmaceutical Biology, Ludwig Maximilians University, Munich, Germany

[2]Palacky University & Institute of Experimental Botany, Czech Republic.

[3]C3 Bio, Munich, Germany.

Annual Meeting 2006, Gesellschaft für Microzirkulation und Vaskuläre Biologie e.V., October 12-14th, 2006, Munich, Germany.

Anti-angiogenic effects of new Cdk-inhibitors LGR561 and LGR849 in vitro and in vivo – first insights into the underlying molecular mechanism.

Johanna Liebl[1], Vladimir Krystof[2], Paul Pechan[3], Angelika M. Vollmar[1], Stefan Zahler[1]

[1] Department of Pharmacy – Center for Drug Research, Pharmaceutical Biology, Ludwig Maximilians University, Munich, Germany

[2] Palacky University & Institute of Experimental Botany, Czech Republic.

[3] C3 Bio, Munich, Germany.

Annual Meeting 2007, Gesellschaft für Microzirkulation und Vaskuläre Biologie e.V., October 4-6th, 2007, Heidelberg, Germany.

8.3 Curriculum vitae

Persönliche Daten

Name	Johanna Liebl
Geburtsdatum	20.02.1980
Geburtsort	Pfarrkirchen
Familienstand	ledig
Staatsangehörigkeit	deutsch

Hochschulstudium

ab 07/2005	Dissertation am Lehrstuhl für Pharmazeutische Biologie Department Pharmazie der LMU München Betreuer: Prof. Dr. A.M. Vollmar, PD Dr. S. Zahler
04/2000-04/2004	Studium der Pharmazie LMU München
03/2002	1. Abschnitt der Pharmazeutischen Prüfung Abschluss des Grundstudiums
04/2004	2. Abschnitt der Pharmazeutischen Prüfung Abschluss des Hauptstudiums
07/2005	3. Abschnitt der Pharmazeutischen Prüfung Approbation zur Apothekerin

Berufsausbildung und Praktika

07/2005-12/2007	promotionsbegleitende Tätigkeit als Apothekerin Amper Apotheke in Fürstenfeldbruck
05/2004-04/2005	Pharmaziepraktikum 1. Halbjahr: Amper Apotheke in Fürstenfeldbruck 2. Halbjahr: Krankenhausapotheke im Klinikum Bogenhausen
07/2002-08/2002	Praktikum am Lehrstuhl für Pharmakologie für Naturwissenschaften Department Pharmazie der LMU München

Schulbildung

1986-1990	Grundschule Triftern
1990-1999	Gymnasium Pfarrkirchen Abschluss: Allgemeine Hochschulreife

8.4 Danksagung

Diese Arbeit wurde an der LMU, Department Pharmazie am Lehrstuhl für Pharmazeutische Biologie von Frau Professor Dr. Angelika M. Vollmar unter Betreuung von Herrn PD Dr. Stefan Zahler angefertigt. Ihnen möchte ich an allererster Stelle meinen herzlichen Dank sagen für die Möglichkeit, in ihrer Gruppe zu promovieren. Ihre hervorragende fachliche Betreuung, die produktiven Diskussionen und die vielen hilfreichen Ratschläge waren essentiell für diese Arbeit. Ganz besonders möchte ich mich auch für die ständige Motivation, die persönliche Unterstützung und ihr immer offenes Ohr für alle meine Fragen und Probleme bedanken. Die Arbeit unter Ihrer Anleitung hat immer sehr viel Spaß gemacht! Danke!

Herzlicher Dank gilt auch allen Mitgliedern meines Prüfungskommitees. Ganz besonders möchte ich mich bei Herrn Prof. Dr. Martin Biel für seine Bereitschaft bedanken, als Zweitgutachter meine Arbeit zu beurteilen.

Ein ganz besonders großes Dankeschön geht an alle Mitglieder der Arbeitsgruppe für die tolle Athmosphäre. Für ihre wertvolle Hilfe und Unterstützung möchte ich mich bei Jana, Conny, Frau Schnegg, Rita und Bianca bedanken. Ein herzliches „Danke“ gilt meinen Laborkollegen Anja, Martin, Bettina und Sabine für die schöne Zeit und die angenehme Zusammenarbeit im „Endothellabor“. Besonders meinem „Boxenbruder“ Martin möchte ich danken für die schöne Zeit in unserer Box, für das geduldige Ertragen meiner Launen und für die selbstgebackenen Geburtstagskuchen. Bei Andrea möche ich mich bedanken für ihre Geduld und Hilfe mit allem, was Computer angeht, vor allem aber auch für die guten Gespräche und ihre Unterstützung gerade während der „Endphase“. Ein weiteres „Danke“ geht an Lila für ihre Hilfsbereitschaft und für „bestes polnisches Wurst“. Mein ganz besonderer Dank gilt Anja, Anita und Kathi für ihre Freundschaft, fürs Zuhören und für die schöne Zeit auch ausserhalb der Arbeit.

Zuletzt möchte ich mich bei meinen Eltern, meiner Schwester Dorle, meiner Oma, meinem Opa und meinem Freund Thomas bedanken, auf die ich mich immer verlassen kann und die mir bei allem immer geholfen haben. Danke für eure ständige Unterstützung, euer Verständnis, und dafür, dass ihr einfach immer für mich da seid. Ihr seid wirklich was ganz Besonderes und ich bin sehr froh, dass ich euch habe.