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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Screening of potential new ROCK inhibitors for future preclinical development

Promotor :
Prof. dr. Jean NOBEN

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Selien Sànchez

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Preface

In the past 8 months I was able to perform my senior internship at Amakem therapeutics. I immediately felt welcome in the jovial group of people working in this company. I have learned a lot in this period, therefore I would like to thank every person that made it possible for me to do my internship at Amakem therapeutics.

First of all, I would like to thank my promotor Dr. Karolien Castermans. Thank you for the guidance, patience and support you gave me while writing this thesis and performing the experiments. I would also like to thank Nicki Boumans for guiding me in the lab, teaching me all the experiments and helping me whenever I needed it. Next, I would like to thank the other members of the biology team: Laura, Jessica, Silke and Nele, to help and support me whenever necessary. In general, I would like to thank to whole Amakem team for making this internship not only educative but also very fun, I enjoyed working with you all.
I would also like to thank my internal promotor Prof. Dr. Jean-Paul Noben for his support and valuable insights during our meetings.
In addition, I would like to thank my fellow-students at Biomed for welcoming and advising me whenever I needed it.

I am very grateful to my parents for the opportunities and support they have given me throughout my education. Finally, I would like to thank you Yentl for your support, for calming me down when I was stressed and for always being there for me.

List of abbreviations

ACN = Acetonitrile

ANOVA = Analysis of variance

BrdU = Bromodeoxyuridine

CAM = Chorio-allantoic membrane

COPD = Chronic obstructive pulmonary disease

CR3 = Complement receptor 3

CRD = Cysteine-rich domain

DMSO = Dimethyl sulfoxide

EBM = Endothelial growth basal medium

EC = Effect concentration

ECM = Extra-cellular matrix

EDTA = Ethylene-diamineteraacetic acid

EGM = Endothelial growth medium

EMEM = Eagle's minimum essential medium

ERM = Ezrin-radiin-moesin

F-actin = Filamentous actin

FGF = Fibroblast growth factor

GTP = Guanosine triphosphate

HDF = Human dermal fibroblast

hiFBS = Heat-inactivated fetal bovine serum

HL-60 = Human Caucasian promyelocytic leukaemia 60 cells

HUVEC = Human umbilical vein endothelial cells

IC = Inhibitory concentration

IPF = Idiopathic pulmonary fibrosis

LC-MS/MS = Liquid chromatography – Tandem Mass spectrophotometry

LPA = Lyophosphatidic acid

MLC = Myosin light chain

MLCP = Myosin light chain phosphatase

MMP = Matrix metalloproteinase

NF-ĸB = Nuclear factor Kappa B

PDGF = Platelet-derived growth factor

PH = Pleckstrin homology

ROCK = Rho-associated coiled-coil kinase

RPMI = Roswell park memorial institute medium

S1P = Sphingosine-1 phosphate

SD = Standard deviation

SEM = Standard error of means

TGF-β = Transforming growth factor-bèta

VEGF = Vascular endothelial growth factor

Summary

Introduction: Rho kinases or ROCKs belong to the Rho family of small GTPases. ROCKs regulate a broad range of cellular processes that involve actin-filament assembly and cell contractility. Because of their function in these events, ROCKs are also involved in processes important in wound healing: inflammation, angiogenesis and fibrosis. Consequently, ROCK inhibitors could have potential therapeutic effects. However, ROCK inhibitors can also cause many side effects such as: lowering the blood pressure and vascular resistance. More selective ROCK inhibitors are necessary to avoid these side effects. For this reason, Amakem therapeutics develops ROCK inhibitors based on their 'localized drug action platform' to minimize systemic effects. These compounds are designed to become metabolically inactive by a controlled conversion, when outside the target organ. This study aimed to screen 10 of these compounds to select potential new ROCK inhibitors as therapeutic candidates for future pre-clinical development.

Material & methods: First, the stability of 10 compounds was tested in different biological fluids by LC-MS/MS. Next, several cell types were used for *in vitro* screening: endothelial cells (HUVEC), fibroblasts (HDF-1), Hela cells and inflammatory cells (HL-60). The compounds were tested on HUVEC and HDF-1 cell migration, HUVEC proliferation and on *in vivo* angiogenesis in a chorio-allantoic membrane (CAM) assay. In addition, the effects of the compounds on cofilin phosphorylation and on HL-60 transmigration were determined.

Results: The stability and cytotoxicity profile of the 10 compounds was first established. Based on these results, 6 compounds were selected for further screening in functional cell based assays. All compounds showed a significant stimulatory effect on HUVEC and HDF-1 migration. However, no effect on *in vivo* angiogenesis was found in the CAM assay. In the HUVEC proliferation assay a stimulatory effect of bFGF and VEGF was observed, yet the compounds did not intervene with this induced proliferation. The ROCK inhibitors showed a concentration dependent effect on cofilin phosphorylation. Finally, the transmigration assay demonstrated an inhibitory effect of compound 6.

Conclusion: Although the ROCK inhibitors had an effect on fibroblst and endothelial cell migration, no effect was found on HUVEC cell proliferation or on *in vivo* angiogenesis. In addition, most ROCK inhibitors also had a concentration-dependent effect on cofilin phosphorylation and compound 6 could inhibit neutrophil transmigration. Further experiments are needed to select potential new compounds for future preclinical development.

Samenvatting

Introductie: Rho kinases of ROCKs maken deel uit van de Rho familie van kleine GTP-ases. ROCKs reguleren een breed gamma aan cellulaire processen die gelinkt zijn aan het samenbrengen van actine filamenten en celcontractie. Doordat ze deze functies uitoefenen, zijn ROCKs ook betrokken bij belangrijke processen voor wondheling: inflammatie, angiogenese en fibrose. Bijgevolg kunnen ROCK inhibitoren potentieel therapeutische effecten hebben. Er zijn echter ook vele mogelijke bijwerkingen verbonden aan ROCK inhibitoren zoals: bloeddrukverlaging en een verlaging van de vasculaire resistentie. Daarom zijn er meer selectieve ROCK inhibitoren nodig om deze bijwerkingen te voorkomen. Dat is de reden waarom Amakem therapeutics ROCK inhibitoren ontwikkelt die enkel lokaal hun werking uitoefenen om bijwerkingen te minimaliseren. Deze moleculen zijn ontworpen om metabool inactief te worden door een gecontroleerde omzetting als ze eenmaal buiten het doelorgaan zijn. Het doel van deze studie is om 10 ROCK inhibitoren van Amakem therapeutics te screenen om dan nieuwe potentiële ROCK inhibitoren te kunnen selecteren als kandidaten voor toekomstig preklinisch onderzoek.

Materiaal en methoden: Eerst werden the 10 ROCK inhibitoren getest op hun stabiliteit in verschillende biologische stalen door de LC-MS/MS. Daarna begon het *in vitro* screeningproces in verschillende celtypes: endotheelcellen (HUVEC), fibroblasten (HDF-1), Hela cellen en inflammatoire cellen (HL-60). The ROCK inhibitoren werden getest op hun effect op HUVEC en HDF-1 celmigratie, HUVEC proliferatie en in een chorioallantoïs membraan (CAM) experiment. Daarnaast, werden de effecten op cofilin fosforylatie en het effect op HL-60 transmigratie bepaald.

Resulaten: Als eerste stap werd de stabiliteit en het cytotoxiciteit profiel van de 10 compounds bepaald. Aan de hand van deze resultaten gebeurde een selectie van 6 compounds voor verdere screening in functionele *in vitro* experimenten. Alle ROCK inhibitoren toonden een significant effect op HUVEC en HDF-1 cel migratie. Er werd echter geen effect gevonden op *in vivo* angiogenese in het CAM experiment. In het HUVEC proliferatie experiment werd er een verhoging van proliferatie waargenomen na behandeling van de cellen met de groeifactoren VEGF en bFGF, maar de compounds hadden geen effect op deze verhoogde proliferatie. Daarnaast werd er een concentratie afhankelijk effect waargenomen van de ROCK inhibitoren op cofilin fosforylatie en compound 6 heeft een inhibitie van neutrofieltransmigratie aangetoond.

Conclusie: Hoewel de ROCK inhibitoren een effect uitoefende op de migratie van endotheel cellen, was er geen effect op HUVEC proliferatie of op *in vivo* angiogenese. De overige experimenten toonde aan dat de meeste ROCK inhibitoren een concentratie afhankelijk effect hebben op de fosforylatie van cofilin en dat compound 6 neutrofieltransmigratie kan inhiberen. Toekomstige experimenten zijn nog nodig om potentieel nieuwe kandidaten te selecteren voor verder pre-klinisch onderzoek.

1.Introduction

1.1 Rho kinases

Rho kinases or ROCKs belong to the Rho family of small GTPases. ROCKs are serine/threonine kinases and two different isoforms exist, ROCK1 and ROCK2. These kinases are important regulators of different cellular processes such as cell motility, cell proliferation and apoptosis.

1.1.1 ROCK structure and localization

Structurally, ROCKs consist of different domains. A kinase domain that can phosphorylate target proteins, a coiled-coil Rho binding domain which facilitates the conversion from inactive to active conformation and the carboxyl domain that forms an auto-inhibitory region which can reduce the kinase activity of ROCKs. The C-terminus contains a pleckstrin homology (PH) domain with a cysteine-rich domain (CRD) (figure 1) [1, 2]. ROCK1 and ROCK2 have 65% overall sequence identity and these proteins show the highest similarity (92%) in the kinase domain [3, 4]. ROCKs are expressed ubiquitously throughout the body [3]. The intracellular location of ROCKs is mostly in the cytosol, however when active Rho is overexpressed, they are partly translocated to the membrane [2, 5].

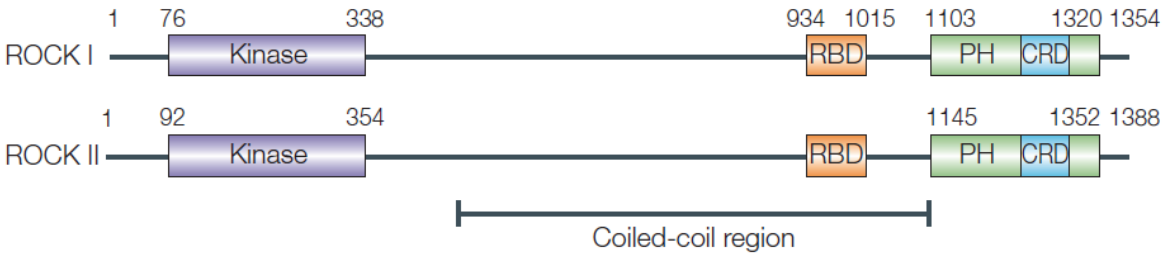

Figure 1 ROCK I and ROCK II protein structure. ROCKs consist of different domains: a kinase domain to phosphorylate target proteins, a coiled-coil Rho binding domain and a carboxyl domain [6].

1.1.2 ROCK pathway

ROCKs become activated when the active GTP-bound Rho interacts with their Rho-binding domain. Rho can be activated in response to different stimuli, e.g. lysophosphatidic acid (LPA) or sphingosine-1 phosphate (S1P). These effectors first stimulate Rho guanine nucleotide exchange factor (GEF) and this results in the formation of GTP-bound active Rho [7]. ROCKS regulate a broad range of cellular processes that involve actin-filament assembly and cell contractility by phosphorylating substrates such as myosin light chain (MLC) phosphatase, LIM kinases, adducin and ezrin-radiin-moesin (ERM) proteins [7-10] (figure 2).

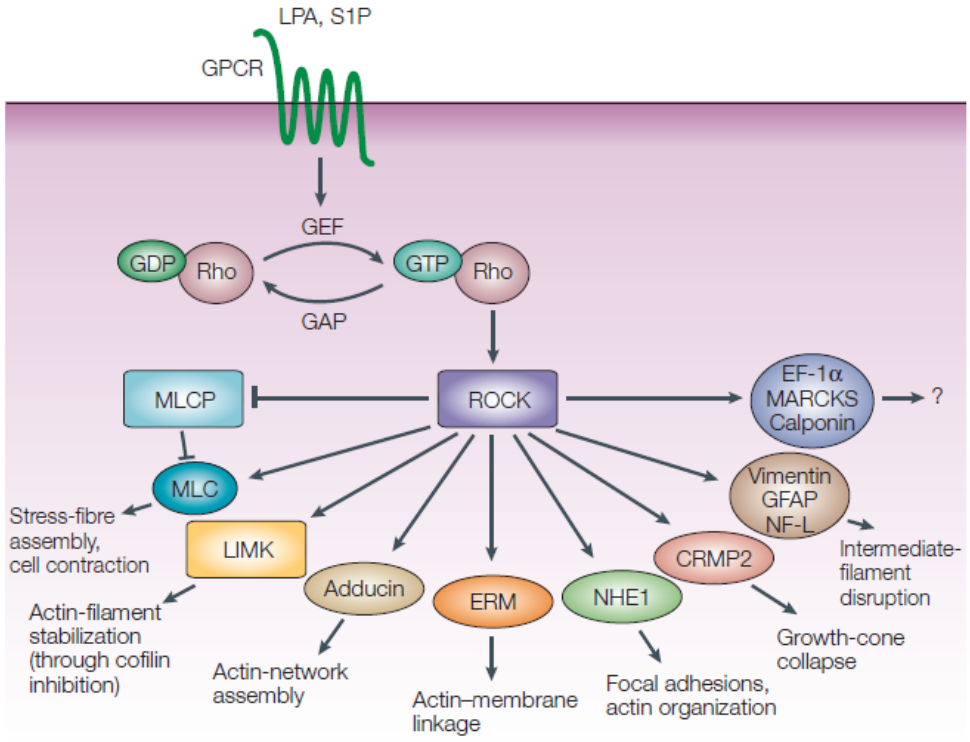

Figure 2 ROCK pathway. ROCK phosphorylates many different targets and in this way influences several cellular processes, such as: cell contraction, stress fiber assembly, actin-filament stabilization, actin reorganization [6].

ROCKs can mediate Ca2+ independent myosin-light-chain (MLC) activation by direct MLC phosphorylation or by phosphorylating MLC phosphatase (MLCP). MLCP is inactivated by phosphorylation, thereby inhibiting its role of inactivating MLC. Active MLC results in enhanced actin binding and in cell contraction (figure 2) [11-13]. LIM kinase (LIMK) 1 and 2, other substrates of ROCKs, are serine/threonine kinases involved in the regulation of actin filament dynamics [14]. Phosphorylation of LIMK 1 and LIMK 2 enhances their ability to phosphorylate cofilin [14]. Cofilin is an actin binding and depolymerizing protein responsible for regulating actin filament turnover [15]. Phosphorylation of cofilin inhibits its actin-filament-disassembly activity and thus results in a higher number of actin filaments (figure 2) [14].

Besides LIM kinases and MLCP, adducin is also phosphorylated by ROCKs. Adducin is a filamentous (F) actin binding protein and when phosphorylated it enhances its association with actin filaments (figure 2) [16, 17]. The regulation of this substrate is important for cell motility [16]. Other substrates of ROCKs are the ERM proteins which are closely related to adducin. They are necessary for the formation of cross-linkers between actin filaments and membrane proteins at the cell surface (figure 2) To summarize, by having a direct effect on different substrates and their functions, ROCKs have an important regulating role in several cellular processes.

ROCKS induce formation and contractility of actin stress fibers in several cell types [18]. These actions are believed to be mediated by the activation of MLC, causing cell contraction [13, 19]. MLC is very effective at cross-linking F-actin, thus increasing the bundling of stress fibers by F-actin and the consequential formation of contact points that link stress fibers to the extracellular matrix, the focal adhesions. Hence, acto-myosin contraction induces the assembly of stress fibers and focal adhesions

[20]. This mechanism is of importance in many cell types to induce shape changes e.g. vasoconstriction in vascular smooth muscle cells or destabilization of endothelial cell-cell junctions necessary for extravasation of lymphocytes [21, 22].

The ability to move requires the organization of several cellular activities: the extension of lamellipodia in front of the cell together with new cell-adhesions with the extra-cellular matrix [23]. ROCKs regulate these processes by rearrangements in these adhesions. This is coordinated by enhancing the formation of stress fibers and focal adhesions [22]. ROCKs also exert an effect on cell-cell adhesions in endothelial and epithelial cells, by regulating the tight and adherent junctions [24].

Rho also participates in phagocytosis where the reorganization of F-actin is involved. However, ROCKs are only needed in the pathway mediated by complement receptor 3 (CR3) but not when mediated by Fcγ receptors [25]. Moreover, in apoptosis the morphological cell changes necessary for blebbing, the loss of focal adhesions sites and subsequently the detachment of the cell from the substratum are all regulated by MLC phosphorylation and therefore also by ROCKs [26]. Cytokinesis also requires the action of ROCKs because of the actomyosin contraction in the cleavage furrow. It has been shown that ROCKs are responsible for the necessary MLC phosphorylation in the furrow during cytokinesis and that they are part of the pericentriolar material [27]. Finally, studies have shown that ROCKs are also linked to the control of cell size and cell differentiation [28].

Due to these numerous cellular functions, ROCKs are involved in important processes such as inflammation, angiogenesis and fibrosis. These processes will be further discussed in the next paragraph.

1.2 ROCKS in wound healing

Processes which generally contribute to wound healing are inflammation, angiogenesis and fibrosis. These processes are highly regulated. However, excessive inflammation, angiogenesis and fibrosis often lead to pathologies, such as COPD, inflammatory bowel disease, chronic kidney disease,… Rho kinases have been found to play a role in these processes.

1.2.1 Inflammation

Inflammation is a complex process that involves, among others, inflammatory cells (leukocytes) blood vessels and secretion of pro-inflammatory cytokines and chemokines.

Leukocytes have to leave the bloodstream in order to perform their function and reach the site of injury or infection [29]. During this transendothelial migration a sequence of events has to be coordinated such as, reorganizing the cytoskeleton and remodeling of cell adhesions. Proper cell movement is not only important during development, but also for wound healing and inflammation [30]. To achieve migration, the adhesive properties of leukocyte cell-surface molecules are dynamically regulated. There are several types of cell-surface molecules involved such as, selectins and integrin's that control the adhesion molecule cross-talk. The development of membrane protrusions and new adhesive contacts in a migrating cell is coordinated by the retraction of the rear-end of the cell and by downregulation of adhesions. The actin cytoskeleton, regulated by the Rho family, responds to extracellular signals by changing the shape and adhesive properties of the cell [31]. Indicating the important role of ROCKs in inflammation.

Next to leukocytes, endothelial cells, that line the blood vessel wall, are evenly important in regulating transendothelial migration. These cells secrete chemokines to attract the leukocytes and express the necessary adhesion molecules by which leukocytes can adhere to the vessel wall. This expression can be induced by inflammatory stimuli. Chemokine and adhesion molecule activation is also necessary for disruption of intercellular junctions, to ensure para-cellular passage of leukocytes [29, 31]. NF-KB is a transcription factor involved in regulating inflammatory cytokines and mediating various immune and inflammatory responses. Studies have shown that ROCKs are implicated in the modulation of the NF-KB pathway and T cell proliferation [32]. LPA, a Rho-activator, is able to induce the expression of adhesion molecules by activating the ROCK and NF-KB pathway in human endothelial cells. LPA is a pro-inflammatory mediator, which is elevated in several diseases [33]. He et al. showed that ROCKs stimulate pro-inflammatory cytokine production [32]. The inflammatory reaction towards injured tissues and microbes is not only required to eliminate these stimuli but also to initiate the repair processes.

Studies have shown that inhibition of Rho or ROCKs leads to an inhibition of the migration of monocytes, neutrophils, HL-60 cells and eosinophils [2, 34, 35]. The ROCK inhibitor Y-27632 impaired the NF-KB activity in leukocytes and intestinal endothelial cells from patients with Crohn's disease, which suggest that ROCK inhibitors might be valuable in the treatment of inflammatory bowel disease [36]. Moreover, Aihara et al. demonstrated that Y-27632 reduced the number of Th1 and Th2 positive cells and suppressed their release of cytokines in the alveolar walls in asthmatic patients [37].

1.2.2 Angiogenesis

Angiogenesis is a process where new blood vessels sprout from pre-existing ones [38]. It is activated by chemokines and growth factors produced by inflammatory cells. In physiological circumstances, angiogenesis is an essential process during development and wound repair. However, when dysregulated and persistent, angiogenesis can be a causal factor in a number of pathologies [39]. Abnormal angiogenesis or endothelial cell function is involved in cancer, ischemic and inflammatory diseases.

Angiogenesis is a multi-step process that involves a complex series of events. First, increased permeability of the basement membrane enables a new capillary to sprout. Second, endothelial cells are activated by angiogenic factors and then migrate through the basement membrane towards the angiogenic stimuli. This migration is driven by enhanced proliferation of the leading migrating cells. The third step involves re-organization of the endothelial cells to assemble into new capillaries. This assembly occurs in cooperation with recruited pericytes and vascular smooth muscle cells [38]. A strict balance of pro- and anti-angiogenic factors is required to regulate angiogenesis. An example of an important pro-angiogenic factor is vascular endothelial growth factor (VEGF), which is a major promotor of physiological and pathological angiogenesis. In this process, VEGF regulates the migration and proliferation of endothelial cells by activating different kinase pathways [40]. One of these pathways is the ROCK pathway. VEGF stimulates the activation of RhoA which is necessary for the reorganization of the F-actin cytoskeleton [38].

Besides growth factors, cell-cell interactions and interactions with extra-cellular matrix (ECM) proteins or tissue enzymes are also involved in the process of angiogenesis. ECM proteins participate in the vessel sprouting process by interacting with the integrin receptors on endothelial cells. Matrix metalloproteinases (MMPs) are enzymes that degrade the ECM to allow remodeling and extension of

the vascular tube [41]. MMP-9, which is primarily activated in tissue repair and remodeling processes, is influenced by ROCK inhibitors. Righetti et al. showed that MMP-9 positive cells are reduced after ROCK inhibition [42].

Emerging evidence shows that LPA, the Rho-activator, is also an important regulator of endothelial cell function and angiogenesis. It is a pro-angiogenic factor and can therefore also promote pathological angiogenesis [38]. Since ROCKs are implicated in all key steps of angiogenesis, ROCK inhibitors have been studied for their potential therapeutic effect in diseases with persistent angiogenesis. H1152, a ROCK inhibitor, increased the sprouting activity and the directional cell motility of human umbilical vein endothelial cells (HUVECs). In contrast, other studies have shown a reduced migration of HUVECs after treatment with ROCK inhibitors. These discrepancies could be explained by using a different type of endothelial cell or a different *in vitro* model. ROCK inhibition with H1152 also reduced intracellular stress fibers without intervening with the formation of lamellipodia. Moreover, cell-cell contacts are loosened after treatment with H1152, which also contributes to a more directional movement of the cells [43].

1.2.3 Fibrosis

As mentioned before, in injured tissues, inflammation is followed by wound repair. Repair by scar formation includes different steps: angiogenesis, deposition of connective tissue by activated fibroblasts and remodeling of the fibrous tissue to produce a stable scar. Deposition of the connective tissue requires activation, proliferation and migration of fibroblasts towards the site of injury, where they produce ECM proteins to form connective tissue. The main ECM proteins are collagen and fibronectin. Fibroblast recruitment and activation is driven by several growth factors. The major source of these growth factors are the inflammatory cells present at the site of injury. The involved growth factors include: fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor-bèta (TGF-β) [41, 44].

FGF is produced by many cell types and also contributes to the stimulation of endothelial cell proliferation during angiogenesis. In addition, it induces the migration of macrophages and fibroblasts towards the damaged area and triggers epithelial cell migration to cover the injured tissue. PDGF is produced by several cell types: endothelial cells, smooth muscle cells, activated macrophages and is stored in platelets. It causes migration and proliferation of fibroblasts. Another important stimulating factor for scar formation is TGF-β. This is an anti-inflammatory cytokine that abolishes the inflammatory reaction by inhibiting lymphocyte proliferation and activity. In the wound-healing process, TGF-β has different functions: attracting neutrophils, stimulating fibroblast migration and production of collagen and fibronectin, and inhibition of the degradation of collagen. Moreover, TGF- β induces the proliferation and differentiation of fibroblasts to its active form, the myofibroblasts, which is the primary cell type for the production of ECM proteins in damaged tissue. Generally, this differentiation occurs to promote wound healing and afterwards apoptosis of the myofibroblasts is induced. In pathological circumstances, TGF-β can be abnormally activated resulting in fibrosis. Fibrosis is defined by the excessive deposition of ECM components in chronic diseases. The mechanism of tissue repair is the same in fibrosis as in scar formation, however fibrosis is induced by persistent injurious stimuli such as infections [41, 44].

The wound healing process including fibroblast recruitment, myofibroblast differentiation, and re-epithelialization, all involve the reorganization of the cytoskeleton of participating cells. This reorganization is directed by the ROCK pathway. ROCK activation has been shown in the lungs of patients with idiopathic pulmonary fibrosis (IPF). Moreover, increased ROCK activity has been established in specific areas of lung fibrosis of mice and humans. In addition, it appears that ROCK signaling is also involved in pro-fibrotic responses of epithelial and endothelial cells in lung tissue. This suggests that ROCKs are major regulators of pulmonary fibrosis and ROCK inhibitors may be good drug candidates for this disease [45]. It has been shown that Rho activates transcription factors targeting different known drivers of fibrosis. Moreover, Tsou et al. demonstrated that LPA has a key role in the development of tissue fibrosis in different organs. The ROCK inhibitor, Y-27632, has been shown to inhibit myofibroblast differentiation and collagen deposition. Also, *in vivo* treatment of mice with the ROCK inhibitor fasudil protected the mice against lung fibrosis [38, 41, 44].

1.3 Research project

Studies have shown that ROCK is involved in several diseases and that inhibition of ROCK shows advantageous effects. Therefore, a lot of effort has been put in the development of ROCK inhibitors. However, ROCK inhibitors can cause many side effects because of the wide abundance of ROCKs. ROCK inhibition can lower blood pressure and vascular resistance on a systemic level. Additionally, high doses can affect other kinases in the body [46]. Consequently, the development of selective ROCK inhibitors might be a way to avoid side effects.

For this reason, the company Amakem therapeutics generates new soft ROCK inhibitors based on their 'localized drug action' platform to minimize systemic effects. This means that the compounds are designed to become metabolically inactive by a controlled conversion, when outside the target organ. This inactivation mechanism is established by the specific design of the compounds which makes them possible substrates for esterases.

In this research project the aim was to screen 10 ROCK inhibitors produced by Amakem therapeutics to select the best ones for future preclinical development. In this screening process, first the stability of the compounds will be determined in cell culture medium and in biological fluids such as human whole blood and plasma. Thereafter, the selection procedure will be based on the functional outcome of the compounds in *in vitro* assays on different cell types. The purpose is to test the effects on endothelial and fibroblast cell migration, endothelial cell proliferation and for a few selected compounds on *in vivo* angiogenesis in a chorio-allantoic membrane (CAM) assay. In addition, the effect on cofilin phosphorylation and transmigration of inflammatory cells will be tested.

2. Material and methods

2.1 ROCK inhibitors produced by Amakem therapeutics

The ROCK inhibitors designed and produced by Amakem therapeutics are soft drugs. This means that they are designed to undergo metabolic inactivation by a controlled conversion into a predictable nontoxic metabolite. This soft property was achieved by the introduction of ester groups that can be hydrolyzed in blood/plasma by esterases, resulting in metabolites with decreased activity and permeability. In this research project compounds 1 to 10 were examined. Compound 1 is not a soft ROCK inhibitor produced by Amakem therapeutics but is actually the reference compound Y27632, often used in literature. This compound has already shown some promising results in different pathologies such as corneal wound healing [47, 48].

2.2 Biochemical analysis: Stability assay

The stability of the ROCK inhibitors was tested in cell culture medium and biological fluids. These fluids were incubated for 10 minutes in a thermomixer at 37°C, 300 rpm. Next, the compounds were added, resulting in a mixture containing 10µM of compound. At different time points, samples were taken from the mixture. These time points differ for each type of fluid (table 1). Next, protein precipitation was initiated by adding them to STOP solution containing acetonitrile (ACN; MerckMillipore, Overijse, Belgium) and the internal standard metoprolol leading to a final compound concentration of 1 µM in the samples. After 10 minutes centrifugation at 14000 rpm and 4°C, the supernatant was transferred to a deepwell plate. The remaining amount of compound in the samples was analyzed by LC-MS/MS (3200 Q Trap, Applied Biosystems, Lifetechnologies, Gent, Belgium). The results were analyzed to determine the remaining percentage of compounds at the different time points. From these percentages the half-life of the compounds was calculated. After performing this assay twice the half-life of the different compounds could be compared.

Table 1 Assay conditions for different tissues

Sample	**Time points**
Medium	0 – 1 – 3 – 5 – 7 – 24 hours
Human plasma	0 – 5 – 10 – 15 – 20 – 60 – 120 minutes
Human whole blood	0 – 15 – 30 – 60 minutes
Chicken plasma	0 – 20 – 60 minutes

2.3 Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switserland) were maintained in EBM-2 endothelial growth basal medium with EGM-2 growth supplements: hydrocortisone, recombinant human fibroblast growth factor-B, recombinant human vascular endothelial growth factor, recombinant long R insulin-like growth factor-1, gentamicin sulfate amphotericin-B, ascorbic acid, heparin, recombinant human epidermal growth factor (all from Lonza) and 10% heat-inactivated fetal bovine serum(hiFBS, Sigma, St. louis, USA). Fresh medium was added every 2-3 days. These cells were cultured in gelatin-coated flasks (0.2% gelatin; Sigma) and subcultured when 80% confluency was reached; to loosen the cells 0.25% trypsin/EDTA solution (Sigma) was used. For the functional assays, cells from passages 5-13 were used.

Human dermal fibroblasts (HDF-1) (Sigma) were maintained in fibroblasts growth medium (Sigma) supplemented with hi-FBS (2%; Sigma) and cultured in cellbind corning cell culture flasks (Sigma). Fresh medium was added every 2-3 days and for the functional assays cells from passage number 3-8 were used. Cells were subcultured when 80% confluency was reached and loosened with 0.25% trypsin/EDTA solution.

Human Caucasian promyelocytic leukaemia cells (HL-60) (Sigma) were maintained in RPMI 1640 medium (Sigma) supplemented with 2mM glutamine and 10% hi-FBS. These cells were subcultured every 4-5 days and passages 10 to 22 were used for functional assays.

HeLa cells were maintained in Eagle's Minimum Essential medium (EMEM; all from LGC standards-ATTC, Molsheim Cedex, France) supplemented with 10% hi-FBS and cultured in cellbind corning cell-culture flasks. Fresh medium was added every 2-3 days and cells were subcultured when 70% confluency was reached. To loosen the cells 0.25% trypsin/EDTA solution was used. For functional assays passages 10 to 24 were used.

All cells were incubated in a humidified incubator at 37°C and 5% CO_2.

2.4 Viability assay

The effect of ROCK inhibitors on cell viability of different cell types was tested using the WST-1 viability assay. Cells were seeded in 96-well plates and incubated for 24 hours with different compound and equivalent (eq.) DMSO (compound solvent) dilutions: 0.01µM, 0.1µM, 1µM, 10µM, 20µM, 40µM, 80µM and 100µM. In addition, the following controls were included: blank (assay medium), cells without compound and a negative control (staurosporine, 1µM; Tocris, Bristol, United Kingdom; table 2). Next, the cells were incubated with the cell proliferation reagent WST-1 (10µl WST-1/100µl cell suspension; Roche-diagnostics, Vilvoorde) for 2 hours. Only for the Hela cells the protocol was slightly different, these cells were incubated for 1h with the compounds and 1h with the WST-1 reagent.

WST-1 is a tetrazolium salt that is converted to formazan by cellular enzymes. An increase in viable cells results in a higher amount of formazan dye. The absorbance of the formazan dye was measured (Fluostar Omega V1.30, BMG labtech, Temse Belgium) at 450nm and at a reference wavelength of 630nm. This reference value shows the background signal and was therefore subtracted from the absorbance value at 450nm. This assay was performed three times and the mean was then used to calculate the EC_{50} value of each compound.

2.5 Apoptosis assay

The effect on cell apoptosis of different ROCK inhibitors was tested by performing a caspase 3/7 assay in different cell types. Cells were seeded in 96-well plates and incubated for 24 hours with different compound and equivalent DMSO dilutions: 0.01µM, 0.1µM, 1µM, 10µM, 20µm, 40µM, 80µM and 100µM. Moreover, the following controls were included: blank (assay medium), cells without compound and a positive control (staurosporine, 1µM; Tocris; table 2). The next day, the cells were incubated with caspase 3/7 reagent (1:1 ratio; Promega, Mannheim, Germany) for 1 hour, protected from light. This caspase reagent contains a caspase 3/7 substrate DVED and is optimized for cell lysis, luciferase activity and caspase activity. Adding the mixture to the cells results in cell lysis and the release of caspase 3 and 7. These caspases cleave the DVED substrate, resulting in a luciferase reaction and production of light. To measure the luminescent signal (Fluostar Omega V1.30) the well content was transferred into a white plate. This assay was performed three times and

the mean was compared to the equivalent DMSO controls to determine at which concentration a significant difference in caspase 3/7 activity could be observed.

2.6 Migration assay

The effect of ROCK inhibitors on the migration of HUVEC and HDF-1 cells was determined via a 2D migration assay. The cells were seeded in 48-well plates, to produce a monolayer of cells (table 2). When a confluent monolayer of cells was obtained, a wound was made in this monolayer with a 200µl pipette tip. Next, the cells were washed with PBS (Invitrogen) and different compound and eq. DMSO dilutions in cell culture medium were added: 0.01µM, 0.1µM, 1µM and 10µM. At different time points (t0-t8) the wound width was measured. This assay was performed three times and the mean percentage of wound closure was calculated by comparison to time point 0. This percentage wound closure was compared to the equivalent DMSO control condition (figure 3).

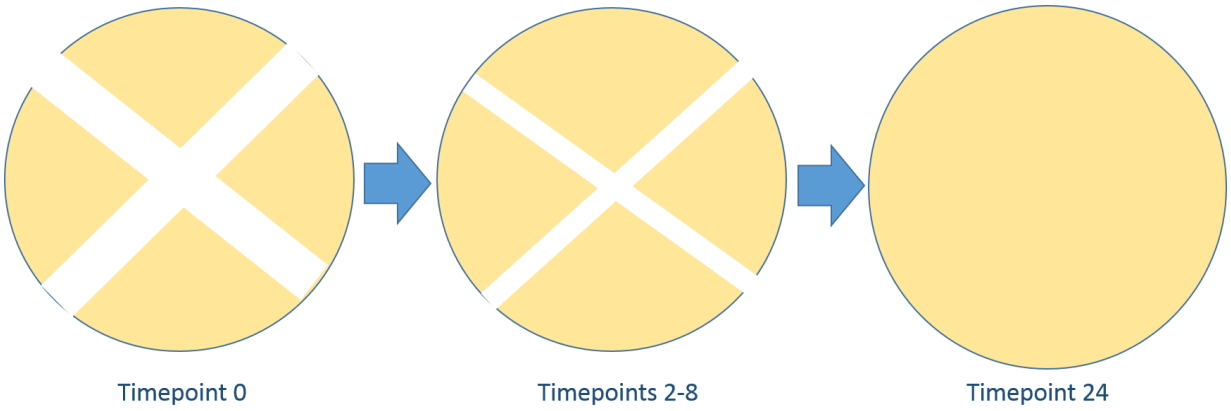

Figure 3 Migration assay. Cells were seeded to create a monolayer. A wound was made in this monolayer to track the migration of the cells. Throughout the day the wound width was measured at different time points, after 24 hours the wound should be closed.

2.7 Experimental conditions

Table 2 Experimental conditions per cell type

	HUVEC	**HDF-1**	**HL-60**	**Hela**
Assay medium	1:10 growth medium in EBM-2 basal medium + 40ng/ml VEGF (Sigma)	Growth medium	RPMI 1640 + 2.5% hiFBS	Growth medium
Viability assay	10.000 cells/well seeded overnight	7.500 cells/well seeded overnight	25.000 cells /well seeded at once with the different conditions	20.000 cells /well seeded overnight
Apoptosis assay	10.000 cells/well seeded overnight	10.000 cells/well seeded overnight	25.000 cells /well seeded at once with the different conditions	-

Migration assay	Seed 30.000 cells for 3 days	Seed 20.000 cells for 3 days	-	-
	Gelatin (0.2%) coated 48-well plates, flat bottom	Cell-binding 48-well plates	-	-

2.8 BrdU assay

To test the effect of ROCK inhibitors on the proliferation of HUVECs, a BrdU assay was performed. For this assay HUVECs were seeded at 5.000 cells/well in a 96-well plate. After at least 4 hours the cells were incubated with serum starvation medium (EBM-2 + 0.1% hiFBS) for 24 hours starting from seeding time. Next, the medium was discarded and different conditions were tested. Two stimuli were compared in this assay: basic FGF (bFGF; Sigma; 10 ng/ml) and VEGF (40ng/ml). The experimental conditions were: serum starvation medium without stimuli, serum starvation medium with stimuli, compound dilutions (0.01µM, 0.1µM, 1µM) and 1µM DMSO. On day 3, 24 hours after adding the conditions, the BrdU labelling reagent (1/1000) was added. BrdU incorporates into the newly formed DNA strands in replicating cells, which enables the detection of cell proliferation. One condition containing serum starvation medium was kept without stimuli and without BrdU to look at the background signal. On the last day, 24 hours after adding the BrdU labelling reagent, the cells were fixated for 30 minutes. Next, blocking buffer was added and incubated for 30 minutes, to limit aspecific binding of the BrdU-antibody to other enzymes. Peroxidase-labeled anti-BrdU working solution, containing the BrdU-antibody, was added for 90 minutes. Afterwards, the plates were washed three times with washing buffer solution to remove all unbound antibodies. Next, the TMB substrate was added to start the colorimetric reaction with the antibody (all buffers were diluted according to the kit protocol; all from GE Healthcare, Buckinghamshire, UK). After approximately 5 minutes the reaction was stopped by adding 1M sulphuric acid. The absorbance was measured at a wavelength of 450nm and a reference wavelength of 630nm to measure the background signal. The condition containing serum starvation medium without stimuli and without BrdU was subtracted from the absorbance values. After repeating the assay three times, the mean was calculated and these values were compared to the stimuli conditions to determine if there was a significant effect of the compounds on induced cell proliferation.

2.9 CAM assay

To test the effect of ROCK inhibitors on angiogenesis, an assay was performed on the chorioallantoic membrane (CAM) of a chicken egg. On day 0, fertilized eggs of white leghorn chickens (Wijverkens Geert Kippenkwekerij, Halle, Belgium) were placed in an incubator (Polyhatch from Brinsea, Titusville, USA) at 38°C with maximum humidity, under rotation. On day 3, 3ml egg white was removed with a needle and a window was made with a little saw to expose the CAM. The viability was checked by determining the presence of a heart-beat and the window and needle hole were closed off with transparent tape to prevent dehydration. The eggs were placed back in the incubator without rotation. On day 10, a silicone ring was placed on the CAM and after 3 hours, treatment was initiated. As a control, seven eggs were treated with 1µM eq. DMSO (spread over 2 experiments) and 6 eggs with 0.3µM eq. DMSO. Compound 2 was tested at 1µM in 10 eggs (spread over 2

experiments) and at 0.3µM in 5 eggs. Compound 6 was tested at 0.3µM in 2 eggs and at 0.1µM in 4 eggs. Compound 8 was tested in 5 eggs at 0.3µM and in 7 eggs at 0.1µM. The compounds and DMSO were diluted in filtered saline and pipetted into the silicone ring on the CAM. The eggs were closed off again and placed back into the incubator. On Days 11, 12 and 13 treatment was repeated with the same concentration of compound or eq. DMSO solutions. On day 14, the day of sacrifice, the windows were made bigger to expose the CAM completely and a white viscous solution, made out of salad oil and zinc oxide, was injected underneath the CAM to visualize the blood vessels on the CAM. Next, pictures were made with an overview of the CAM and the ring, inside the ring and outside the ring. The vascular density was analyzed by projecting five concentric circles onto the picture inside the ring and counting the blood vessels crossing these circles, this was done by three independent blinded persons. The mean vascular density of the compound conditions were compared to the mean of the control conditions with eq. DMSO concentrations.

2.10 Cofilin assay

To test the effect of ROCK inhibitors on LIM kinase activity and their ability to phosphorylate cofilin, a cofilin assay was performed on Hela cells, according to the protocol of PerkinElmer (PerkinElmer, Zaventem). The cells were seeded at 20.000 cells/well in a 96-well plate and incubated for 1h with different compound dilutions: 1pM, 0.01nM, 0.1nM, 1nM, 0.01µM, 0.1µM, 1µM, 10µM, 25µM and 50µM. The cells were lysed for 10 minutes while shaking at 350rpm. Next, the lysates were transferred to a 384-well plate, incubated with acceptor beads, shaken for 2 minutes at 350 rpm and incubated for 2h at room temperature. Finally, the donor beads were added under low light conditions, the plate was shaken at 350 rpm for 2 minutes and further incubated for 2h in the dark. The donor and acceptor beads are brought in close proximity of each other by binding to antibodies that specifically recognize phosphorylated cofilin (figure 4). By excitation of the donor beads, the acceptor beads were activated to emit light. The amount of light was proportional to the amount of phosphorylated cofilin present in the cell lysates. This signal was measured (EnSpire Multimode Plate Reader, PerkinElmer) and the average value of two experiments were used to calculate an $EC_{50,}$ value.

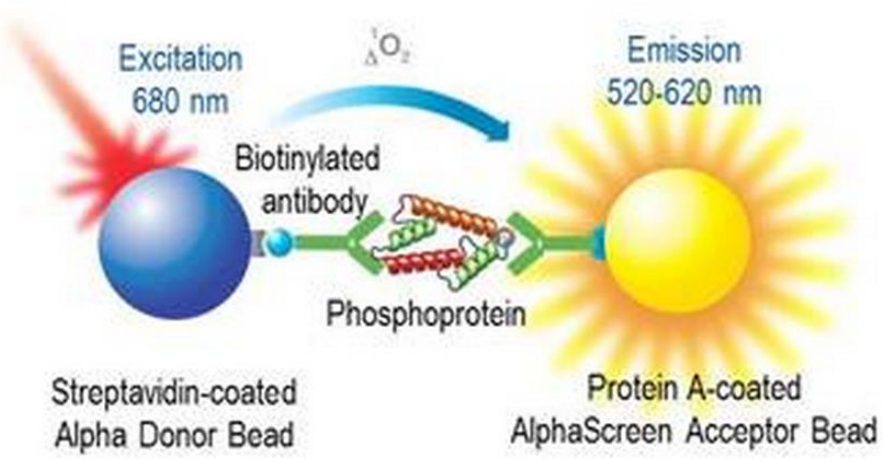

Figure 4 Cofilin assay prinicple. The donor and acceptors beads are brought in close proximity of each other by binding antibodies that recognize phosphorylated cofilin. Excitation of the donor beads activates the acceptor beads to emit light [(49)].

2.11 Transmigration

To test the effect of ROCK inhibitors on the transmigration of HL-60 cells, a 3D transmigration assay was performed. This assay mimics the transendothelial migration of inflammatory cells towards the site of injury. First, the HL-60 cells were differentiated to neutrophils by incubating them with DMSO (1.3% per 150 000 cells/ml (Sigma) for 5-6 days. To test whether these cells have differentiated, their caspase 3/7 activity was measured, which should be 200% compared to undifferentiated HL-60 cells [(50)]. Secondly, the differentiated HL-60 (dHL-60) cells were incubated with compound or DMSO dilutions (0.01µM, 0.1µM, 1µM and 10µM), or assay medium (control), for 30min at 37°C. These solutions were put in the upper chamber of the transwell system. The chemoattractant conditions (10nM, fNLPNTL, Bachem AG) were put in the lower chamber of the transwell system. Here, the same conditions as the upper chamber were tested, next to a control condition with assay medium without chemoattractant [(35, 51)]. The transwell membrane had a pore size of 0.5µm (figure 5; Transwell Costar 5µm polycarbonate membrane, Sigma). After 4h incubation of the cells in the transwell system, the cells in the lower chamber were counted manually. This experiment was performed three times and results were compared to the equivalent DMSO control, to detect a significant effect.

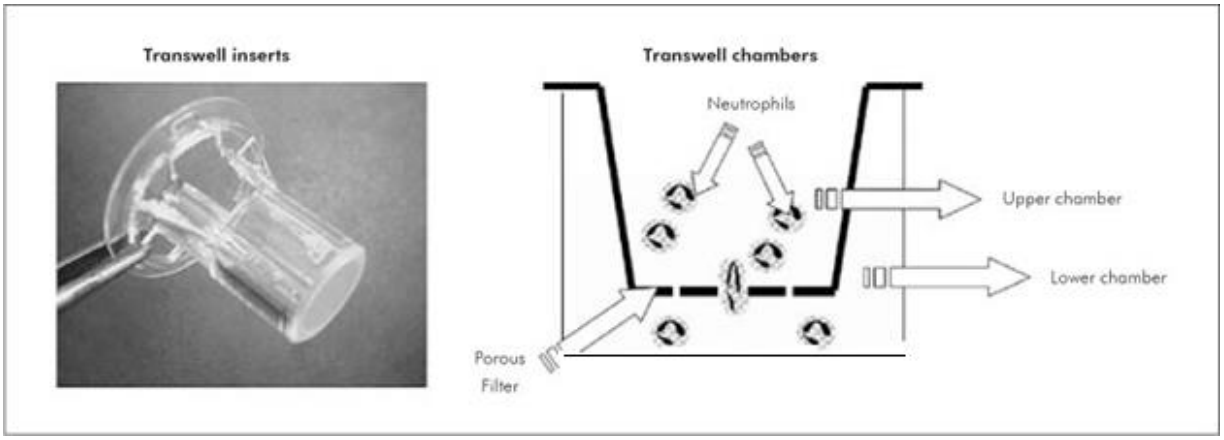

Figure 5 Transwell system. In the upper and lower chamber the same compounds dilutions were added. The cell conditions were placed in the upper chamber and the chemoattractant conditions in the lower chamber [(52)].

2.12 Statistics

Data are presented as means ± SD or SEM of n independent experiments. A paired t-test was used to compare 2 groups in the apoptosis, BrdU, transmigration and CAM assay or a 2-way ANOVA was used to compare multiple measurements in the migration assay (Graphpad Prism 5). A p-value of <0.05 was considered significant and was indicated with *, a p–value of <0.005 and <0.0005 were indicated as ** and ***.

3. Results

3.1 The On target potency of ROCK inhibitors from Amakem therapeutics

The compounds used in this research project were tested for their on-target potency; this was measured externally at Reactions Biology Corporation (RBC; Mavern, PA, USA). The results in table 3 show the IC_{50} for ROCK2, this is the concentration in nM where 50 percent of ROCK2 was inhibited while 1µM ATP was also present. The ROCK inhibitors go in competition with ATP to bind to ROCK. Moreover, the effect of the compounds on the most important substrate of ROCK, MLC, was tested. This was also measured externally at Proquinase GmbH (Freiburg, Germany). Here, the results show the $EC_{50,}$ which is the concentration needed to reduce 50 percent of phosphorylated MLC (MLCP-PP).

Table 3 On-target potency and effect on MLC phosphorylation of the tested compounds. The IC_{50} for ROCK2 shows the concentration where 50% of ROCK2 is inhibited while 1µM ATP was present. The $EC_{50,}$ for phosphorylated MLCPP shows the concentration where a reduction of 50% of MLC-PP was obtained.

Compound	IC_{50} ROCK2 (nM)	EC_{50} MLC-PP (nM)
1	53.7	1427
2	<1	5.4
3	<1	15
4	<1	15.9
5	<1	5.3
6	<1	4.7
7	<1	19
8	3.6	16
9	3.9	15

3.2 The stability profile of ROCK inhibitors from Amakem therapeutics

Amakem therapeutics produces soft ROCK inhibitors based on their 'localized drug action' platform. To test this property, the compounds were analyzed in a stability assay in human whole blood and plasma.

In human whole blood compounds 1, 4, and 10 were stable and had a half-life of more than 60 minutes. In contrast, compound 6 had the lowest half-life of 9 minutes. Compounds 2, 3, 5, 7, 8 and 9 had a half-life between 28.5 and 58.2 minutes (table 4). In human plasma, compounds 1 and 10 were stable and had a half-life of more than 60 minutes. Compound 7 had the lowest half-life of 24.1 minutes and all other compounds had a half-life between 35.6 and 59.5 (table 4). The stability profile for some of the compounds was different in plasma compared to whole blood. For example: compounds 5, 6 and 8 were more stable in plasma compared to whole blood and compounds 4, 7 and 9 were more stable in whole blood than in plasma.

Table 4 Stability test human whole blood and human plasma. Half-life in minutes, data are presented as means± standard deviation (SD) of 5 donors and two pooled plasma samples n=2.

	Human whole blood		Human plasma	
Compounds	Average (half-life in minutes)	SD	Average (half-life in minutes)	SD
1	>60,0	0,0	>60,0	0,0
2	56,7	7,4	56,4	7,4
3	58,2	3,9	59,5	0,9
4	>60,0	0,0	58,8	3,1
5	34,8	11,4	51,8	17,0
6	9,0	3,1	47,2	19,1
7	33,1	16,4	24,1	21,1
8	28,5	13,6	46,9	15,6
9	52,5	10,6	35,6	23,2
10	>60.0	0,0	>60,0	0,0

3.3 The effects of ROCK inhibition on HUVEC and HDF-1 cell migration

Before testing the effect of the ROCK inhibitors on endothelial cell (HUVECs) migration, the effects on the cell viability and apoptosis with different compound concentrations were assessed. The results of this viability assay are shown as the concentration of compound where cell viability is reduced to 50 percent, the EC_{50} value. This EC_{50} differed between compounds, e.g. 1 has a high value compared to the low EC_{50} of 10 (table 5).

Next, the effect on apoptosis was tested. The result of the apoptosis assay is shown as the concentration of compound from which there is a significant difference compared to the equivalent DMSO control condition. Some compounds showed no significant difference due to a large variation between experiments. Others showed a significant difference with the equivalent DMSO control at a relatively high compound concentration e.g. 6 and 7 (table 5).

Based on the results of these assays, 6 compounds were selected for further testing in a 2D-migration assay: compounds 1, 2, 3, 4, 6 and 8.

Table 5 Viability and apoptosis assay HUVEC cells. Results of the WST-1 viability assay are shown as an EC_{50} value: the concentration where cell viability is reduced to 50%. The results of the apoptosis assay are shown as the concentration from which on there is a significant difference with equivalent DMSO control. Non-significant (Ns), n=3.

Compounds	**WST-1 (EC_{50})**	**Caspase 3/7 activity**
1	>100µM	≥40µM
2	15µM	Ns
3	43µM	≥40µM
4	25µM	Ns
5	12µM	≥40µM
6	23µM	≥80µM
7	~10µM	≥100µM
8	14µM	≥80µM

9	~10µM	≥80µM
10	~1.5µM	Ns

In a 2D-migration assay, the migration of HUVEC cells over a coated surface was analyzed after treatment with the compounds. Different concentrations of compounds were tested and compared to an equivalent DMSO control. These concentrations were determined based on the results of the viability and apoptosis assay, which disclosed the non-toxic range of concentrations of each compound. All compounds showed a significant reduction in wound width after 8 hours compared to the DMSO control (figure 6). However, compounds did not stimulate migration at similar concentrations. Compound 1 only showed a significant difference ($p=0.0402$) in wound width at the highest concentration (10µM), compared to compound 2 which only demonstrated a significant effect in lower concentrations (0.01µM ($p=<0.0001$) and 0.1µM ($p=0.0001$)). In addition, compound 6 and 8 also had a significant effect at 0.01µM ($p=0.015$ and $p= 0.0377$). These results suggest that compounds 2, 6 and 8 are more potent in this particular assay compared to the other compounds.

Migration assay HUVEC

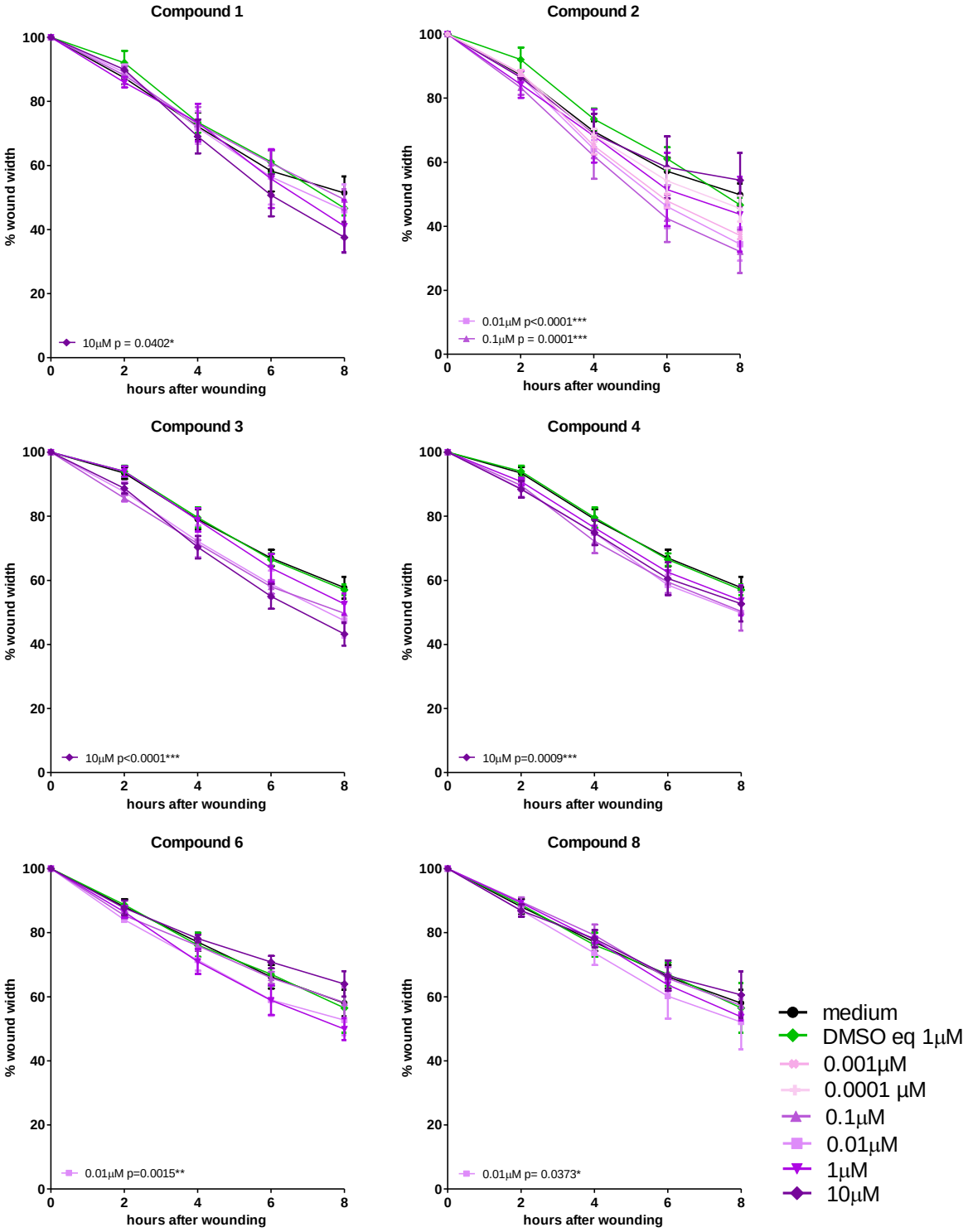

Figure 6 Migration assay HUVEC cells. The HUVEC cells were seeded to produce a monolayer of cells and subsequently a wound was made. The wound width was measured throughout the day after 2, 4, 6 and 8 hours. Different compound concentrations were tested: (0.0001µM, 0.001µM) 0.01µM, 0.1µM, 1µM and 10µM. The results show significant differences for all compounds compared to the equivalent DMSO controls. Data are presented as means ±SEM, n=3.

Next to endothelial cells, the effect of ROCK inhibitors on fibroblast (HDF-1) migration was also assessed. Similar to the HUVEC cells, first, effects on cell viability (WST-1) and apoptosis (caspase 3/7 activity) were determined.

The EC_{50} values differed between compounds, e.g. 1 again showed a high EC_{50} value for these cells compared to 10 which has a low EC_{50}. In the apoptosis assay these cells showed little significant effects because of a large variation between experiments. Only compound 9 demonstrated a significant difference at 80µM (table 6). Based on these results, the same 6 compounds as for the HUVEC cells were selected for testing in the 2D-migration assay with HDF-1 cells.

Table 6 Viability and apoptosis assay HDF-1 cells. Results of the WST-1 viability assay are shown as an EC_{50} value: the concentration where cell viability is reduced to 50%. The results of the apoptosis assay are shown as the concentration from which on there is a significant difference compared to the equivalent DMSO control. Non-significant (Ns), n=3.

Compounds	**WST-1 (EC_{50})**	**Caspase 3/7 activity**
1	>100µM	Ns
2	29.44µM	Ns
3	17.55µM	Ns
4	42,78µM	Ns
5	19.05µM	Ns
6	29.95µM	Ns
7	14.99µM	Ns
8	22.14µM	Ns
9	34.53µM	≥80µM
10	~9.96µM	Ns

A similar protocol as for the HUVEC 2D-migration assay was used for this assay with the HDF-1 cells. The wound width was measured throughout the day and compared to an equivalent DMSO control. Similar to HUVEC, almost all compounds showed a significant difference in wound width after 8 hours in the monolayer of HDF-1 cells (figure 7), except for compound 4. However, the concentration by which this significant difference occurred varied between compounds. Compounds 1 and 2 only induced a significant stimulation of migration at 1µM (p=0.0234 and p=0.0082) compared to compounds 3, 4 and 8 which only showed a significant difference at 10µM (p=0.0044, p=0.0575 and p=0.0263). In contrast, compound 6 generated a significant reduction in wound width at 0.1µM, 1µM and 10µM (p=0.0012, p=0.0059 and p=0.0268). These results demonstrate that compound 6 is the most potent in this assay.

Migration assay HDF-1

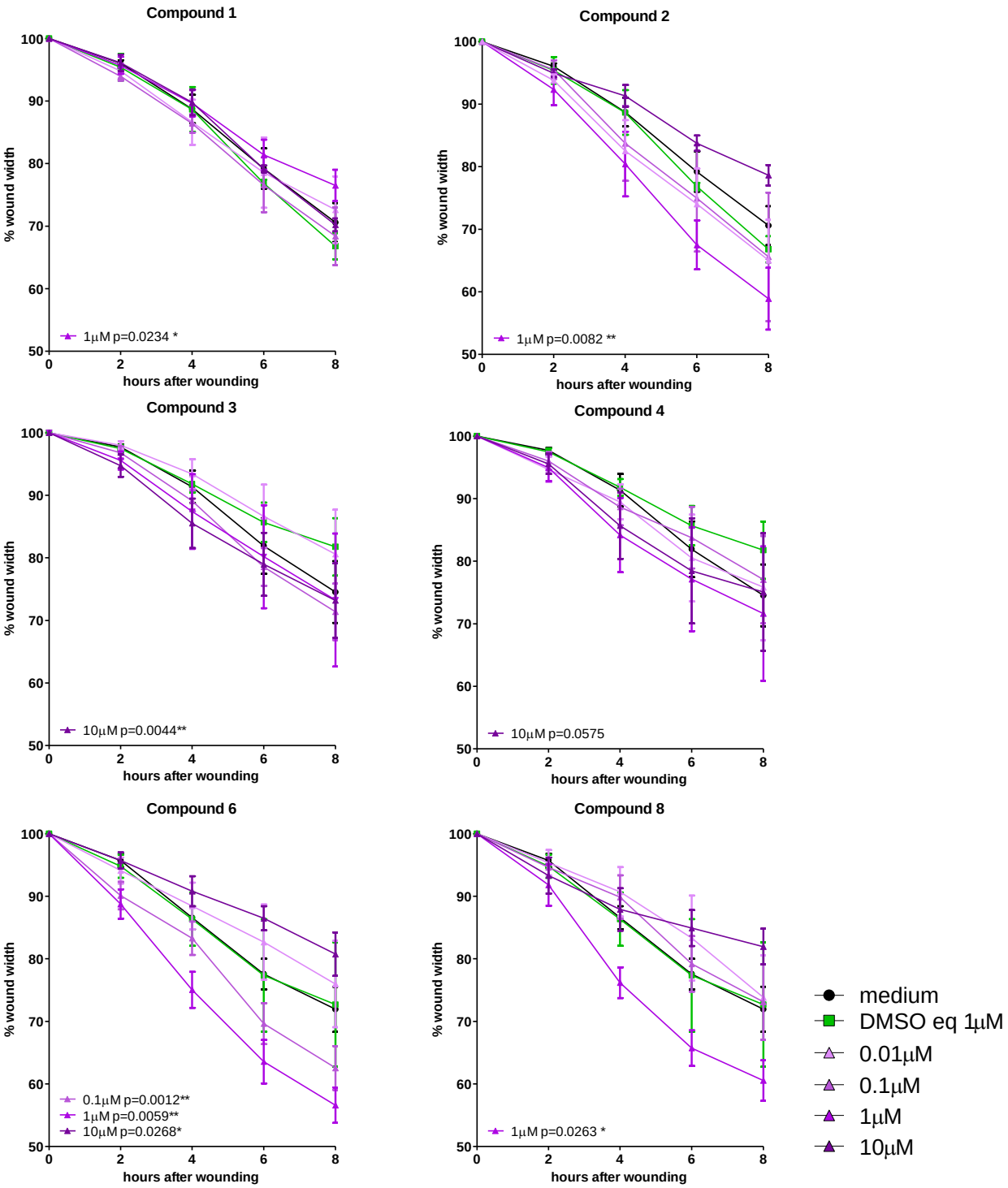

Figure 7 Migration assay HDF-1 cells. The HDF-1 cells were seeded to produce a monolayer of cells and subsequently a wound was made. The wound width was measured throughout the day after 2, 4, 6 and 8 hours. Different compound concentrations were tested: 0.01µM, 0.1µM, 1µM and 10µM. The results show significant differences for all compounds compared to the equivalent DMSO controls. Data are presented as means ± SEM, n=3.

3.4 The effect of ROCK inhibitors on HUVEC cell proliferation

To test the effect of ROCK inhibitors on endothelial cell proliferation a BrdU assay with HUVEC cells was performed. The results of the HUVEC migration assay suggested that compounds 2, 6 and 8 were the most potent. Therefore, these three ROCK inhibitors were chosen to test in the BrdU assay. The results of these compounds were compared to the control condition, containing only serum starvation medium with stimuli. These results show that bFGF has a significant effect on HUVEC proliferation (p=0.0293) compared to the medium control. Regarding VEGF there is a trend towards stimulation of proliferation. However, compounds 2, 6 and 8 did not indicate an effect on this induced proliferation (figure 8).

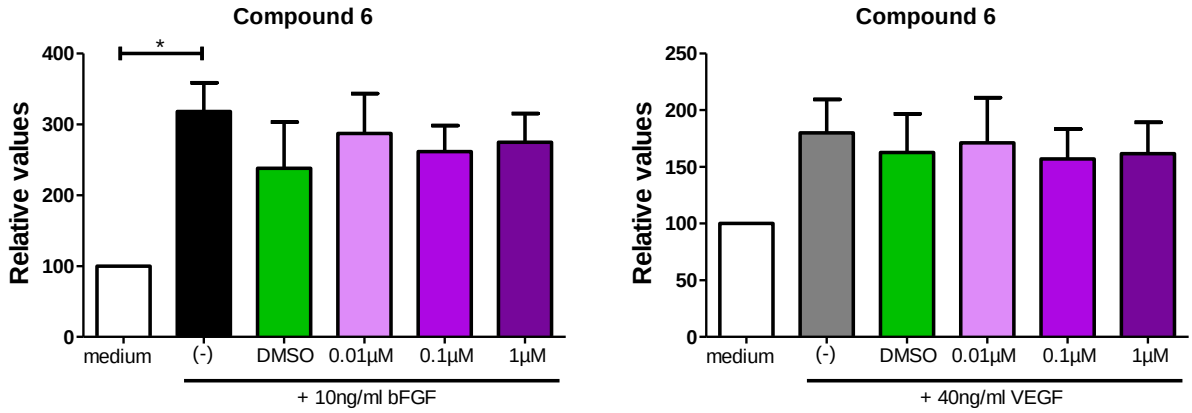

Figure 8 BrdU assay HUVEC cells. bFGF and VEGF were tested as stimuli of proliferation and compounds 2, 6 and 8 were analyzed at 0.01µM, 0.1µM and 1µM. bFGF showed a significant effect on HUVEC proliferation (p=0.0293) compared to the medium control. Regarding VEGF, there is a trend towards stimulation of proliferation. However, the selected compounds showed no effect on this induced proliferation. Data are presented as means ± SEM, n=3.

3.5 The effect of ROCK inhibition on *in vivo* angiogenesis

Following the results of the HUVEC migration assay, a CAM assay was performed to test the effect of ROCK inhibitors on *in vivo* angiogenesis. Compound 2, 6 and 8 appeared to be the most potent and were therefore chosen to test in this CAM assay. However, in advance the stability of the compounds was tested in chicken plasma. In table 7 these results are displayed, all compounds showed a half-life of more than 60 minutes except for compound 6.

Table 7 Stability test chicken plasma. Average and standard deviation. Half-life in minutes is shown. n=2.

Chicken plasma	Average (Half-life in minutes)	SD
1	>60.0	0.0
2	>60.0	0.0
3	>60.0	0.0
4	>60.0	0.0
6	58.7	1.9
8	>60.0	0.0

For the CAM assay, fertilized eggs were treated from day 10 to day 13 with DMSO or compound 2, 6 or 8. Different concentrations of the ROCK inhibitors were tested. The treatment occurred inside a silicone ring, placed on the CAM. On day 14, pictures were taken to count the vascular density. The amount of blood vessels was determined by 3 independent, blinded persons. None of the tested compounds showed a significant effect on vascular density compared to the eq. DMSO control (figure 9). An example is shown in figure 9A. However, treatment of the CAM with compounds did show some morphological differences at the application site, as depicted by figure 9B and C.

Figure 9 CAM assay. Fertilized eggs were treated from day 10 to day 13 with compounds inside a silicone ring with either DMSO or compound 2, 6 or 8 in different concentrations. Results showed no significant effects of these compounds on the vascular density. ***A****: as an example, 0.3µM compound 8 is compared to 0,3µM DMSO treatment* ***B****: the CAM shown outside the silicone ring.* ***C****: the CAM shown inside the silicone ring. B and C show some morphological differences. Data is shown as means ± SD, n=5.*

3.4 Inhibition of the phosphorylation of cofilin by ROCK inhibitors

To test the effect of the ROCK inhibitors on LIM kinases and thus on the phosphorylation of cofilin, a cofilin assay was performed. The obtained signal represents the amount of phosphorylated cofilin. The same 6 compounds, selected based on the HUVEC and HDF-1 data, were analyzed. The viability assay demonstrated no effect of the compounds on the cell viability after 1 hour incubation (data not shown). In this cofilin assay a concentration range of the compounds was tested to determine an EC_{50} value, this is the concentration where the amount of phosphorylated cofilin is reduced to 50%. However, it seems that for most compounds the EC_{50} values were higher than 50µM, the highest concentration tested, therefore the EC_{50} could not be exactly calculated. This type of result is shown for compound 3 in figure 10. Compound 8 was the only tested compound that did show an S-shaped curve (figure 10) and for this compound an EC_{50} value of 0.19µM was calculated. Compound 4, did not show a concentration-dependent effect on phosphorylated cofilin.

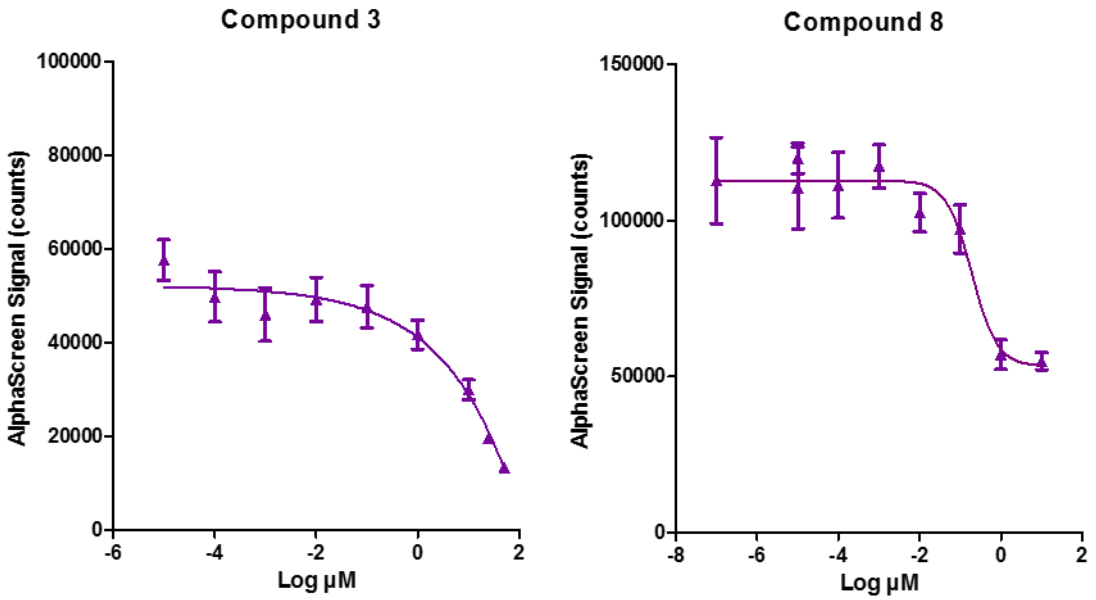

Figure 10 Cofilin assay compounds 3 and 8. A concentration range of compounds was tested, represented here as a logarithmic scale on the X-axis. The Y-axis is the measured signal, this represent the amount of phosphorylated cofilin, decreasing with increasing concentration of compound. Most compounds showed to have an EC_{50} higher than 50µM, the highest tested concentration. An example is compound 3. Compound 8 was the only tested compound that did show an S-shaped curve, an EC_{50} value of 0.19µM was calculated. Data are presented as means ± SEM, n=2.

3.7 Inhibition of neutrophil transmigration by ROCK inhibitors

Before testing the effect of ROCK inhibitors on HL-60 transmigration, the effects on HL-60 cell viability (WST-1) and apoptosis (caspase 3/7 activity) were assessed with different compound concentrations. Similar to the results of the HUVEC and HDF-1 cells, the EC_{50} value differed between compounds. In the apoptosis assay these cells also demonstrated some differences. For example, compound 1 showed a significant difference at a relatively high concentration of more than 100µM, compared to compounds 3, 7, 8 and 9 that generated this difference already at a low concentration of 10µM. Overall, the results of the HL-60 cells showed significant differences at quite low concentrations compared to the HUVEC cells. During the screening process, already 6 of the 10 compounds were selected for further testing following the HUVEC and HDF-1 data. Hence, these 6 ROCK inhibitors were chosen to test in the transmigration assay.

Table 8 Viability and apoptosis assay HL-60 cells. Results of the WST-1 assay are shown as an EC50 value: the concentration where cell viability is reduced to 50%. The results of the caspase 3/7 assay are shown as the concentration from which on there is a significant difference with the DMSO control. Non-significant (Ns.) n=3.

Compounds	WST-1 (EC50)	Caspase 3/7 (n=3)
1	37.0µM	≥100µM
2	11.2µM	≥0.1µM
3	11.4µM	≥10µM
4	16.4µM	≥40µM
5	~9.1µM	Ns
6	11.9µM	≥100µM
7	1.3µM	≥10µM
8	1.6µM	≥10µM

9	1.5µM	≥10µM
10	1.3µM	Ns

In the transmigration assay, the effect of ROCK inhibitors was determined by using a transwell system (figure 5). Different concentrations of the compounds were tested and compared to an equivalent DMSO control. The chosen concentrations were determined based on the results of the viability and apoptosis assay. Compound 6 showed a significant reduction of cells that transmigrated to the lower chamber, (p=0.0187) at a concentration of 0.1µM, compared to the equivalent DMSO control (figure 11). The other tested compounds showed no significant effect. Compound 6 is therefore the most potent in this assay.

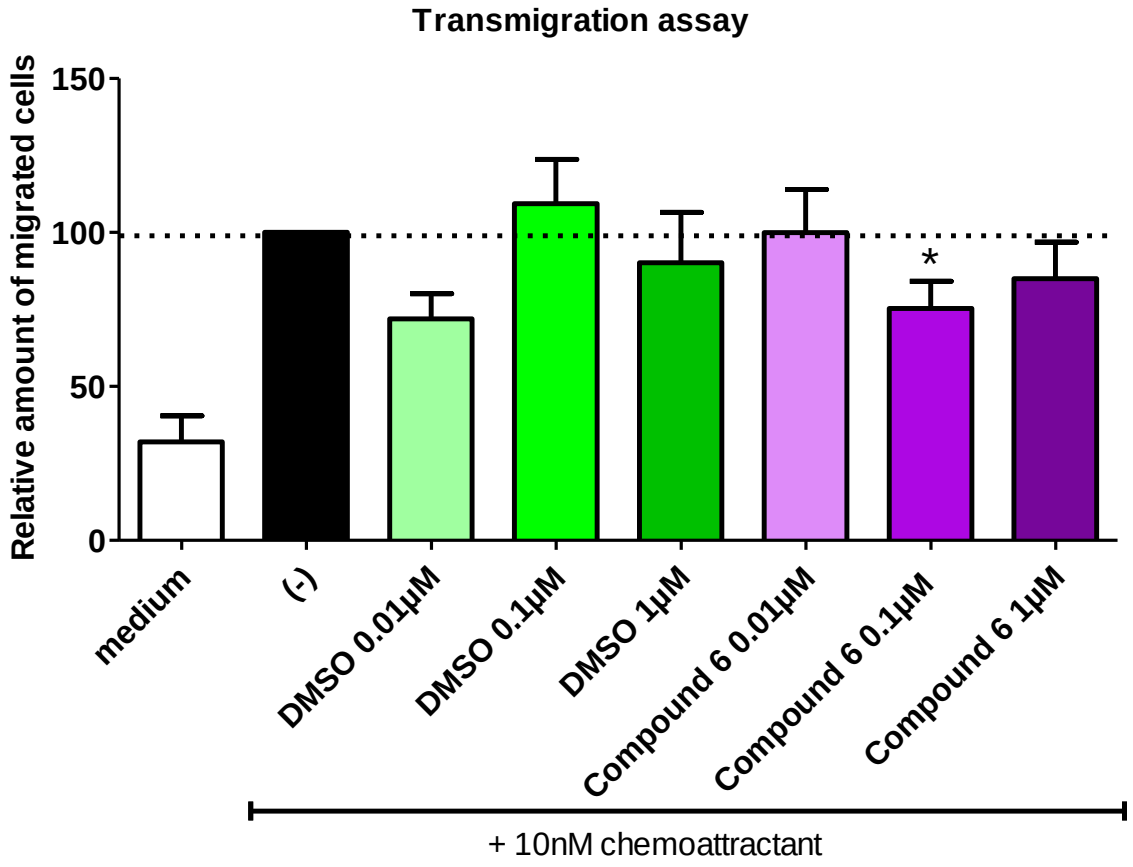

Figure 11 Transmigration assay differentiated HL-60. A transwell system was used. Different compounds and equivalent DMSO concentrations were tested: 0.01µM, 0.1µM and 1µM. The compound conditions were compared to the equivalent DMSO controls. Compound 6 showed a significant reduction of migrated cells at a concentration of 0.1µM (p=0.0187). Data are presented as means ± SEM, n=4

4. Discussion and outlook

The goal of this research project was to screen 10 new potential ROCK inhibitors, produced by Amakem therapeutics, to select compounds for further preclinical screening. The results of this project showed that ROCK inhibitors can have various effects on different cell types. However, further research is needed to select potential new candidates for future pre-clinical screening.

The compounds produced by Amakem therapeutics are based on their 'localized drug action' platform, to reduce side effects. These ROCK inhibitors are designed to become inactivated in blood by esterases. However, this property still has to be evaluated for each produced compound. Hence, this project started by determining the stability profile of the 10 compounds. The stability of the compounds was first tested in cell culture medium, this is important because in the *in vitro* assays the compounds should be stable to know at which concentration they have an effect. The results show that all compounds were stable for at least 24 hours, which is the longest period of time of incubation in the functional assays. Next, the stability of the compounds was tested in human whole blood and in human plasma. Not all compounds were broken down at the same rate; some were even stable at the last time point of 60 minutes. This could be due to a lower concentration of the specific esterases responsible for the conversion of those compounds to their inactive metabolite. There was also a difference in metabolism in whole blood and plasma. Some compounds were shown to be more stable in plasma than in whole blood. Possibly not all esterases which metabolize the compounds are present in plasma. Other compounds were more stable in whole blood compared to plasma which could be explained by a higher concentration of certain esterases. During this research project, a difference in stability between single-donor plasma samples and pooled plasma samples has been detected. Because both types of samples were used for the results shown here, a higher standard deviation was found for some compounds. The explanation for this is currently still under investigation. The fact that some compounds demonstrated a longer half-life than others is important in later steps of the pre-clinical screening, where the therapeutic potential is important to consider. The *in vitro* screening was important to prove cellular activity of the compounds. A very stable but very effective compound could still be slightly altered to become a softer compound. Moreover, a very unstable ROCK inhibitor might be more unpredictable in the *in vitro* assays since it might be unclear whether the compound has no cellular activity or if it is converted to an inactive metabolite. Therefore, in the selection procedure of this research project, the results of *in vitro* testing were as important as the results of the stability tests.

The *in vitro* selection procedure started with testing the effect of the ROCK inhibitors on the cell viability and apoptosis of HUVEC and HDF-1 cells. These results showed some differences between the two cell types. In the apoptosis assay the results of the HDF-1 cells showed more variation, which could be due to the cells being more sensitive to environmental changes, such as a difference in passage number or a reduced activity of the caspase reagent. Next, 6 compounds were selected based on these results to test their effect on HUVEC and HDF-1 cell migration. These results showed that all compounds significantly increased the cell migration of HUVEC and HDF-1 cells. Also the results of the migration assay displayed some differences between HUVEC and HDF-1 cells. Overall, the compounds demonstrated significant effects on cell migration at lower concentrations in HUVEC cells than in HDF-1 cells. This could mean that the ROCK inhibitors were more potent in HUVEC cells. Compounds 2, 6 and 8 seemed to be the most potent compounds in the migration assay on HUVEC

and HDF-1 cells. If compounds have an effect already at lower concentrations it is more likely a specific ROCK inhibitory effect and less side effects would be generated. This assay indicated that these compounds are potent ROCK inhibitors in these cell types. However, Breyer et al. reports that the change in motility of cells after ROCK inhibition is dependent on the environment and the assay that is used [43]. In literature, it has been described that sprouting activity is increased in HUVEC cells after ROCK inhibition [53]. In contrast, another article reported that ROCK inhibitors reduce HUVEC migration [54]. Therefore, more experiments are needed to translate this stimulation of migration into an effect on angiogenesis or fibrosis.

Next, the effect of ROCK inhibition on HUVEC cell proliferation was tested. The results of this BrdU assay showed that stimulation with growth factors, VEGF and bFGF, induced cell proliferation of these macro-vascular endothelial cells. However, there was no effect of the compounds on induced proliferation. Yin et al. showed that the ROCK inhibitor fasudil inhibited VEGF-induced HUVEC proliferation. Similarly, they tested the effect in HUVEC cells and with 10µM fasudil. However, they used a Ki-67 immunofluorescence staining instead of a BrdU assay. Moreover, their HUVECs were primarily isolated [54]. Therefore, these differences could be an explanation for the difference in results.

To test the effect of ROCK inhibitors on *in vivo* angiogenesis, a CAM assay was performed. Before, the stability of the compounds was tested in chicken plasma. Here, all compounds, except 8, showed a half-life of more than 60 minutes. Compound 8 showed a half-life of 58.7 minutes. However, this compound was still chosen for the CAM assay, to detect a possible difference in effect of a softer compound. The results of the CAM showed no effect of 2, 6 or 8 on the vascular density compared to the DMSO control. Compound 6 at 0.3µM seemed to be too toxic for the chicken embryos, after 1 day treatment more than half of the chicken embryos died. After treating the CAM with 6 and 8, analysis of the pictures suggested that although no differences in blood vessel density could be observed, sprouting of small vessels occurred without clear direction. In addition, the CAM sometimes appeared to be cloudier. Although compounds 2, 6 and 8 did not affect blood vessel formation *in vivo*, previous results of Amakem therapeutics showed that two of their compounds (11 and 12) did show inhibition of angiogenesis in the CAM assay. The difference could be explained by a difference in chemical structure. Compounds 2, 6 and 8 in this research project are from a different series than 11 and 12. A difference in physicochemical properties might result in more aspecific enzyme-binding. Also, a difference in activity profile might be the reason that 2, 6 and 8 had no effect; they might also react with other kinases. Moreover, the compounds have only proven to be stable in chicken plasma. It is possible that there is a difference in stability between compounds in the eggs. In literature, it has been described that the ROCK inhibitor fasudil can suppress VEGF-induced *in vivo* neovascularization. However, this was tested in another type of experiment than the CAM assay [55]. From the results of the HUVEC migration, the BrdU and the CAM assay, compounds 2, 6 and 8 are not very potent anti-angiogenic compounds. Consequently, to select a new potential ROCK inhibitor that has the potential to affect angiogenesis a new screening of other compounds must be done.

As mentioned earlier, LIM kinases are also substrates of ROCK and their role is to phosphorylate cofilin which regulates actin filament turnover [14]. ROCK inhibitors should therefore indirectly also inhibit LIM kinases and accordingly phosphorylation of cofilin. To test this, a concentration range of compound was examined to determine the effect on phosphorylated cofilin. However, the EC_{50} value

of most compounds could not be calculated because it was higher than the highest tested concentration (50µM). Only for 8 the EC_{50} value could be calculated (EC_{50}= 0.19µM). Nonetheless, except for compound 4 all other compounds showed a concentration-dependent effect on phosphorylation of cofilin. However, for most compounds this effect was only obvious at high concentrations suggesting an aspecific effect.

To test the effect of ROCK inhibitors on inflammation, a HL-60 transmigration assay was performed. This assay mimics the transendothelial migration necessary for inflammatory cells to reach the site of infection. The results of this assay demonstrated a significant inhibition of transmigration by compound 6 at 0.1µM. The other tested compounds showed no effect. The effect is not concentration-dependent since 1µM of compound 6 did not affect transmigration of the dHL-60. The results obtained by the viability assay did show that HL-60 cells are more sensitive for the compounds compared to the HUVEC and HDF-1 cells. Therefore, 1µM could have been too close to the EC_{50} value. In literature it has been shown that treatment with the ROCK inhibitor fasudil could decrease transendothelial migration of neutrophils in LPS- induced lung injury [56]. In addition, studies have described that pretreatment with the ROCK inhibitor Y-27632 reduced the transmigration of neutrophils [35, 57]. This is contradictory to our results since we were unable to determine a significant reduction in transmigration of neutrophils after 10µM Y-27632 (compound 1; n=21) preincubation. An explanation might be the differences in the experimental conditions; Shimizu et al. used primary neutrophils and IL-1β chemoattractant. However, Hauert et al. used the same HL-60 cells, the same chemoattractant and 10µM Y-27632 [35, 57].

The aim of this research project was to screen 10 ROCK inhibitors from Amakem therapeutics to select new potential ROCK inhibitors for future pre-clinical screening. However, further experiments with these compounds or a screening with other compounds are necessary to establish this aim. The compounds were tested for their potency to affect processes important for angiogenesis, fibrosis and inflammation. Regarding angiogenesis, only the HUVEC cell migration was affected by the compounds, but no effect was shown on HUVEC proliferation and *in vivo* angiogenesis. A future experiment could be to perform a tube formation assay, to determine the effect of the compounds on this process. Concerning fibrosis, due to the limited time only a migration assay was performed on HDF-1 cells. Therefore, future experiments could include looking at the effect on differentiation to myofibroblasts. In addition, the effects of ROCK inhibition on important properties of the myofibroblasts, such as proliferation and production of cytokines e.g. IL-6, could be determined. Finally, the effect on inflammation was examined by the transmigration assay. dHL-60 cells (neutrophils) were used and only compound 6 had an inhibitory effect at 0.1µM. However, preliminary results of Amakem therapeutics using THP-1 cells (monocytes) instead of dHL-60 cells, displayed an inhibitory effect by several compounds. Thus, a future experiment might be to test these ROCK inhibitors in THP-1 cells.

5. References

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