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DIPLOMARBEIT

Effect of Indirubin-3'-monoxime
on Platelet Derived Growth Factor-Induced
Vascular Smooth Muscle Cell Migration

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ABSTRACT

The accumulation of vascular smooth muscle cells (VSMC) within the intima of the arterial walls and the increase in extracellular matrix they produce, contributes to vessel narrowing in the pathogenesis of vasoproliferative pathologies, including atherosclerosis and restenosis. The underlying mechanisms are increased proliferation and migration of VSMC from the media in the intima, caused by different growth factors and cytokines. An important proliferative stimulus, and likely the most potent chemoattractant for VSMC, is the platelet derived growth factor-BB (PDGF-BB).

Indirubin is an ingredient of a traditional Chinese medicine preparation, Dang Gui Long Hui Wan, which had been used in the treatment of various chronic diseases, including leukemia. Previous work in our lab has shown that indirubin-3'-monoxime (I3MO), a more soluble derivation, is able to inhibit PDGF-BB-induced VSMC proliferation. Furthermore, the compound was able to reduce neointima formation *in vivo* in a mouse model (experiment performed in cooperation with Prof. Binder, Medical University of Vienna). In addition, I3MO blocked phosphorylation of STATs without interfering with other common early signaling events. Here we show that in addition to its antiproliferative properties, I3MO is able to inhibit VSMC migration dose-dependently after stimulation with PDGF-BB. Furthermore it is able to interfere with early cytoskeletal rearrangements. In addition, we found that I3MO reduced STAT3 and STAT5b phosphorylation after stimulation with 10 ng/ml of PDGF-BB, a concentration leading rather to VSMC migration than to their proliferation. Moreover, I3MO reduced expression of promigratory STAT3 and STAT5b target genes after stimulation with 10 ng/ml of PDGF-BB. To further elucidate the underlying mechanisms of I3MO-effects, we compared I3MO to AG490, a chemical JAK2 inhibitor, and to LiCl, a GSK-3 inhibitor. We found considerable similarities between AG490 and I3MO regarding their intracellular actions. Although the exact cellular target remains to be investigated, our studies affirm I3MO as a promising compound in the treatment of atherosclerosis and restenosis.

ZUSAMMENFASSUNG

Atherosklerose und Restenose gehören zu den vasoproliferativen Erkrankungen, die sich durch eine Anhäufung von glatten Gefäßmuskelzellen in der Intima auszeichnen. Außerdem produzieren diese glatten Gefäßmuskelzellen mehr extrazelluläre Matrix. Dies kann zu Gefäßverengung führen. Der Grund für die Anhäufung sind die erhöhte Proliferation und die Migration der glatten Gefäßmuskelzellen von der Media in die Intima, die von bestimmten Signalmolekülen ausgelöst werden. Dazu zählt Platelet derived growth factor-BB (PDGF-BB), ein Faktor, der stark mitogen wirkt und zudem der stärkste bekannte chemotaktische Stimulus für glatte Gefäßmuskelzellen ist.

Indirubin ist ein Inhaltsstoff aus einer Zubereitung der traditionellen chinesischen Medizin, Dang Gui Long Hui Wan. Die Zubereitung wird in der Behandlung verschiedener chronischer Erkrankungen eingesetzt, darunter Leukämie. In unserem Labor ist bereits gezeigt worden, dass Indirubin-3'-monoxime (I3MO), ein besser wasserlösliches Derivat, PDGF-BB induzierte Proliferation von glatten Gefäßmuskelzellen hemmen kann. Ebenfalls konnte in Zusammenarbeit mit Prof. Binder von der Medizinischen Universität Wien gezeigt werden, dass I3MO die Bildung einer Neointima *in vivo* in einem Mausmodell vermindert. Schließlich hemmt I3MO die Phosphorylierung einiger Transkriptionsfaktoren aus der STAT-Familie, ohne dabei verschiedene andere bekannte Proteinkinasen zu hemmen. In dieser Arbeit zeigen wir nun, dass I3MO konzentrationsabhängig zusätzlich zur Proliferation auch die Migration von glatten Gefäßmuskelzellen hemmt. Weiters kann es frühe Umbauten im Zellskelett beeinflussen. Zusätzlich fanden wir heraus, dass I3MO die Phosphorylierung von STAT3 und STAT5b auch nach Stimulation mit 10 ng/ml PDGF-BB hemmen konnte, also in einer Konzentration die vorwiegend zu Migration von glatten Gefäßmuskelzellen führt als zu deren Proliferation. I3MO reduzierte weiters die Expression von migrationsfördernden Zielgenen nach Stimulation mit 10 ng/ml PDGF-BB. Schließlich verglichen wir die intrazellulären Effekte von I3MO mit denen eines JAK2 Inhibitors (AG490) und mit denen eines GSK-3 Inhibitors (LiCl). Wir fanden deutliche Ähnlichkeiten in den Effekten von I3MO und AG490. Obwohl das intrazelluläre Zielprotein von I3MO noch aufgeklärt werden muss, bekräftigt unsere Arbeit, dass I3MO ein vielversprechendes Molekül ist, das in der Behandlung von vasoproliferativen Erkrankungen wie Atherosklerose und Restenose eingesetzt werden könnte.

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B. INTRODUCTION

B. INTRODUCTION

1. Background

The accumulation of vascular smooth muscle cells (VSMC) within the intima of arterial walls and their increased production of extracellular matrix (ECM) leads to a reduction of the vessel lumen. This pathological process, called intimal thickening, occurs in atherosclerosis and restenosis after angioplasty, organ transplantation and in vein grafts. The underlying mechanisms are proliferation of VSMC and their migration from the media to the intima.¹ Therefore, inhibiting migration and proliferation of VSMC might be a rational treatment for vasoproliferative diseases, including atherosclerosis and restenosis.²

2. Atherosclerosis

Atherosclerosis is characterized by intimal lesions called fibrofatty plaques, which protrude into and obstruct vascular lumens.³ The plaques cause flow-limiting stenoses and, after rupture of a plaque, thrombotic occlusions of arteries. This can lead to myocardial infarction, ischemic stroke and transient ischemic attacks.⁴ As myocardial infarction and stroke are leading causes of death, atherosclerosis, in westernized societies, is the underlying cause of about 50 % of all deaths.⁵ The genesis of atherosclerosis is still under investigation, but it has become clear that together with the pathological accumulation of lipids within the artery wall, also immune and inflammatory processes might participate in the development of atherosclerosis.⁶

2.1. Development of atherosclerosis

A normal artery consists of three layers. The innermost layer, named intima, is composed of a monolayer of endothelial cells and of elastic fibres. The middle layer, named media, consists of VSMC. The adventitia, the outer layer, is mainly composed of connective tissue.⁵

In lesion-prone areas, such as coronary arteries, atherosclerotic lesions begin to develop under a damaged, dysfunctional endothelium.⁷ Dysfunctional endothelium is characterized by a decreased bioavailability of vasoprotective mediators, mainly nitric oxide (NO), whereas pro-atherosclerotic factors are increased.⁸ Possible risk factors for endothelium damage include high levels of low density lipoproteins (LDL), cigarette

smoking, hypertension, diabetes mellitus, and inherited diseases like tangier disease. Furthermore, elevated plasma homocysteine concentrations and infectious microorganisms are thought to play a role in the genesis of dysfunctional endothelium.⁹ As a primary event, LDL and other lipoproteins, diffuse through the ,leaky endothelium and accumulate within the subendothelial space ⁵, where they become oxidized. Oxidized lipoproteins stimulate the endothelial cells to produce adhesion molecules and growth factors. Monocytes and T-lymphocytes adhere and migrate within the subendothelial space. There, the monocytes differentiate into macrophages and internalize oxidized lipoproteins. Subsequently, they convert into so-called foam cells.^{5,7} Macrophages or foam cells fail to return to blood circulation¹⁰ and accumulate. Together with VSMC they form fatty streaks.¹¹ Macrophages internalize oxidized LDL until they die by apoptosis or necrosis, leaving the lipid behind as extracellular lipid droplets. The advanced lesion, called atheroma, is characterized by a confluent core of extracellular lipid, caused by dead foam cells and atherogenic lipoproteins.⁷ With disease progression, layers of fibrous connective tissue can replace the intima and the lesions can calcify. The most advanced types of atherosclerosis are fissures, hematoma and thrombi.^{11,12}

2.2. The role of vascular smooth muscle cells in atherosclerosis

Whereas in a healthy vessel only few VSMC reside in the intima, they become present in early fatty streaks. In advanced lesions they constitute the principal cell type in the intima.¹³

Normally, VSMC are fully differentiated and show a low proliferation rate,¹⁴ but in response to atherogenic stimuli they can switch from the quiescent “contractile” state to the active “synthetic” state. Synthetic muscle cells migrate from the media to the intima and begin to proliferate.¹⁵ If the stimuli continue for years, vascular smooth muscle cells can thicken the artery wall, which is compensated by dilation of the vessel, a term called “remodeling”.¹³ The thickening caused by proliferation of VSMC leads to loss of vessel lumen and limited blood flow. Hence, ischemia might follow.⁷

Furthermore, synthetic VSMC seem to promote atherosclerosis by synthesizing more extracellular matrix (ECM). Cultured synthetic VSMC were seen to produce between 25 and 30-fold the amount of ECM as contractile-state cells.¹⁶ The increased deposition of ECM makes the vessel wall more susceptible to changes in the flow characteristics of the blood and to fissuring or ulceration.¹³ Furthermore, the constitution of the ECM in atherosclerotic lesions is altered: whereas within a healthy

artery type I and type III fibrillar collagen are predominant, atherosclerotic vessels contain proteoglycans with scattered type I collagen fibrils and fibronectin.¹⁷ Proteoglycans bind several molecules necessary for lipid accumulation, calcification, and thrombosis.^{13,18} Additionally, proliferation of VSMC is up-regulated when VSMC bind to fibronectin and proteoglycan.¹⁷

Moreover, VSMC take up lipids and form foam cells like macrophages. They seem to contribute to the retention of monocytes and macrophages within the lesion via expression of adhesion molecules and to have an antiapoptotic effect on them.^{16,17} Finally, smooth muscle cells produce a lot of atherogenic cytokines that attract and activate leukocytes, induce proliferation of SMCs, promote endothelial cell dysfunction and stimulate production of extracellular matrix components. The most important of which are platelet derived growth factor (PDGF), transforming growth factor- β (TGF- β), macrophage inhibitory factor (MIF), interferon-gamma (IFN- γ) and monocyte chemoattractant protein-1 (MCP-1).¹⁷

However, some aspects of the role of VSMC in atherosclerosis are still being controversially discussed. The fibromuscular cap, created by VSMC and their extracellular matrix in advanced lesions, seems to be important for the so-called plaque-stability: stable plaques with small lipid core and covered by a thick fibromuscular cap tend to create occlusion and stenosis. Unstable, vulnerable plaque (characterized by a large lipid core and a thin fibrous cap) usually leads to rupture and thrombosis.¹⁰ VSMC apoptosis might represent a further cause for plaque rupture.¹⁹ Thus, VSMC seem to play a protective role in mature plaque.⁷ Recently, also the origin of VSMC in the intima has been newly hypothesized: experimental data suggested that they may not derive from the media, but from progenitor cells originated in the bone marrow.²⁰ Finally, the magnitude of proliferation of VSMC in human atherosclerotic lesions has been discussed: some studies report abundance of proliferation while others report very low proliferation rates.²¹ The low proliferation rate may be concordant with a greater role for migration from the media to the intima.¹³ Fig. 1 gives an overview of the development of atherosclerosis and the migration of VSMC.

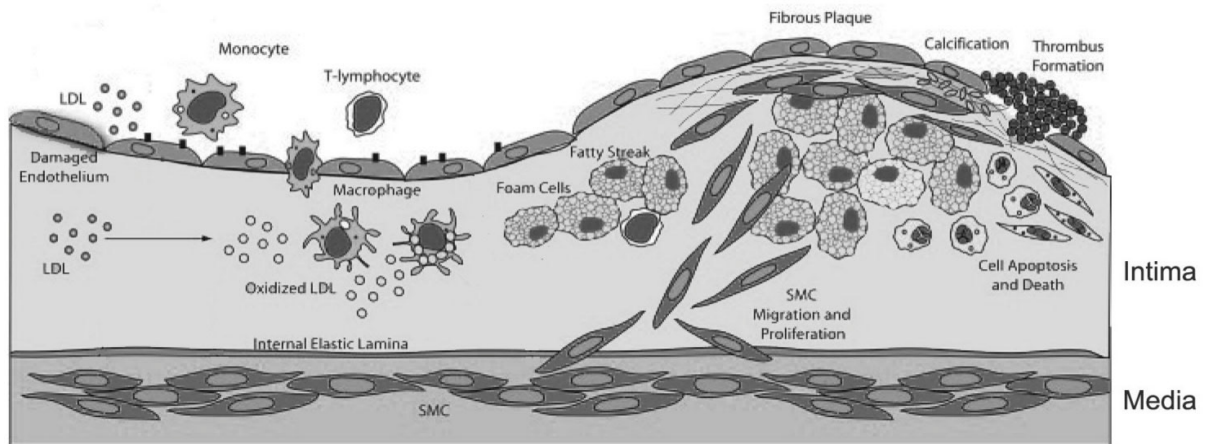


Fig. 1. The development of atherosclerosis.

LDL particles diffuse through dysfunctional endothelium and become oxidized. Macrophages and T-lymphocytes migrate within the intima, ingest oxidized LDL, and convert into foam cells. VSMC move from the media to the intima and proliferate, leading to advanced lesions and stenosis. Lesions may calcify and thrombi may form. Figure adopted from Madamanchi et al ²², for abbreviations see section 2.

2.3. Diagnosis and treatment of atherosclerosis

Advanced atherosclerotic lesions can be diagnosed with invasive coronary angiography or intravascular ultrasonography. Noninvasive technologies, like cardiac computed tomography or positron emission tomography, allow to detect the atherosclerotic plaques.²³

Up to now, no specific drug therapy has been found to prevent the formation of atherosclerosis lesions. Therefore, the primary and secondary prevention of atherosclerosis includes risk factor modification with therapeutic lifestyle changes (diet improvement, smoking cessation, and physical activity) as well as drug therapy with non-atherosclerosis-specific drugs, including acetylsalicylic acid, statins, angiotensin-converting enzyme inhibitors and β -blockers. Thrombolysis is used in patients with acute cardiovascular events.²⁴

Furthermore, physically invasive methods are used in case of vessel constriction. The method utilized in case of moderate constriction is percutaneous transluminal angioplasty (PTA).²⁵ In PTA, a catheter is advanced towards the stenosis and then inflated to press the plaque into the arterial wall.²⁴ It is used in coronary stenosis as well as in peripheral stenosis, including renal and carotid stenosis.²⁶ Furthermore, a bare metal stent or a drug-eluting stent, can be deployed in the stenosed section. The first PTA was conducted on a carotid artery by Grüntzig and colleagues in 1977. Since

then, PTA has revolutionized revascularization. In coronary artery disease, percutaneous coronary intervention (PCI) has become the most common intervention.²⁷

Other mildly invasive treatments are the removal or pulverization of the plaque with atheroablative technologies. The fully invasive approach, the coronary artery bypass grafting, is used for treating the most severe coronary artery blockages or after a heart attack.²⁵

As described above, VSMC and their migration are highly involved in the progress of atherosclerosis. Thus, the development of strategies to inhibit VSMC migration has been a major focus in the search for specific drugs capable of preventing atherosclerosis and restenosis, which is described in the next section.¹⁵

3. Restenosis

During all invasive treatments of atherosclerosis a mechanical injury of the vessel wall occurs. A common vessel response is lumen loss, a process called restenosis. Restenosis is defined as a loss of more than 50 % of the lumen diameter²⁸, but the process can even lead to complete reocclusion of the vessel.²⁹ Restenosis is the major drawback after PCI,³⁰ occurring between 30 % and 50 % in patients treated with balloon angioplasty and in 10-30 % of patients who receive a bare-metal stent.²⁷

In balloon angioplasty, the three stages of restenosis are early passive elastic recoil, followed by negative vascular remodeling with adventitial thickening and, subsequently, neointima formation due to VSMC migration and proliferation. Although stents prevent recoil and adventitial thickening, they are unable to prevent neointima formation.²⁷

The molecular mechanisms leading to restenosis are incompletely understood, but they are thought to be a maladaptive response of the vessel to trauma.²⁷ The process begins with thrombus formation after injury. Subsequently, the pathology develops similarly to atherosclerosis: leucocytes adhere and release factors to stimulate VSMC migration, proliferation, and ECM production.²⁸ Balloon angioplasty of the rat carotid artery is a common animal model to investigate the consequences of neointimal formation after mechanical injury. The arteries are distended with a balloon and the endothelium is removed. Mural thrombi are formed and the VSMC proliferate within 48

hours of injury. Subsequently, they migrate to the intima and proliferate again, leading to a thickened intima consisting of VSMC and connective tissue matrix.¹³

To prevent neointimal formation, drug-eluting stents have been developed. They deliver antimitotic or antimicrotubular agents such as sirolimus and paclitaxel, thereby inhibiting proliferation and migration.³¹ As they are directly placed in the stenosed section, the local drug level is maximized, while the systemic toxicity is minimized.³⁰ Numerous clinical data clearly showed drug-eluting stents superior to bare-metal stents in reducing restenosis and the number of new revascularization even in high-risk patients.^{28,32}

Therefore, inhibiting proliferation and migration represents a critical importance in neointima formation. Consequently, the search for new substances represents a challenge of active research.

4. Migration of vascular smooth muscle cells

VSMC migration is regulated by numerous stimuli released by macrophages, T-lymphocytes, platelets, endothelial cells and VSMC.³³ Three terms are used to describe cell migration after stimulation: chemokinesis is defined as an undirected increase in motility independent of a concentration gradient, whereas a directed motion toward a concentration gradient is called chemotaxis. Finally, haptotaxis is defined as directed migration along a path of varying adhesiveness of the ECM.³¹

Cell migration is a common phenomenon in organisms. In addition to atherosclerosis and restenosis, it also occurs in embryonic development, in renewal of skin and intestine, in tissue repair, immune response and in several pathologic processes, including cancer.^{34,35} Different cell types show different characteristics of migration but share some basic mechanisms. Basic features are valid also for VSMC.

4.1. Cell migration

Migration can be seen as a cyclic process, which starts with the polarization of the cell after stimulation of cell surface receptors by migration-promoting factors.^{34,36} Polarization implies that signaling events at the front of the cell (the leading edge) and the back of the cell (the trailing edge) differ.³⁴ It is accompanied by remodeling of microtubules and localization of the microtubule organizing centre (MTOC) and Golgi apparatus in front of the nucleus.^{34,37} The small G protein Cdc42 and its effector complex PAR6/PAR3/aPK, active at the leading edge, are involved in these

processes.^{34,37,38} Another polarized molecule is PIP₃: while it is produced by phosphatidylinositol 3-kinase (PI₃K) at the front (see section 5.1.1.), it is removed by the phosphatase PTEN at the rear of the cell.^{34,39}

Once the cell is polarized, it starts to protrude toward the stimulus. Protrusions of cells are based on changes in their cytoskeleton, which is thought to occur by a “treadmilling mechanism”. Whereas the barbed end of an actin filament grows fast by forced actin polymerization, the pointed end grows slower due to increased depolymerization. This mechanism allows inherent membrane protrusion. The cells can form large, broad lamellipodia, where filaments organize in a branching network, or spike-like filopodia, where filaments form long parallel bundles.^{34,36,40} The functions of the two forms are different: filopodia act as sensors, whereas lamellipodia provide a strong foundation for the directional movement of the cells.³⁷ In a later phase of migration, actin filaments organize also into stress fibres, which may serve for maintaining cells straight under tension.⁴¹

Several proteins are involved in actin elongation. Protrusion at the barbed end is mediated by the actin-related protein 2/3 complex (Arp2/3), which binds to the side of actin filaments and induces the formation of new daughter filaments. Arp2/3 is activated by WASP/WAVE (Wiskott-aldrich syndrome protein/Verprolin-homologous protein), which in turn are targets of the small G proteins Rac and Cdc42, respectively.^{34,36,38,40} In addition, formins (mDia1/mDia2) promote linear filament extension, they are activated by the small G proteins RhoA and Cdc42.^{38,39} Profilin binds actin monomers and targets them to the barbed end, whereas cofilin induces depolymerization at the pointed end. Capping proteins terminate filament elongation, providing the flux of actin monomers to non-capped filaments.^{34,36,40} As Ena/Vasp-proteins and the formin mDia2 are enriched at the tip of filopodia; they seem to have a crucial role in the unbranched continuous elongation of filaments.^{34,36}

For driving the cell forward, a newly formed protrusion must adhere by focal contacts to the surroundings.³⁴ During migration, adhesions assemble at the leading edge and disassemble at the trailing edge. Furthermore, the new focal contacts at the leading edge can either disassemble during advancement of protrusion, a process called adhesion turnover, or mature into larger focal adhesions, as observed in less motile cells.³⁸ Rac and Rho seem to be strongly involved in these processes.³⁵

Integrin-receptors are crucial in the genesis of new adhesions.^{34,36} They link actin filaments to the ECM.³⁵ Integrin activation is bidirectional; whereas components of the

ECM bind to integrins from outside, other molecules, such as talin, are intracellular activators.^{15,34} The binding leads to activation of signaling cascades and to integrin clustering.^{34,37} Integrin clustering promotes a sequential addition of adhesion proteins, including vinculin, tensin, paxillin and, later, α -actinin.^{31,36}

It is not clear which mechanisms regulate the focal contact either to mature or to disassemble, but adhesion turnover seems to be regulated by interaction of focal adhesion kinase (FAK) with Src family tyrosine kinase (Src) and the adaptor proteins Cas and Crk, by small G proteins and by extracellular signal-regulated kinase (ERK).^{34,38} Although the major components of focal contacts have been described also in VSMC, the exact regulation of proteins remains mostly undefined.³¹

Whereas focal contacts are necessary as traction sites, the traction force to drive the cell forward derives from the actin filaments interacting with myosin II. Myosin II is activated by myosin light chain kinase (MLCK) or Rho-associated kinase (ROCK) and negatively regulated by MLC phosphatase (MLCP). MLCK is activated by intracellular Ca^{2+} concentration and by a number of kinases, which are mostly regulated by Ras, Rac and Cdc42. ROCK is activated by the small G protein RhoA.^{34,38,39} In VSMC, basic features in traction force generation are known, but regulating details are still to be defined.³¹

The migration cycle is concluded as the rear retracts and adhesions at the trailing edge disassemble. It is thought that the same mechanisms as in the front act also at the rear, including FAK, Src and ERK.^{34,35,38} Moreover, the physical tension created by myosin II may be enough to break the linkage between actin filaments and ECM.³⁴ The phosphatase calcineurin, the protease calpain as well as endocytosis of adhesion molecules also seem to be involved in rear disassembling.^{34,38} Fig. 2 shows the model of a migrating cell.

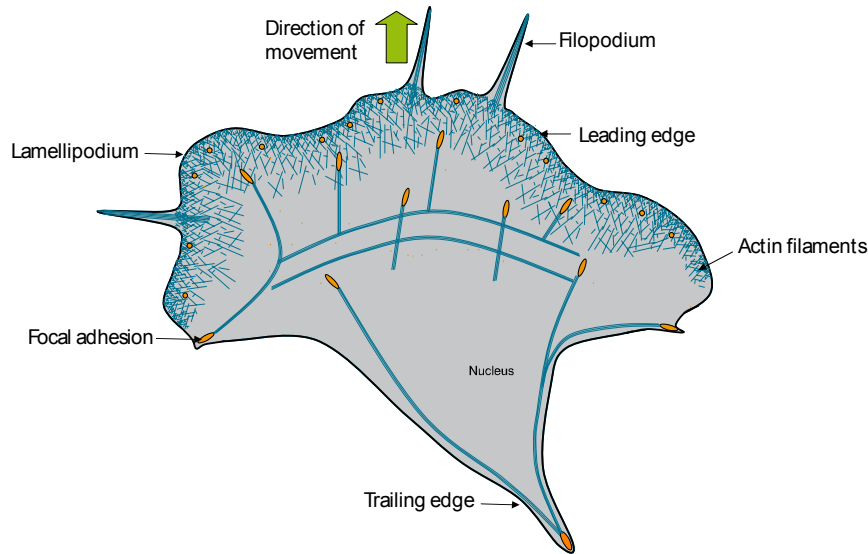


Fig. 2. Schematic illustration of a migrating cell.

During migration, cells form spike-like filopodia and broader lamellipodia for membrane protrusion. Stress fibres stabilize the cell body and focal contacts serve as traction sites. For further explanations, see section 4.1. Figure adopted from Le Clainche and Carlier.³⁶

4.2. Signaling pathways involved in VSMC migration

Migration is promoted by multiple signaling pathways activated by receptor tyrosine kinases (RTK), G protein-coupled receptors and integrins.^{31,34} Among others, small G proteins, Src, PI₃K, mitogen-activated protein kinases (MAPK), and FAK seem to play a pivotal role in migration-activating signaling. They are presented in the next section. (5.1.)

Furthermore, the small G protein family, including Ras, Rho, Rac and Cdc42, seems to be crucial for migration.³⁹ In VSMC, especially the role of Rho and Rac as promoters of cell migration is well described.⁴²⁻⁴⁷ Small G proteins can be activated by both RTK and G protein-coupled receptors. Activation is promoted by guanine nucleotide exchange factors (GEFs), which change GDP in GTP, whereas inactivation is mediated by GTPase-activating proteins (GAPs).⁴⁸ The most important functions of small G proteins were highlighted above. (See section 4.1.)

Several of the signaling proteins involved in cell migration play also a role in VSMC migration. The detailed mechanisms of regulation of migration are not known, but some of the pathways are now understood. They are presented in fig. 3.

urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs), are responsible for VSMC migration, as they degrade the ECM barrier.¹⁵

Most of the listed molecules have been found to stimulate migration *in vitro*. Thus, defining their role *in vivo* and completing the list of responsible agents, still remains a topic of research.³¹

5. The role of PDGF in VSMC migration

As mentioned above, PDGF-BB is the most potent chemoattractant and mitogen for VSMC *in vitro*, and inhibiting the interaction between PDGF and its receptor prevents PDGF-induced migration.⁴⁹ Several studies confirm its important role also *in vivo* in atherosclerosis and restenosis.³³ A major source of PDGF-BB in atherosclerotic lesions are activated macrophages, but it is secreted also by endothelial cells and VSMC.⁵⁰

PDGF-BB is one of the three biologically active forms of the PDGF family.⁵¹ These proteins are composed of two highly homologous peptide chains, PDGF-A and PDGF-B; they form PDGF-AA, PDGF-AB and PDGF-BB.⁵² Recently, PDGF-CC and PDGF-DD, which have to be activated by proteases after secretion, have been discovered.⁵¹ Two PDGF-receptors have been described so far: α - and β -receptor. As they dimerize after PDGF-binding, three possible combinations emerge: $\alpha\alpha$ -, $\alpha\beta$ - and $\beta\beta$ -receptor dimers. PDGF-AA induces solely $\alpha\alpha$ -receptor dimers, PDGF-AB and PDGF-CC induce $\alpha\alpha$ - and $\alpha\beta$ -receptor dimers and PDGF-DD induces $\alpha\beta$ - and $\beta\beta$ -receptor dimers. PDGF-BB is the universal inducer of all three combinations.^{51,52}

The PDGF family has several important functions *in vivo*, including embryonic development, wound healing, maintenance of the interstitial fluid pressure, tonus regulation of blood vessels, and probably also the regulation of feedback mechanisms in platelet aggregation.⁵² In addition to its role in atherosclerosis and restenosis, PDGF is involved in many pathological processes, including rheumatoid arthritis, pulmonary fibrosis, myelofibrosis, and abnormal wound repair.⁵⁰

As PDGF mediates proliferation, migration and cell survival in many different cell types⁵¹, evidence is growing that the different isoforms of PDGF ligand and receptor may mediate distinct cellular functions.³³ For example, in VSMC PDGF-BB and PDGF-AB have been reported to be chemotactic, whereas the effects of PDGF-AA have been less clear. At least *in vitro*, PDGF-AA showed no chemotactic effects on

VSMC of several species. It even seemed to antagonize migratory effects of PDGF-BB.^{33,53} These features and the finding that VSMC in atherosclerotic lesions show higher levels of β -receptors, support the idea that β -receptors promote directed migration, whereas α -receptors may negatively regulate chemotaxis.^{33,50}

Interestingly, it has been suggested that PDGF-BB principally leads to VSMC migration rather than to their proliferation. This has been confirmed by several studies.¹³ For instance, infusion of PDGF-BB and tritiated thymidine in rats subjected to carotid injury led to neointima formation. Within the neointima, the number of labeled, proliferated cells was less than the number of unlabeled, thus migrated cells.⁵⁴

5.1. Signaling pathways of PDGF

The PDGF-receptor becomes activated when PDGF binds to two receptors simultaneously and brings them together. The receptors are part of the RTK family, and thus approximation leads to transphosphorylation between the two receptor chains. Through these autophosphorylation events, the receptors activate downstream signaling molecules. They interact with specific domains of proteins, mainly the src homology 2 (SH2) domains, which recognize phosphorylated tyrosine residues.^{50,52}

Several SH2 domain containing molecules have been shown to interact with either α - and/or β -PDGF-receptors. Among the most important are PI_3K , phospholipase C- γ (PLC- γ), Src, the tyrosine phosphatase SHP-2, GTPase-activating protein for Ras, the Janus kinases (JAKs), the signal transducers and activators of transcription (STATs) and Grb2. All of them have been shown to be important for proliferation, migration and/or cell survival. Furthermore, several SH2-domain-containing adaptor molecules with partly unknown functions bind to the PDGF-receptor, including Fer, Shc, Nck, Grb7, Grb10, Grb14 and Crk.⁵² Fig. 4 gives an overview of some important signaling proteins activated by PDGF.

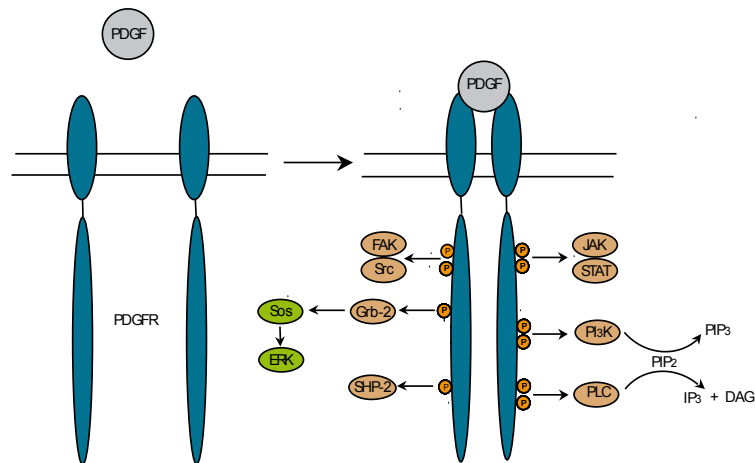


Fig. 4. Overview of important signaling pathways activated by the PDGF-receptor.

Binding of PDGF leads to PDGF-receptor (PDGFR) dimerization and to the activation of several signaling cascades. For further explanations and abbreviations see section 5.1. Figure adopted from Kang.⁵⁵

Although most of the proteins are activated by both α - and β -PDGF-receptors, some have a higher affinity for or bind only one receptor type.⁵² Furthermore, Ca^{2+} mobilization is much higher after stimulation of β -receptors than of α -receptors. Ca^{2+} activates MLCK and myosin II and might therefore be important in VSMC migration.³¹ Most of the signaling pathways induced by PDGF were found also in VSMC⁵⁶, and there were attempts to classify them in migration- or proliferation-inducing signaling proteins. However, it is clear that not one single pathway, but rather a cross-talk between different signaling molecules and pathways leads to the different cell responses.⁵⁰ Until now, the processes are not completely understood.

5.1.1. Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI_3K) consists of a catalytic and a regulatory subunit. Both, the α - and the β -PDGF-receptor, can activate some isoforms of this kinase, which are able to phosphorylate the inositol ring of phosphatidylinositol-4,5-bisphosphate (PIP_2) at position three, producing phosphatidylinositol-3,4,5-trisphosphate (PIP_3).⁵⁷ PI_3K is implicated in the stimulation of cell growth, inhibition of apoptosis and cell migration.⁵² It activates different proteins important for cell migration, including members of the small G protein family like Rho, Rac and Cdc42, the serine/threonine kinase Akt, members of PKC family, the c-Jun N-terminal kinase (JNK) and FAK.^{15,33,52,58} PI_3K interacts with Ras, and therefore seems to be an upstream activator of MAPK.¹⁵

In VSMC, the role of PDGF-induced PI_3K activation is not clear. It seems to be necessary in PDGF-induced VSMC proliferation^{57,59}, but inhibition of PI_3K was hardly or not capable of antagonizing PDGF-induced migration of some VSMC types.^{31,57-60} In his review, Gerthoffer supposed that the highly efficacious stimulus PDGF-BB activates a variety of signaling pathways and thus the inhibition of one component might not be enough to keep a VSMC from migrating.³¹

5.1.2. Phospholipase C- γ

Both, the α - and the β -PDGF-receptor, are able to bind members of the phospholipase C- γ (PLC- γ). Like PI_3K , PLC- γ uses PIP_2 as substrate and converts it into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), an enzyme phosphorylating other intracellular proteins, while IP_3 leads to calcium release from stores located in the endoplasmic reticulum.⁶¹ Furthermore, PLC- γ seems to be involved in the activation of phospholipase D (PLD)^{52,56}, Na^+/H^+ exchanger⁵² and FAK⁵⁸.

PLC- γ has been shown to be important in both migration and proliferation.^{50,52} Also in VSMC, binding of PDGF leads to phosphatidylinositol turnover; PIP_2 levels decrease, whereas the concentrations of DAG and Ca^{2+} rise.⁵⁰ Nevertheless, it is still an open question whether PLC- γ is important in VSMC proliferation or migration. In rabbit aortic smooth muscle cells, PDGF-BB-induced migration was reported to occur at concentrations lower than those needed for detectable PLC- γ activation.⁶² On the other hand, Liu et al. showed that an inhibitor of the phosphatidylinositol-specific PLC family antagonized PDGF-induced proliferation and migration.⁵⁷

5.1.3. Src family of tyrosine kinases

Src family is characterized by a non-receptor kinase domain, which normally is inactive because of an intrinsic suppression mechanism.⁶³ Binding to different receptors, including the α - or the β -PDGF-receptor, activates the kinase.^{52,63} The Src family is associated with PDGF-mediated proliferation⁵², but their contribution to cell migration is not entirely clear.⁵⁸

VSMC express several members of the Src family, including Src, Fyn, Lyn and Yes.³¹ Interestingly, in VSMC PDGF-induced activation of Src seems strongly related to migration and proliferation, and inhibition of Src blocks PDGF-induced VSMC migration and proliferation.^{64,65} Possible downstream targets of Src include the MAPK

signaling, FAK, Cas and paxillin.³¹ In colonic smooth muscle cells, Src and FAK were shown to promote PDGF-mediated Ca^{2+} influx and contraction.⁶⁶

5.1.4. SH2-containing protein-tyrosine phosphatase

The SH2-containing protein-tyrosine phosphatase (SHP-2) can be activated by the α - or the β -PDGF-receptor. It dephosphorylates the autophosphorylated receptor and is therefore part of the negative feedback mechanism. However, in certain conditions, SHP-2 also promotes positive signaling.⁵² PDGF-stimulated cells do not enter the cell cycle when SHP-2 is blocked⁵¹, and SHP-2 may be involved in the activation of the MAPK signaling pathway.⁵² Furthermore, several studies suggest a role in PDGF-induced-chemotaxis.⁵⁸

Studies regarding the role of SHP-2 in PDGF-stimulated VSMC are rare, but together with other phosphatases, SHP-2 was seen to have a negative impact on VSMC growth and migration.⁶⁷

5.1.5. Mitogen-activated protein kinases

MAPK are a family of cytoplasmic serine threonine kinases, which are involved in transduction from extracellular signals to the nucleus.¹⁵ Their activation leads to cell proliferation, differentiation, survival, inflammation and apoptosis.⁶⁸⁻⁷⁰ Furthermore, evidence is growing that they are also essential for cell migration.⁷⁰ They might be directly involved in vascular remodeling and diseases. In VSMC, three classes of MAPK have been shown to be activated by PDGF: ERK 1/2, p38 MAP kinase (p38) and JNK.⁶⁸

Extracellular signal-regulated kinases 1 and 2

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) become activated by their MAPK/ERK kinases MEK1 and MEK2, which in turn are activated by Raf. Activation of Raf usually requires Ras.⁷¹ Activation of Ras was shown to play a central role in proliferation and in migration⁵². In addition to ERK 1/2, several other molecules have been reported to be downstream effectors of Ras, including p38 and JNK.^{58,70} In PDGF-stimulated cells, Ras is activated by the nucleotide exchange factor Sos-1, which in turn becomes activated by the adaptor molecule Grb2. Grb2 binds directly to the β -PDGF-receptor and probably also to the α -PDGF-receptor.⁵²

In VSMC, the activation of ERK1/2 is associated rather with proliferation than with migration.⁵⁰ Nevertheless, their importance in migration has been shown in several studies in the last years. For example, antisense oligonucleotides against ERKs as

well as the infection of VSMC with a dominant negative mutant of ERK resulted in decreased VSCM migration after PDGF stimulation.^{68,72} The downstream signals of ERK providing VSMC migration are not clear; they may include MLCK, FAK and calpain.⁷⁰

p38 MAP kinase

PDGF induces also the activation of p38 MAP kinase (p38)⁶⁸, which becomes directly activated by MAPK kinases (MKK), including MKK3, MKK6 and others.⁶⁹ P38 has been associated with inflammation (for example via increased production of proinflammatory cytokines), proliferation and apoptosis.⁷³ In addition, several studies suggest an important role in migration.⁷⁰

Also in VSMC, the activation of p38 is thought to contribute to migration. Infection of VSMC with a dominant negative mutant of p38 as well as the treatment with p38 inhibitors reduced PDGF-induced VSMC and other smooth muscle cell migration.^{68,74} However, not all p38 inhibitors have been reported to inhibit PDGF-stimulated migration of VSMC.⁷⁵ MAPK activated protein kinase 2 (MK2) and its effector protein, the heat shock protein 27, have been reported to be necessary elements in downstream signaling of p38 in PDGF-induced smooth muscle cell migration.⁷⁴

c-Jun N-terminal kinase

In addition, PDGF and other extracellular stimuli activate c-Jun N-terminal kinase (JNK) via JNK kinases, including MKK4 and MKK7. Interestingly, also Rac, FAK, Src and Cas seem to activate JNK.⁶⁹ Like other MAPK, JNK has been associated with inflammation, cell survival, proliferation and apoptosis.⁷⁶ Several studies demonstrate the important role of JNK also in cell migration.⁷⁰

In VSMC, the role of JNK is less investigated than the role of p38 and ERK.³¹ Infection of VSMC with a dominant negative mutant of JNK as well as the treatment with JNK inhibitors reduced PDGF-induced VSMC migration⁶⁸, but further studies are needed to address the role of JNK. Also the downstream effectors remain to be elucidated; paxillin has been proposed as a possible effector protein.³¹

5.1.6. Focal adhesion kinase

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase localized at focal contacts and adhesions.⁷⁰ As part of a cytoskeleton-associated protein complex, including Src, Cas, Grb2 and Shc, it links integrin receptors to intracellular signaling

pathways.^{58,77} (See also section 4.1.) The exact function of FAK is unknown, but its implication in cellular migration was investigated in numerous studies.^{60,62,77-80} FAK has been reported to be phosphorylated after PDGF stimulation⁶², and its phosphorylation has been found to be essential for PDGF-induced migration.⁷⁷ The downstream effectors of FAK are incompletely understood, they may include paxillin.⁶⁰

Also in VSMC, FAK seems to play an important role in migration. FAK and paxillin have been found to be phosphorylated at lower concentrations than those needed for stimulation of other PDGF targets, like PLC- γ and PI₃K. Increased phosphorylation correlated with the chemotactic response to PDGF.⁶² Furthermore, overexpression of the endogenous inhibitor FAK-related-nonkinase suppressed PDGF-induced migration in VSMC.⁸¹

5.1.7. Janus kinase/ Signal transducers and activators of transcription

The signal transducers and activators of transcription (STATs) are a family of seven structurally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6.⁸² The phosphorylation of specific tyrosine residues leads to their homo- and heterodimerization. After activation, STATs are rapidly translocated to the nucleus, where they bind to target promoters to increase expression of several genes,⁸³ including immediate early genes, such as c-myc and c-fos, as well as cell cycle regulatory genes, such as cyclins.⁸⁴

A large number of cytokines, growth factors and ligands for G protein-coupled receptors are able to induce STAT activation.⁸² In contrast to most growth factor receptors, cytokine receptors often do not have an intrinsic tyrosine kinase activity. Therefore, receptor-associated Janus kinases (JAKs) provide the phosphorylation of STATs.⁸³ After ligand binding, the receptors undergo a conformational change, which brings two JAKs together allowing autophosphorylation and consecutive activation of STATs. Four classes of JAKs are known so far: JAK1, JAK2, JAK3 and TYK2.⁸²

At least STAT1, STAT3 and STAT5 can be directly activated by the PDGF-receptor.^{52,85} Furthermore, several studies suggest that PDGF-receptors can activate STATs via JAKs.⁸² For example, Vignais et al. showed that JAK1, JAK2 and TYK2 became phosphorylated after PDGF stimulation and were found to be associated with the receptor.⁸⁶ Furthermore, it has been suggested that Src⁵² and reactive oxygen species⁸⁴ could be involved in activation of STATs after PDGF stimulation. Finally, several MAPK phosphorylate STATs on serine residues leading to their full activation; therefore they are supposed to unify both JAK- and MAPK-signaling cascades.⁸⁴ The

JAK/STAT-signaling cascade is inactivated by classical feedback loops; STATs up-regulate the expression of suppressor of cytokine signaling (SOCS), which inactivate JAKs.⁸³

Due to the fact that STATs are transcription factors, they are crucial for a wide array of biological functions, including the regulation of proliferation, survival, differentiation and transformation.⁸⁴ Primarily in knock-out mice studies it has been shown that all STATs are very important in promoting an efficient immune response after cytokine stimulation.⁸² Constitutive activation of STAT3, STAT5a and STAT5b and the loss of activation of STAT1 has been found to be involved in cancer development.⁸⁷

Furthermore, STATs seems to be involved in the development of vascular diseases.⁸⁸ For instance, it has been shown that balloon injury of a rat carotid artery induced JAK2-, TYK2-, STAT1- and STAT3 expression and STAT3 phosphorylation.⁸⁹ Moreover, overexpression of a dominant negative STAT3 in a balloon injured artery resulted in reduced proliferation and neointima formation.⁹⁰ A similar study with similar results was done with STAT5b, indicating that also STAT5b might be involved in VSMC proliferation and migration.⁹¹

A few studies exist regarding the effects of STAT activation after PDGF stimulation. In VSMC, it has been demonstrated that the JAK2/STAT3-axis is important in vascular smooth muscle growth. Induction of cytosolic phospholipase A₂ (cPLA₂) was increased after PDGF stimulation and this seemed to be mediated by the STAT3/JAK2-axis. cPLA₂ is thought to be a major source of eicosanoid metabolites, which play an important role in cell proliferation and survival.⁹² In addition, the Jak2/STAT3-axis was shown to be necessary for VSMC migration. Transfection of VSMC with dominant negative JAK2 and STAT3 resulted in significantly decreased motility. Also in this study, cPLA₂ was found to be an important effector molecule.⁹³ Although no studies exist regarding the role of STAT3 in human vascular smooth muscle cells, it was demonstrated that PDGF stimulates proliferation of human airway smooth muscle cells via JAK/STAT3 activation.⁸⁴

Also STAT5b has been found to be activated after PDGF stimulation of VSMC. Transfection with dominant negative STAT5b reduced VSCM proliferation and motility. In addition, expression of cyclin D1 was seen to be induced by STAT5 after PDGF stimulation.⁹¹ Taken together, these studies suggest that STAT3 and STAT5b are important in VSMC migration, but further studies are needed to clarify the exact pathways, the upstream activators and the downstream effectors.

6. Indirubin

The discovery of indirubin goes back to a traditional Chinese medicine preparation, Dang Gui Long Hui Wan, a mixture of eleven herbal components. In the traditional Chinese medicine it is used as a therapeutic principle to “purge the fire of liver and gallbladder”. It has been used in the treatment of various chronic diseases, including hypertension, mania, convulsions, hypochondriac pain, and leukemia.⁹⁴ In 1967, the efficiency of Dang Gui Long Hui Wan in the treatment of chronic myelocytic leukemia was shown in a clinical study; Qing dai was discovered as the active ingredient.⁹⁵

Qing Dai, the Chinese name of indigo naturalis, is listed in the Chinese pharmacopoeia as a haemostatic, antipyretic, anti-inflammatory and sedative medicine. Furthermore, it is used in the treatment of bacterial and viral infections. It can be obtained from various dyed plants including *Baphicacanthus cusia*, *Indigofera suffruticosa*, *Indigofera tinctoria*, *Polygonum tinctorium* or *Isatis indigotica*.^{96,97} Initially, the main constituent of Qing Dai, the well known blue dye indigo, was associated with its antileukemic properties, but further investigations revealed the red colored indirubin as the responsible principle.⁹⁸ Clinical trials followed showing indirubin as an effective agent in the treatment of chronic myelocytic and chronic granulocytic leukaemia.⁹⁶

6.1. Origin of indigoids

In addition to plants, the bis-indoles indigo and indirubin can be found in mollusks and in the urine of patients suffering from various diseases, including acute myelomonocytic leukemia, porphyrinuria, and purple urine bag syndrome.^{99,100} Both compounds can also be detected in the urine of healthy persons.¹⁰⁰ In plants, indigoids form from indoxyls in dead organic material. The hydroxy-groups of indoxyl-precursor substances are released by enzymatic or acidic hydrolysis. Subsequently, free indoxyl is oxidized to isatin; isatin and indoxyl dimerize and form the blue indigo, the red-brown isoindigo and the red indirubin. At present, indigo and indirubin are mainly obtained by synthesis.¹⁰¹

6.2. Derivatives of indirubin

Although indirubin was generally well-tolerated in clinical trials, some patients had to stop the treatment because of severe side effects in the gastrointestinal tract.⁹⁵ Hence, a lot of indirubin analogues have been designed in the last years, with the aim to create derivatives with increased solubility, cellular efficacy and selectivity. Figure 5

presents the structures of indirubin and a range of the most frequently used indirubin derivatives.¹⁰²

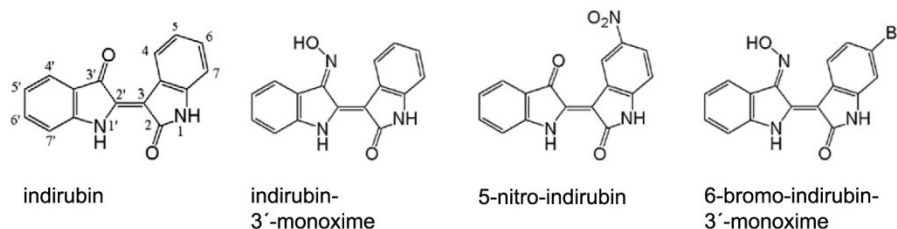


Fig. 5. Structures of indirubin and some of the most frequently used indirubin derivatives.

Figure adopted from Meijer et al.¹⁰²

6.3. Molecular targets of indirubin

Several molecular targets of indirubin have been found in the last years. Among the most important are cyclin-dependent kinases (CDKs), glycogen synthase kinase-3 (GSK-3), aryl hydrocarbon receptor (AhR), glycogen phosphorylase, JNK, Src, and STAT3.¹⁰²

6.3.1. Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are the catalytic subunits of a family of serine/threonine kinases, which are essential for the control of cell cycle events, transcription, and neuronal function.¹⁰³ A small group of the numerous CDK family triggers cell cycle progression and cell cycle regulation.¹⁰³ They are activated by associated proteins, the cyclins, which are synthesized and degraded during each cell cycle.

Briefly, the cell cycle can be divided into G1-phase, where proteins needed for DNA replication are synthesized, S-phase, where DNA is duplicated, G2-phase, where the cell prepares for mitosis, and M-phase, where two identical nuclei form.¹⁰⁴ Transition from the resting state G0-phase into G1-phase and the early G-phase are mediated by cyclin D/CDK4-complex and cyclin D/CDK6-complex.^{96,103} Transition to S-phase is mediated by cyclin E/CDK2-complex.^{96,103} During S-phase-progression, CDK2 associates with cyclin A.^{97,103} Cyclin A, together with CDK1, triggers then S-phase and G2-phase.^{97,103} Subsequently, cyclin B associates with CDK1 to drive the cell through M.^{96,103} CDK-activating kinase (CAK) is believed to phosphorylate all cell cycle CDKs. It is a protein complex formed of CDK7, Cyclin H and Mat1.¹⁰³

Indirubin and three derivatives, including indirubin-3'-monoxime (I3MO), were shown to inhibit CDK1, CDK2, CDK4 and CDK5 in cell-free kinase assays. The crystal structure of CDK2 in complex with indirubin derivatives showed that the kinases were inhibited via interaction of indirubins with their ATP-binding site, through van der Waals interactions and three hydrogen bonds.¹⁰⁵ Other studies confirmed this finding.¹⁰² A plentitude of studies investigating the effects of I3MO and its derivatives on numerous cancer cell lines^{98,105-108} and *in vivo* studies¹⁰⁹ followed. It has been found that I3MO and its derivatives can induce cell cycle arrest in all cancer cell lines investigated, with exception of adenocarcinoma cells.¹⁰⁵ Furthermore, 5'-substituted I3MO derivatives have been found to induce significant inhibition of tumor growth in rat tumor models.¹⁰⁹ The cells have been found to be mainly arrested in G2/M-phase^{98,105,107}, at least at higher concentrations of I3MO and derivatives; but also the arrest in other cell cycle phases has been shown.^{98,105-107} In addition, I3MO, and especially its derivatives, induced apoptosis in several cell lines and *in vivo* tumor models, but the rate differed from cell type to cell type.^{98,105-109}

Decreased activity of CDK1 and decreased concentration of cyclin B/CDK1 complex has been found in mammary carcinoma cells after incubation with I3MO⁹⁸, while reduced levels of cyclin B/CDK2 and cyclin D/CDK4 have been reported in oral carcinoma cells after incubation with 5-nitro-I3MO.¹⁰⁷ In addition, I3MO and derivatives have been shown to induce also CDK inhibitors, for example p16¹⁰⁷ and p21.¹⁰⁶⁻¹⁰⁸ Also, indirubins have been found to activate classical apoptotic signaling molecules, including p53, which mediates cell cycle arrest through induction of CDK inhibitors¹⁰⁸ and members of the caspase-cascade.^{106,107,109} On the other hand, 7-bromo-I3MO has been shown to induce apoptosis independent of classical pathways.¹¹⁰ Taken together, these results indicate that I3MO and its derivatives may be interesting anticancer agents. Their exact action on CDK inhibition remains to be elucidated.

In addition to its anticancer activity, I3MO was found to inhibit CDK9, the catalytic subunit of a transcription factor essential for HIV-1 replication, in a cell-free kinase assay. Furthermore, it inhibited HIV-1 replication in leucocytes at concentrations that did not affect cell proliferation.¹¹¹

6.3.2. Glycogen synthase kinase-3

The glycogen synthase kinase-3 (GSK-3) family is evolutionarily related to CDKs and consists of three members discovered so far: GSK-3 α , GSK-3 β and a splice variant of GSK-3 β .^{99,112} The isoforms show close homology in their kinase domains, and little is known about isoform-specific functions.¹¹² The ubiquitously expressed serine-threonine kinase shows four interesting properties. First, it is constitutively active.¹¹³ Second, phosphorylation of an N-terminal serine by several proteins, including Akt, Protein kinase A or C and others, leads to inactivation of GSK-3, whereas phosphorylation of a tyrosine residue leads to its activation. The tyrosine residue activation might occur by autophosphorylation or by other kinases, but little is known about them.¹¹² Third, phosphorylation of substrates by GSK-3 leads generally to their inactivation.¹¹³ Finally, GSK-3 substrates need to be “pre-phosphorylated” by other kinases to be phosphorylated by GSK-3.^{112,113}

GSK-3 has been reported to phosphorylate more than 50 proteins *in vitro*¹¹⁴, including metabolic and signaling proteins, structural proteins and transcription factors.¹¹² Thus, it contributes to the regulation of crucial cellular functions, such as apoptosis, cell cycle, cell polarity and migration, gene expression, and others.¹¹⁴

For many years, the kinase has been thought to be strongly related to glycogen metabolism. It phosphorylates and thus inactivates glycogen synthase, an enzyme implicated in the conversion of glucose to glycogen.^{113,115} Among other mechanisms, it is thought that increased GSK-3 activity causes insulin resistance and diabetes, by reducing the clearance of glucose from blood.¹¹⁵ Today, GSK-3 is also known to be involved in other diseases, including Alzheimer’s disease, cancer and inflammation.^{113,114,116} GSK-3 inhibitors are currently being developed for various diseases.¹¹³

In Alzheimer’s disease, GSK-3 seems to be responsible for the hyperphosphorylation of tau, a protein implicated in the formation of neurofibrillary tangles.¹¹⁷ Furthermore, β -amyloid, a protein responsible for the formation of neuronal plaques, is thought to activate GSK-3.¹¹⁸

The role of GSK-3 in cancer formation is less clear. It is known that GSK-3 is centrally involved in the Wnt signal transduction pathway. Wnts play essential roles in cell proliferation, differentiation and motility. Within this pathway, GSK-3 serves to phosphorylate β -catenin, thereby targeting it for degradation and inhibiting transcription of Wnt target genes.^{116,119} Mutations of proteins in the Wnt signaling

pathway have been linked to numerous types of human cancer. Thus, it has been thought that GSK-3 inhibition could mimic tumorigenesis.¹¹⁹ However, studies on the role of GSK-3 in oncogenesis are contradictory and its action may be cell type-dependent.¹²⁰ In their review, Patel and Woodgett even proposed GSK-3 inhibitors as selective anticancer agents.¹¹⁶

Moreover, GSK-3 seems to have an important role in inflammation. It was shown to be necessary for proinflammatory cytokine production in peripheral immune cells¹²¹ and to stimulate the immune response of microglia, including migration.¹²²

Generally, GSK-3 has been found to influence all stages of cell migration, including dynamics of actin cytoskeleton, microtubule and adhesion turnover. For instance, GSK-3 has been seen to activate small G proteins, including Rac, Rho and ADP-ribosylation factor 6 (Arf6).¹²³ However, the specific signaling pathways are not clear. Its effects may depend from time and place of action and may be also cell type specific.¹¹⁴ In contrast, some studies reported a promigratory effect of inhibited GSK-3: it was proposed that Cdc42 phosphorylates and thus inhibits GSK-3 locally in astrocytes, thereby allowing the adenomatous polyposis coli protein (APC) to promote microtubules dynamics.¹²⁴ On the other hand, inhibition of total cellular GSK-3 suppressed migration of several cell types, including intestinal epithelial cells¹²⁵, kidney epithelial cells¹²⁶, epidermal keratinocytes¹²⁷, and axon elongation in neurons.¹²⁸ Due to these results it could be expected that inhibition of GSK-3 may also suppress VSMC migration.

However, expression of a constitutively active, nonphosphorylatable mutant of GSK-3 reduced VSMC migration toward growth factor stimuli, including PDGF.¹²⁹ In the few existing studies and reviews about the role of GSK-3 in VSMC, GSK-3 was found to suppress proliferation and to induce apoptosis. Growth factors, including PDGF, were found to phosphorylate and to inactivate GSK-3, thus reversing these effects.^{130,131} Finally, expression of a constitutively active mutant of GSK-3 in rats was reported to reduce neointima formation in rats.¹³²

Indirubin and some of its derivatives, especially I3MO and 5-iodo-I3MO have been shown to inhibit GSK-3 even more potently than CDKs in cell-free kinase assays. Similar to CDK inhibition, I3MO acts as a competitive inhibitor of ATP binding to GSK-3.⁹⁹ In Alzheimer's disease models, I3MO was demonstrated to suppress tau phosphorylation *in vitro* and *in vivo*, likely due to inhibition of GSK-3 and CDK5.¹⁰⁵ Furthermore, a GSK-3 selective I3MO derivate, 6-bromo-I3MO, decreased

phosphorylated tau protein levels, and total tau protein levels.¹³³ In addition, the blockage of GSK-3 by I3MO prevented loss of dopamine neurons in a Parkinson disease mouse model, suggesting an important role of the kinase also in this disorder.¹³⁴ Finally, I3MO suppressed Notch-1 activation, a protein implicated in cell-fate decisions, in a GSK-3-dependent manner in human embryonic kidney cells.¹³⁵

6.3.3. Other targets of indirubin

Although indirubins have been mostly described as GSK-3 and CDK inhibitors, there are some reports about other molecular targets. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that regulates genes involved in xenobiotic metabolism, cellular proliferation, and differentiation. Although a number of xenobiotic compounds, including dioxin, have been found to interact with AhR, the physiological ligands are unknown. In a yeast reporter assay, indigo and indirubin have been identified as AhR ligands present in normal human urine.¹⁰⁰ A subsequent *in vivo* study confirmed this finding, but indirubins have been found to be clearly less potent than *in vitro*.¹³⁶ A number of AhR target genes up-regulated after indirubin stimulation have been identified, including cytochrome p 450-enzymes^{136,137} and the CDK inhibitor p27.¹³⁸ Up-regulation of p27 has been found to induce cell cycle arrest. Interestingly, the effects of indirubin on AhR and CDK seemed to be independent, as kinase-inactive indirubin derivatives strongly interacted with AhR and induced cell cycle arrest in G1-phase.¹³⁸ Thus, the interaction with AhR might further contribute to the antiproliferative properties of indirubins.

Moreover, crystal structure analyses have demonstrated that indirubin-5-sulphonate binds to the purin inhibitor site of glycogen phosphorylase, an enzyme implicated in glycogen degradation.¹³⁹ In cell-free kinase assays, I3MO has been found to inhibit serum- and glucocorticoid-induced kinase (SGK), AMP-activated protein kinase (AMPK) and lymphocyte kinase (LCK).¹⁴⁰ In computational approaches, 7-substituted I3MOs have been shown to inhibit Aurora B and C, kinases involved in mitosis.¹⁴¹ JNK has been shown to be inhibited by I3MO in cell-free kinase assays and the inhibition of JNK seemed to be responsible for increased c-Jun phosphorylation and thus apoptosis in cerebellar granule neurons.¹⁴² Finally, I3MO inhibited Src kinase and JAK-1 kinase *in vitro* and suppressed constitutive STAT3 signaling due to inhibition of Src kinase in human cancer cells.¹⁴³

7. Aim of the work

In her PhD thesis Andrea Schwaiberger showed that I3MO inhibits PDGF-induced VSMC proliferation dose-dependently, arresting them in G0/G1-phase. Furthermore, she found a selective complete inhibition of STAT1/STAT3/STAT5b phosphorylation and a partial inhibition of STAT6 phosphorylation after stimulation with PDGF-BB. Other early signaling protein kinases, including p38, ERK and Akt were not affected.

In addition, in collaboration with Univ.-Prof. Dr. Bernd Binder from the Department of Vascular Biology and Thrombosis Research (Medical University of Vienna), she showed a significantly reduced neointima formation in a cuff-induced neointima mouse model.¹⁴⁴

However, some interesting questions about I3MO regarding its cellular actions remained open. Therefore, we asked whether the compound is able to inhibit PDGF-BB-induced VSMC migration as well. Additionally, we wanted to investigate whether inhibition of STATs by I3MO might be responsible for impaired VSMC migration. To further elucidate the underlying signaling pathways, we compared cellular actions of I3MO to other protein inhibitors, including the JAK2 inhibitor AG490 and the GSK-3 inhibitor LiCl.

C. MATERIALS AND METHODS

C. MATERIALS AND METHODS

1. Materials

Unless otherwise noted, all cell culture reagents were obtained from Lonza Group Ltd. (Basel, Switzerland). All other reagents were obtained from Carl Roth (Karlsruhe, Germany). Laurent Meijer from CNRS (Roscoff, France) kindly provided indirubin-3'-monoxime (I3MO).

Growth factors

Name	Conc. used	Provider
recombinant human PDGF-BB	10 ng/ml	Bachem (Weil am Rhein, Germany)

Table I. Growth factors

PDGF-BB was dissolved in sterile aqua bidest. (10 %) and sterile PBS + 0.2 % BSA (90 %) to a concentration of 10 ng/μl, and stored at -20 °C. Prior of use it was diluted with DMEM to a final concentration of 1 ng/μl. For abbreviations see also chapter H, section 1.

Protein inhibitors

Name	Conc. used	Provider
I3MO	3 μM, 5 μM	Meijer (CNRS, Roscoff, France)
LiCl	10 mM, 20 mM	Carl Roth (Karlsruhe, Germany)
AG490	25 μM, 50 μM	Calbiochem (La Jolla, CA, USA)

Table II. Protein inhibitors

Protein inhibitors were dissolved in DMSO and stored at -80 °C.

1.3. DNA cross linker

Name	Conc. used	Provider
Mitomycin C	2.5 nM–25 μM	Calbiochem (La Jolla, CA, USA)

Table III. DNA cross linker

Mitomycin C was dissolved in DMSO.

1.4. Antibodies

Primary Antibodies	Source	Dilution used	Provider
α -tubulin	Mouse, mc	1:2500	SC
Cyclin A	Rabbit, pc	1:1000	SC
Cyclin D1	Mouse, mc	1:1000	NEB
cPLA ₂	Rabbit, pc	1:500	NEB
Phospho-ERK1/2 Y ^{202/204}	Rabbit, pc	1:1000	NEB
Phospho-p38 Y ^{180/182}	Rabbit, pc	1:1000	NEB
Phospho-STAT3 Y ⁷⁰⁵	Rabbit, pc	1:1000	NEB
Phospho-STAT5 Y ⁶⁹⁴	Rabbit, pc	1:1000	CS
Secondary Antibodies	Source	Dilution used	Provider
HRP-linked rabbit IgG	goat	1:2500	CS
HRP-linked mouse IgG	goat	1:2500	US
<i>Abbreviations</i>			
mc	Monoclonal		
pc	Polyclonal		
SC	Santa Cruz (Santa Cruz, CA, USA)		
NEB	New England Biolabs (Beverly, MA, USA)		
CS	Cell signaling (Danvers, MA, USA)		
HRP	horseradish peroxidase		
US	Upstate (Charlottesville, VA, USA)		

Table IV. Antibodies

Antibodies were diluted in 5% BSA in TBS-T.

2. Methods

2.1. Cell culture

Cell culture media		
Growth medium	Calf serum	10 %
	L-glutamine	2 mM
	Penicillin	100 U/ml
	Streptomycin	100 µg/ml
	in DMEM	
Starvation medium	Calf serum	0.1 %
	L-glutamine	2 mM
	Penicillin	100 U/ml
	Streptomycin	100 µg/ml
	in DMEM	
Freezing medium	Calf serum	30 %
	L-glutamine	2 mM
	Penicillin	100 U/ml
	Streptomycin	100 µg/ml
	DMSO	10 %
	in DMEM	
Cell culture buffers		
PBS, pH 7.4	NaCl	123.2 mM
	Na ₂ HPO ₄	10.4 mM
	KH ₂ PO ₄	3.5 mM
	in aqua dest.	
Cell culture reagents		
Trypsin/ EDTA in PBS	Trypsin (Invitrogen, Carlsbad, CA, USA)	0.05 %
	EDTA	0.02 %
	in PBS	
Abbreviations		
DMEM	Dulbecco's Modified Eagle Medium	
DMSO	Dimethyl sulfoxide	
PBS	Phosphate buffered saline	
EDTA	Ethylenediaminetetraacetic acid	

Table V. Cell culture media, buffers and reagents

Cell isolation

VSMC were kindly provided by Irene Sroka. They originate from rat thoracic aortas of sibling Sprague-Dawley rats. For isolation protocol see reference.¹⁴⁵ Cells obtained from early passages (3-6) were frozen in liquid nitrogen.

Cell cultivation

VSMC were cultured in growth medium at 37 °C and 5 % CO₂. They were grown to a nearly confluent cell layer and passaged twice per week. For passaging, cells were washed with PBS and trypsinized with Trypsin/EDTA for three minutes. Subsequently, growth medium was added and the cells were centrifuged for 4 min at 1400 rpm (373xg). The cell pellet was re-suspended in growth medium and the cells were counted with a trypan-blue staining based cell viability analyzer (Vicell™ XR, Beckmann Coulter, Fullerton, CA, USA). Cells were seeded in flasks, in 6- or 12-well plates or in 6 mm dishes and grown to designated density. Passages 6-14 were used for experiments. Prior to experiments, VSMC were cultured with starvation medium for 24 h-48 h.

Storage

Cells were trypsinized, centrifuged at 1400 rpm (373xg/4min/4 °C), and re-suspended in freezing medium. Cryovials with 1x10⁶ cells were kept at 4 °C for 20 min. Subsequently, they were frozen at -20 °C for 1 day and at -80 °C for 2-3 days and then transferred into liquid nitrogen. For unfreezing cells, they were thawed, put in growth medium and centrifuged (1400 rpm/4 min). The cell pellet was dissolved in growth medium again and seeded in cell culture flasks.

2.2. Protein extracts

Protein extract buffers

Lysis buffer

Stock solution, pH 7.5	HEPES	50 mM
	NaCl	50 mM
	NaF	50 mM
	Na ₄ P ₂ O ₇ ·10H ₂ O	10 mM
	EDTA	5 mM
	Na ₃ VO ₄	1 mM
	in aqua dest.	
Prior to use	Stock solution	430 µl
	PMSF (0,1 M in isopropanol)	5.0 µl
	Complete™ 25x	20 µl
	Triton X-100 (10% in aqua dest.)	50 µl

SDS sample buffer (3x buffer)

Stock solution	TRIS- HCL pH 6.8 0,5 M	0.19 M
	SDS	0.21 M
	Glycerol	4.10 M
	Bromophenol blue	0.22 mM
	in aqua dest.	
Prior to use:	Stock solution	85%
	2-Mercaptoethanol	15%

Protein quantification solution	Rotiquant© (20 %in aqua bidest.)
--	----------------------------------

Abbreviations

HEPES	Hydroxyethylpiperazineethanesulfonic acid
PMSF	Phenylmethanesulphonylfluoride
SDS	Sodium dodecyl sulfate

Table VI. Protein extraction reagents

Protein extraction

0.9 x 10⁶ cells were seeded in 60 mm dishes, cultured for 24 h at 37 °C, 5 % CO₂ and serum-starved for 24 hours prior to experiment. Subsequently, they were preincubated with protein inhibitors (I3MO, LiCl, AG490) or vehicle (DMSO 1 %) for 30 min and stimulated with 10 ng/ml of PDGF-BB for 10 min. Cells were washed three times with ice-cold PBS and treated with lysis buffer (10 min/4 °C). Cells were scraped off and lysates were centrifuged (16000xg/10 min/4 °C). Supernatants were diluted with sample buffer (1:3) and heated to 95 °C (5 min). Samples were stored at -20 °C.

Protein quantification

For protein quantification after extraction a Bradford assay was performed.¹⁴⁶ The method is based on the shift of the absorbance maximum of Commassie Brilliant Blue G-250 from 470 nm to 595 nm after binding to basic or aromatic amino residues. A small amount of supernatant of each protein sample was diluted 1:10 in aqua bidest and triplicates of 10 µl were transferred in a 96-well plate. Subsequently, 190 µl of diluted Rotiquant® was added and absorbance was measured at 595 nm with a Tecan sunrise™ microplate reader. For protein quantification, a standard curve with increasing concentrations of BSA (50-500 ng/µl) was prepared.

2.3. SDS-polyacrylamide gel electrophoresis

Electrophoresis puffer	TRIS-base	250 mM
	Glycine	1.92 M
	SDS	35 mM
	in aqua dest.	
SDS page gels		
Stacking gel		
Freshly prepared:	PAA	640 µl
	(stock: 30 % acrylamid, 0.8 % bisacrylamid)	
	TRIS HCl pH 6.8	375 µl
	SDS (10 %)	37.5 µl
	Aqua dest.	2.62 ml
Addition prior to use:	TEMED	7.5 µl
	APS (10 %)	37.5 µl
Resolving gel		
Freshly prepared:	PAA (stock: see stacking gel)	1.875 ml
	TRIS HCl pH 8.8	1.875 µl
	SDS	75 µl
	Aqua dest.	3.05ml
	TEMED	7.5 µl
Addition prior to use:	APS (10 %)	37.5 µl
Abbreviations		
PAA	Polyacrylamide	
TRIS	Trihydroxymethylaminomethane	
TEMED	Tetramethylethylenediamine	
APS	Ammonium persulfate	

Table VII. SDS-PAGE reagents

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli was used for separating proteins.¹⁴⁷ Hereby, proteins move through a PAA-gel towards the anode in an electric field. The proteins are denatured and negatively charged because of their previous dissolution in SDS. They are separated almost only due to their size. Resolution gel and stacking gel were prepared in a gel chamber and an equal amount of protein samples (20 µg) was loaded. The gel chamber was put into electrophoresis chambers (Mini-Protean™, Biorad laboratories, Hercules, CA, USA) filled with electrophoresis buffer. Electrophoresis was performed at constant voltage (100 V), until the dye front reached the anode.

2.4. Western blotting

Western Blotting buffers

Blotting buffer (5x buffer)	TRIS-base	125 mM
	Glycine	0.97 M
	in aqua dest.	
Dilution prior to use:	Blotting buffer	20 %
	MeOH	20 %
	Aqua dest.	ad 100 %

Table VIII. Western blotting buffers and solutions

Proteins were transferred from the PAA-gel to an Immun-Blot™ PVDF membrane (Biorad Laboratories, Hercules, CA, USA) using tank blotting technique. Thereby, gel and membrane were placed into gel holder cassettes and put into cooled blotting chambers (Mini-Trans-Blot™, Biorad, Laboratories, Hercules, CA, USA), filled with blotting buffer. They were run at constant current (110 mA/ gel for 120 min).

2.5. Protein detection

Protein detection buffers and solutions

TBS-T, pH 8 (10 x)	TRIS-base	25 mM
	NaCl	190 mM
	Tween-20 (Addition after pH adjustment) in aqua dest.	0.08 mM
Dilution prior to use:	TBST 10 x	10 %
	Aqua dest.	90 %
ECL solution	TRIS-HCl pH 8.5	0.1 M
	Luminol (stock: 250 mM in DMSO)	1.25 mM
	p-Coumaric acid (stock: 91 mM in DMSO) in aqua dest.	0.2 mM
	H ₂ O ₂ (stock: 30 %)	0.01%
Addition prior to use		

Table IX. Protein detection buffers and solutions

After blotting, membranes were washed with TBS-T (3 x 10 min), blocked with 5% milk powder in TBS-T and incubated with primary antibody-solutions overnight at 4 °C. Subsequently, membranes were washed with TBS-T (3 x 10 min), incubated with secondary antibody-solutions and washed again with TBS-T (3 x 10 min). Proteins were detected with a luminescent image analyzer (LAS-3000™, Fujifilm, Tokyo, Japan), using freshly prepared ECL solution.

2.6. Wound healing assay

For measuring migration, a wound healing assay (also named scratch assay) was used. For this assay, cells were first grown to a confluent cell monolayer in 6-well plates and serum-starved for 48 h. Using a sterile pipette tip, a small scratch was created. Subsequently, cells were pretreated with protein inhibitors (I3MO, LiCl, and AG490) or vehicle (DMSO 1 %) for 30 min and then stimulated with PDGF-BB (10 ng/ml). Migration rate was determined by photographing the cell free area of each scratch immediately before and 22 h after PDGF stimulation (Olympus Live View Digital SLR Camera E-330, Olympus Europe GmbH, Hamburg, Germany). For evaluation, Cell Profiler software was used. (Cell Profiler, Broad Institute, Cambridge, MA, USA, www.cellprofiler.org) To ensure that only migration, but not proliferation contributes to scratch re-narrowing, the assay was stopped after 22 h, whereas the VSMC used had a generation time of 26-28 h.¹⁴⁵

2.7. Phalloidin staining

Phalloidin staining reagents

collagen I-solution	collagen I in 0.02 N acetic acid (BD Bioscience Pharmingen, San Diego, CA, USA).	
Cytoskeleton buffer, pH 6.1	NaCl	150 mM
	EGTA	5 mM
	MgCl ₂	5 mM
	Glucose	5 mM
	MES	10 mM
	NaOH (pH adjustment to 6.1)	
Extraction buffer	Triton X-100	0.25 %
	Glutaraldehyde	50 %
	in cytoskeleton buffer	
Fixation buffer	Glutaraldehyde	1%
	in cytoskeleton buffer	
Phalloidin solution	FITC-labeled phalloidin solution (Sigma Aldrich, St. Louis, MO, USA) in PBS	0.66%

Abbreviations

EGTA	Ethylene glycol tetraacetic acid
MES	2-N-morpholino-ethanesulfonic acid
FITC	Fluorescein isothiocyanate

Table X. Phalloidin staining reagents

The phallotoxin phalloidin binds specifically to the actin cytoskeleton of cells.¹⁴⁸ Therefore, in this assay fluorescent-labeled phalloidin was used to visualize changes in actin cytoskeleton of VSMC after PDGF stimulation. For this assay, coverslips were placed in 12 well-plates and covered with collagen I-solution (350 µl/ml) under sterile conditions for 2 h at room temperature. Then, the coverslips were washed twice with PBS and carefully dried. 0.2×10^6 cells per coverslip were seeded on the 12 well-plate for 24 h, and serum-starved for another 24 h. Subsequently, cells were pretreated with protein inhibitors (I3MO, LiCl) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for one hour. Cells were briefly rinsed with PBS, extracted with preheated extraction buffer for 1 min and postfixed with fixation buffer at room temperature for 15 min. Cells were washed with PBS for a few minutes. Subsequently, cells were stained with 20 µl of phalloidin solution in a dark and humid chamber for 30 min. Samples were kept in the dark from this step onwards.

Coverslips were washed with PBS three times for 5 min and then mounted with mounting medium (Sigma Aldrich, MO, USA) on a glass slide. Samples were analyzed with a fluorescence microscope (Olympus BX51, Olympus Europe GmbH, Hamburg, Germany) and pictures were taken with cell imaging software (cell[^]F software, Europe GmbH, Hamburg, Germany).

2.8. Technical equipment and software

Technical equipment		Provider
Vi-Cell™ XR	Cell viability analyzer and cell counter	Beckmann Coulter, Fullerton, CA, USA
TECAN Sunrise™	96-well-plate reader	TECAN, Mannedorf, Switzerland
LAS-3000™	Luminescent image analyzer	Fujifilm, Tokyo, Japan
Olympus CKX31	Light microscope	Olympus Europe GmbH, Hamburg, Germany
Olympus Live View Dig SLR Camera E-330	Camera	Olympus Europe GmbH, Hamburg, Germany
Software		
www.cellprofiler.org	Cell migration software	Broad Institute, Cambridge, MA, USA
GraphPad PRISM™ 4.03	Statistical software program	GraphPad Software Inc, San Diego, CA, USA

Table XI. Technical equipment and software

2.9. Statistics

Statistical analyses were made using GraphPad PRISM™, version 4.03 (GraphPad Software Inc, San Diego, CA, USA). Experiments were carried out at least three times and data were expressed as means ± standard error (SEM). For statistical evaluation, one-way ANOVA followed by Dunnett post-test was used. P-value < 0.05 was considered statistically significant.

D. RESULTS

D. RESULTS

In her PhD thesis, Andrea Schwaiberger showed an antiproliferative effect of I3MO on VSMC in cell culture. Furthermore, neointima formation was reduced by I3MO in a cuff-induced neointima mouse model.¹⁴⁴ We wanted to learn more about the influence of I3MO on VSMC, concentrating on PDGF-induced migration and the signaling proteins inhibited by I3MO. Our interest was especially focused on a possible role of JAK2 and GSK-3 as targets of I3MO.

1. Influence of I3MO, AG490 and LiCl on PDGF-BB-induced VSMC migration

1.1. Wound healing assay as a model to study PDGF-BB-induced migration

To examine the influence of I3MO on VSMC migration, we used a wound healing migration assay (see chapter C, section 2.6.). For migration stimulation, we utilized 10 ng/ml of PDGF-BB, since previous results identified this concentration as optimal to trigger VSMC migration.¹⁴⁵ Fig. 6 shows the concentration-dependent influence of PDGF-BB on VSMC migration. Migration was stimulated from concentrations of 1 ng/ml of PDGF-BB onwards. The migration peak was reached at 10 ng/ml, whereas 20 ng/ml led to decreased migration. Data were generated by Mario Kumerz and Irene Sroka (PhDs).

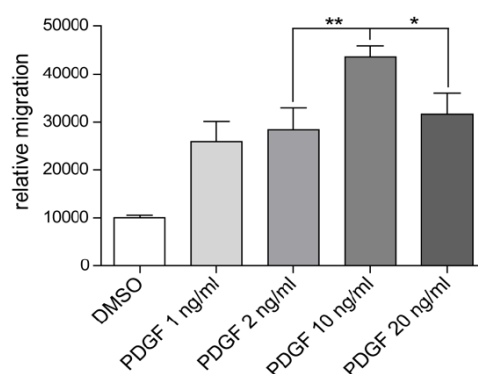


Fig. 6. Concentration-dependent increase in PDGF-BB-induced VSMC migration.

Serum-starved VSMC were scratched, pretreated with DMSO 1 % for 30 min and stimulated with increasing concentrations of PDGF-BB (1-20 ng/ml). Cells were captured at 0 h and after 20 h. Cell free areas were calculated using Cell Profiler software (see chapter C, section 2.8.) Graphs show means of three independent experiments $\pm$ SEM. (, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by Bonferroni post-test versus PDGF treatment). Data generated by Mario Kumerz and Irene Sroka, PhDs.*

In order to exclude that PDGF-BB-induced proliferation contributes to re-narrowing of the scratched area within the timeframe of the experiment, we used the DNA cross linker Mitomycin C, an antiproliferative agent¹⁴⁹

Fig. 7 shows that 100 nM of Mitomycin C did not reduce PDGF-stimulated VSMC migration. Concordant with previous work¹⁵⁰, the same concentration of Mitomycin C led to an increase in S-phase-arrested cells after 24 h (Fig. 7). Therefore we concluded that our experimental setup allowed us to study exclusively cell migration phenomena. Data were generated by Irene Sroka, PhD.¹⁴⁵

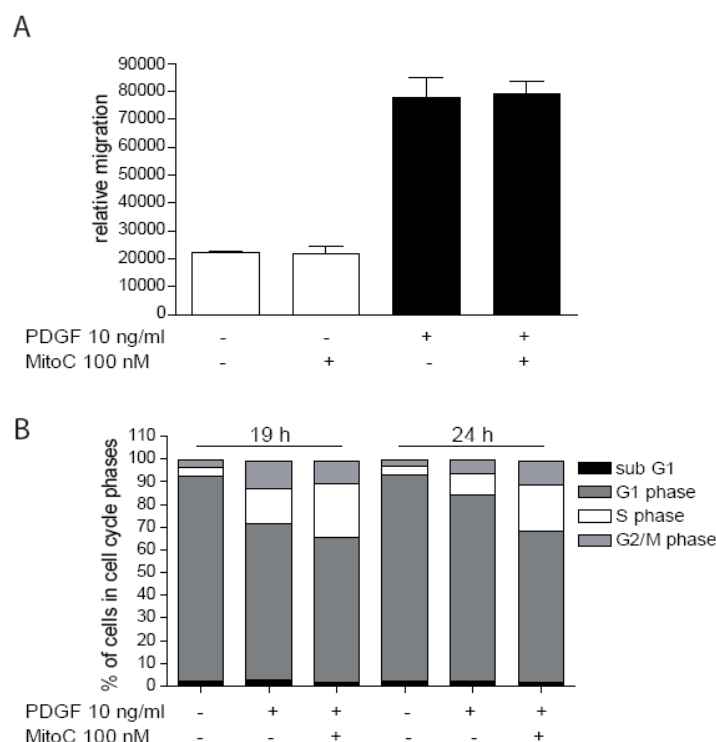


Fig. 7. Influence of Mitomycin C on PDGF-BB-induced VSMC migration and proliferation.

A. Serum-starved VSMC were scratched, pretreated with Mitomycin C or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml). Cells were captured at 0 h and after 20 h. Cell free areas were calculated using Cell Profiler software (see chapter C, section 2.8.) Graphs show means of three independent experiments $\pm$ SEM. (***, $p < 0.001$, one-way ANOVA followed by Bonferroni post-test versus PDGF treatment). Data were generated by Irene Sroka, PhD. **B.** Serum-starved VSMC were pretreated with Mitomycin C or vehicle (DMSO 1%) for 30 min and stimulated with PDGF-BB (20 ng/ml). The percentage of cells in each cell cycle phase was determined after 20 h, using flow cytometric analysis of PI-stained nuclei. Graphs show means of three independent experiments. Data were generated by Irene Sroka, PhD.

1.2. I3MO inhibits PDGF-BB-induced migration of VSMC

I3MO was shown to effectively reduce PDGF-induced proliferation.¹⁴⁴ Since migration of VSMC also plays an important role in the onset of atherosclerosis and restenosis, we aimed to investigate a potential antimigratory effect of I3MO. We therefore subjected I3MO to a wound healing assay. Fig. 8 shows that I3MO (in concentrations of 3 μ M and 5 μ M) effectively reduced VSMC migration in a wound healing assay after stimulation with 10 ng/ml of PDGF-BB.

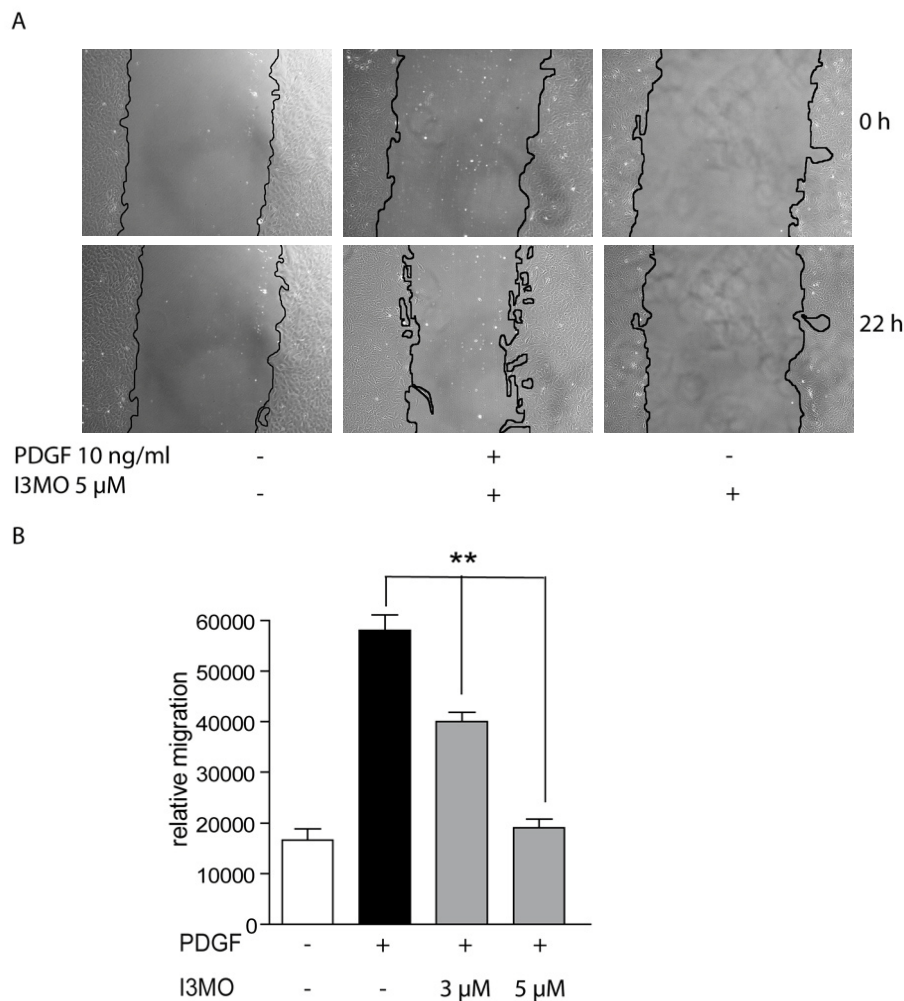


Fig. 8. Influence of I3MO on PDGF-BB-induced migration.

Serum-starved VSMC were scratched, pretreated with I3MO (3 μ M or 5 μ M) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml). Cells were captured at 0 h and after 22 h. Cell free areas were calculated using Cell Profiler software (see chapter C, section 2.8.). (A) Representative photographs of scratches. (B) Graphs show means of three independent experiments $\pm$ SEM. (**, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

1.3. AG490 and LiCl inhibit PDGF-induced migration of VSMC

Previous work in our lab has shown that I3MO selectively reduced STAT3 phosphorylation on Y⁷⁰⁵ upstream of STAT3 without affecting other early-signaling kinases.¹⁴⁴ The direct target of I3MO, however, has not been elucidated so far.

STAT3 has been reported in a few studies to promote VSMC migration; and this might depend on its phosphorylation by an upstream kinase, JAK2.^{93,151} Hence, we hypothesized that also I3MO might reduce VSMC migration after PDGF-BB stimulation via inhibition of JAK2 and subsequent STAT3 phosphorylation. For investigating this hypothesis, we tested the effects of the tyrphostin AG490, a selective JAK2 inhibitor¹⁵²⁻¹⁵⁴ on VSMC migration after stimulation with 10 ng/ml of PDGF-BB. We used concentrations from 25 to 50 μ M, as described in the literature.^{93,155,156}

Furthermore, I3MO has already been shown as a GSK-3 inhibitor in different cell types and GSK-3 has been described to be involved in migration (see chapter B, section 6.3.2.). Therefore, we additionally examined whether LiCl, a known GSK-3 inhibitor¹⁵⁷⁻¹⁶³, influenced VSMC migration after stimulation with 10 ng/ml of PDGF-BB in a wound healing assay. According to several other studies, we used concentrations of LiCl from 10 to 20 mM.^{128,164-166} Fig. 9 shows that in addition to I3MO also AG490 and LiCl were able to potentially inhibit VSMC migration.

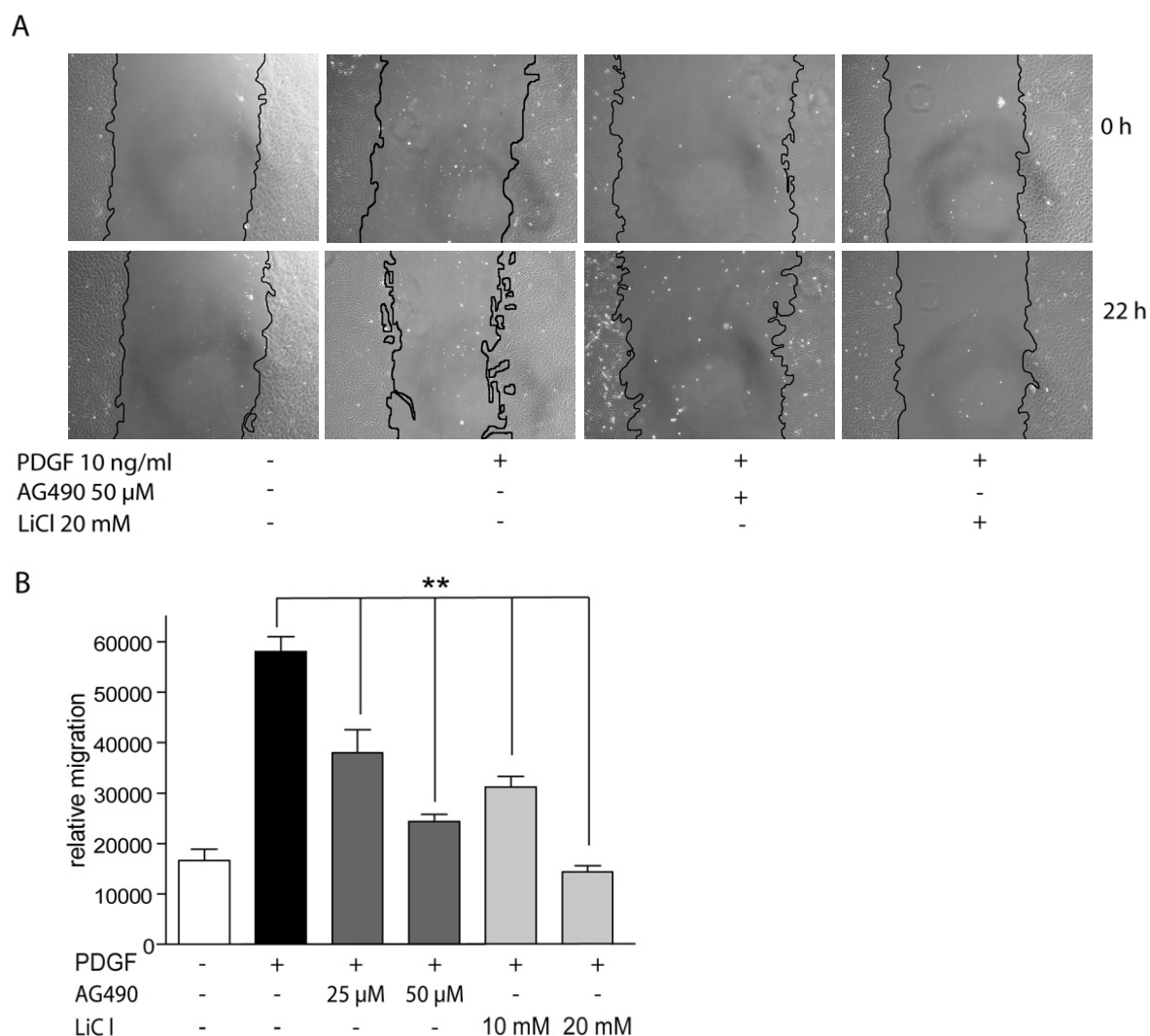


Fig. 9. Influence of LiCl and AG490 on PDGF-BB-induced migration.

Serum-starved VSMC were scratched, pretreated with AG490 (25 μM or 50 μM), LiCl (10 mM or 20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml). Cells were captured at 0 h and after 22 h. Cell free areas were calculated using Cell Profiler software (see chapter C, section 2.8.). (A) Representative photographs of scratches. (B) Graphs show means of three independent experiments ± SEM. (**, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

To exclude that only osmotic influences contribute to a decrease in VSMC migration after treatment with LiCl, we repeated the experiment with NaCl and MgCl₂ at the same concentrations (10 and 20 mM). Fig. 10 shows that neither the treatment with NaCl nor with MgCl₂ led to a similar reduction in VSMC migration after stimulation with 10 ng/ml of PDGF-BB.

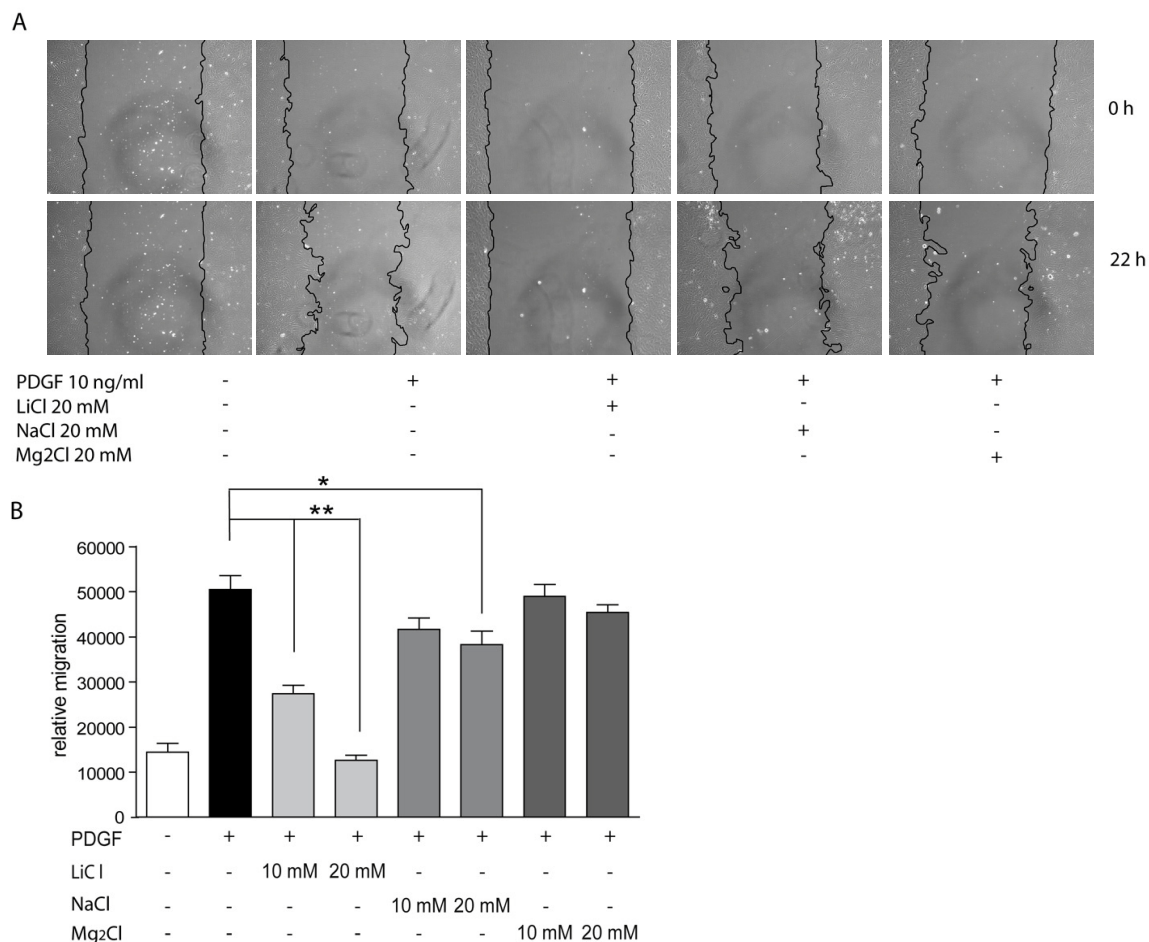


Fig. 10. Influence of LiCl, NaCl and MgCl₂ on PDGF-BB-induced migration.

Serum-starved VSMC were scratched, pretreated with LiCl (10 mM or 20 mM), NaCl (10 mM or 20 mM), MgCl₂ (10 mM or 20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml). Cells were captured at 0 h and after 22 h. Cell free areas were calculated using Cell Profiler software (see chapter C, section 2.8.) (A) Representative photographs of scratches. (B) Graphs show means of three independent experiments $\pm$ SEM. (*, $p < 0.05$; **, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

Overall, these data show that both, an inhibitor of JAK2 and an inhibitor of GSK-3 elicit an antimigratory effect in VSMC which is comparable to I3MO.

2. Influence of I3MO, AG490 and LiCl on STAT3/STAT5b phosphorylation

In her thesis, Andrea Schwaiberger reported I3MO as an inhibitor of phosphorylation of STAT1 on Y⁷⁰¹, STAT3 on Y⁷⁰⁵ and STAT5 on Y⁶⁹⁴ after stimulation with 20 ng/ml of PDGF-BB. Furthermore, phosphorylation of STAT 6 on Y⁶⁴¹ was partially inhibited after 10 min. Phosphorylation of several protein kinases downstream of the PDGF-BB-receptor, including ERK, p38 and Akt was not affected.¹⁴⁴

STAT3 and STAT5b have been shown in several studies and reviews to regulate cell migration¹⁶⁷⁻¹⁷¹, including VSMC migration.^{91,93,151,172} We therefore repeated the experiment of Andrea Schwaiberger using 10 ng/ml of PDGF-BB, the concentration we had used to examine influences of I3MO on VSMC migration. Fig. 11 (A and B) shows that I3MO inhibited phosphorylation of STAT3 on Y⁷⁰⁵ and phosphorylation of STAT5b on Y⁶⁹⁴ also after stimulation with 10 ng/ml of PDGF-BB.

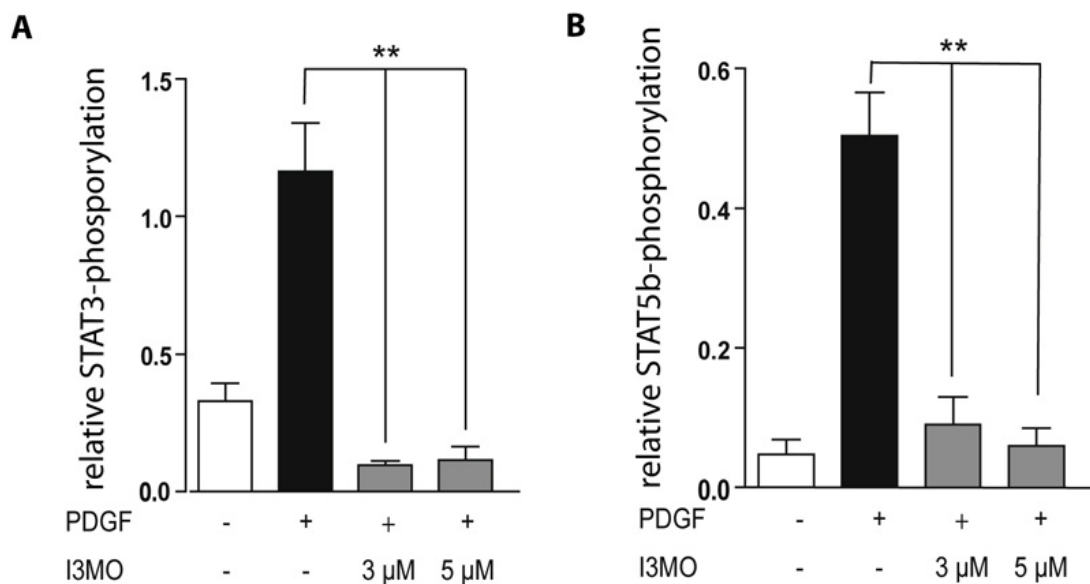


Fig. 11. Influence of I3MO on PDGF-BB-induced STAT3 and STAT5b phosphorylation after 10 min. Serum-starved VSMC were pretreated with I3MO (3 μM or 5 μM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 10 min. Cell lysates were prepared and subjected to western blot analysis for phosphorylation of STAT3 on Y⁷⁰⁵ (A) and phosphorylation of STAT5b on Y⁶⁹⁴ (B). Graphs show means of densitometrically analysed protein/tubulin ratios of three independent experiments ± SEM. (**, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

In VSMC, it has been shown that STAT3 phosphorylation after PDGF-BB stimulation was JAK2-dependent.^{92,93} We therefore used the JAK2 inhibitor AG490 to verify whether phosphorylation of STAT3 on Y⁷⁰⁵ might be reduced in an I3MO-like manner. In addition, we investigated whether the phosphorylation of STAT5b on Y⁶⁹⁴ after PDGF-BB stimulation might be influenced by AG490.

Furthermore, GSK-3 was reported to be involved in STAT3 and STAT5b phosphorylation in different cell types and *in vivo*.^{173,174} Therefore, we investigated the influence of the GSK-3 inhibitor LiCl on phosphorylation of STAT3 on Y⁷⁰⁵ and phosphorylation of STAT5b on Y⁶⁹⁴ after PDGF-BB stimulation for 10 min in VSMC.

Fig. 12 (A and B) shows that LiCl was unable to reduce phosphorylation of STAT3 on Y⁷⁰⁵ and STAT5b phosphorylation on Y⁶⁹⁴ after PDGF-BB stimulation for 10 min. AG490 reduced STAT3 phosphorylation, and the reduction was significant at higher concentration as we had expected from literature.⁹³ Furthermore, AG490 was able to reduce STAT5b phosphorylation significantly also in lower concentrations. However, AG490 did not abrogate STAT3 and STAT5b phosphorylation as potent as I3MO.

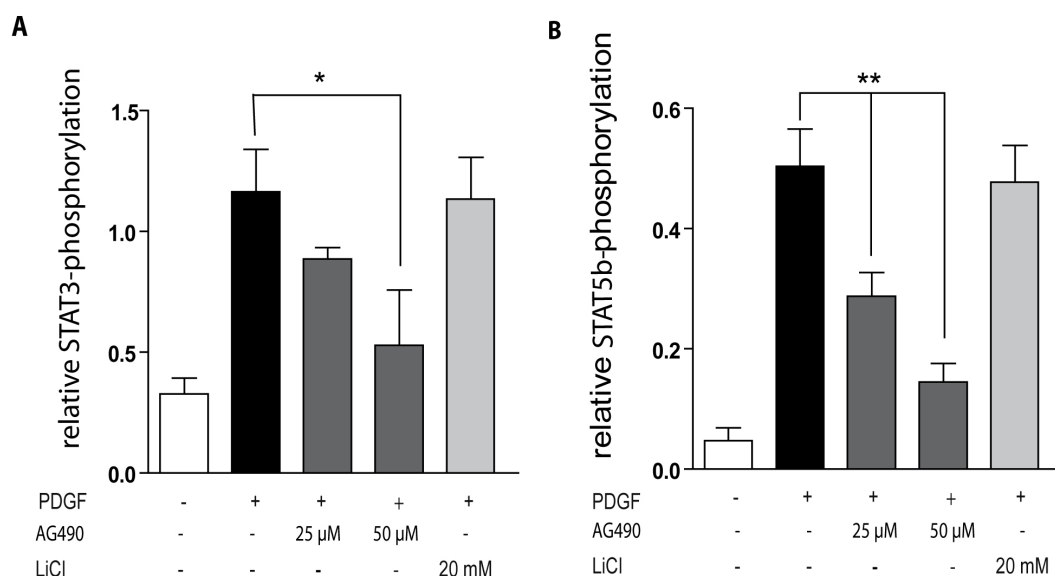


Fig. 12. Influence of LiCl and AG490 on PDGF-BB-induced STAT3 and STAT5b phosphorylation after 10 min.

Serum-starved VSMC were pretreated with AG490 (25 μ M or 50 μ M), LiCl (20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 10 min. Cell lysates were prepared and subjected to western blot analysis for phosphorylation of STAT3 on Y⁷⁰⁵ (A) and phosphorylation of STAT5b on Y⁶⁹⁴ (B). Graphs show means of densitometrically analysed protein/tubulin ratios of three independent experiments $\pm$ SEM. (*, $p < 0.05$; **, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

Beurel et al. showed that the complete inhibition of STAT3 phosphorylation by LiCl after stimulation with interferon- γ (IFN- γ) and lipopolysaccharide (LPS) in primary astrocytes was reached after 1 h.¹⁷³ We therefore further examined the effects of I3MO, LiCl and AG490 on phosphorylation of STAT3 on Y⁷⁰⁵ and STAT5b on Y⁶⁹⁴ after stimulation with 10 ng/ml of PDGF-BB for 1 h.

Levels of phosphorylated STAT3 were reduced by all three compounds, but AG490 and LiCl were not as potent as I3MO. Furthermore, the difference between levels of phosphorylated STAT3 in PDGF-BB-treated and vehicle-treated samples was not significant anymore (Fig. 13, A). Also levels of phosphorylated STAT5b were reduced by all three compounds and the phosphorylation of STAT5b in PDGF-BB treated sample was still significantly higher than in vehicle-treated sample. Interestingly, all three compounds showed about the same potency in reducing the phosphorylation of STAT5b after 1 h (Fig. 13, B).

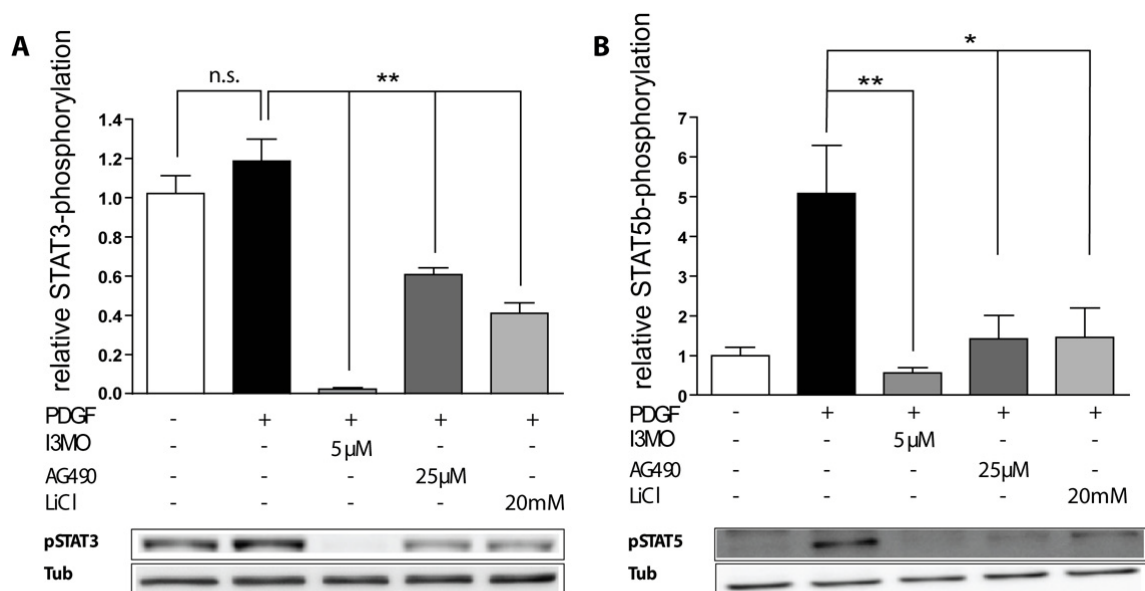


Fig. 13. Influence of I3MO, LiCl and AG490 on PDGF-BB-induced STAT3 and STAT5b phosphorylation after 1h.

Serum-starved VSMC were pretreated with I3MO (5 μ M), AG490 (25 μ M) LiCl (20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 1 h. Cell lysates were prepared and subjected to western blot analysis for phosphorylation of STAT3 on Y⁷⁰⁵ (A) and phosphorylation of STAT5b on Y⁶⁹⁴ (B). Graphs show means of densitometrically analysed protein/tubulin (Tub) ratios of three independent experiments $\pm$ SEM. (n.s., not significant; *, $p < 0.05$; **, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

Finally, we examined the influence of all three compounds on phosphorylation of other protein kinases after stimulation with 10 ng/ml of PDGF-BB for 10 min, including phosphorylation of ERK and p38, which was found not to be affected by I3MO by Andrea Schwaiberger, who used 20 ng/ml of PDGF-BB.¹⁴⁴

Fig. 14 (A and B) shows that none of all three compounds was found to reduce phosphorylation of ERK and p38 after PDGF-BB stimulation for 10 min.

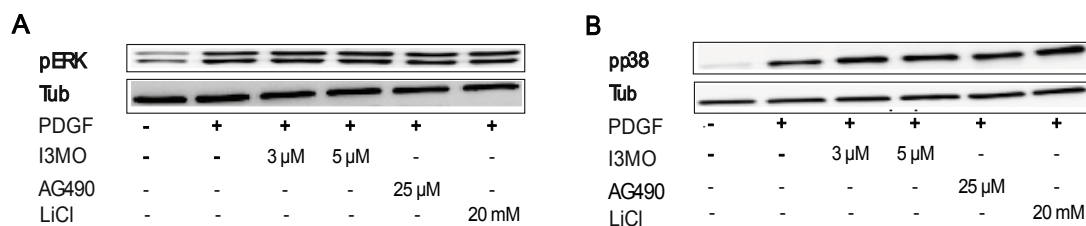


Fig. 14. Influence of I3MO, LiCl and AG490 on PDGF-BB-induced ERK and p38 phosphorylation after 10 min.

Serum-starved VSMC were pretreated with I3MO (3 μM or 5 μM), AG490 (25 μM), LiCl (20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 10 min. Cell lysates were prepared and subjected to western blot analysis for ERK (A) and p38 (B) phosphorylation. Tubulin (Tub) was used for loading control. Photographs show one representative blot out of three independent experiments.

3. Influence of I3MO, AG490 and LiCl on STAT3/STAT5b target gene expression

We showed that I3MO was able to inhibit phosphorylation of STAT3 on Y⁷⁰⁵ and phosphorylation of STAT5b on Y⁶⁹⁴ after stimulation with 10 ng/ml of PDGF-BB. To examine whether this is the pathway by which I3MO is reducing VSMC migration, we investigated the influence of I3MO on STAT3 and STAT5b target genes that are knowingly involved in migration.

Recently, the expression of cPLA₂ after PDGF-BB stimulation has been shown to directly depend on STAT3 activation in a JAK2-dependent manner.⁹² Furthermore, cPLA₂ has been implicated in PDGF-BB and thrombin-induced VSMC migration.^{93,151} Yellaturu et al. showed that expression of cPLA₂ was highest after 16 h.⁹²

Hence, we chose a similar timeframe to check whether I3MO, AG490 and LiCl could reduce cPLA₂ expression after stimulation with 10 ng/ml of PDGF-BB. Fig. 15 shows that AG490 reduced levels of cPLA₂ as expected from the literature.⁹² In addition, I3MO and LiCl also reduced expression of cPLA₂.

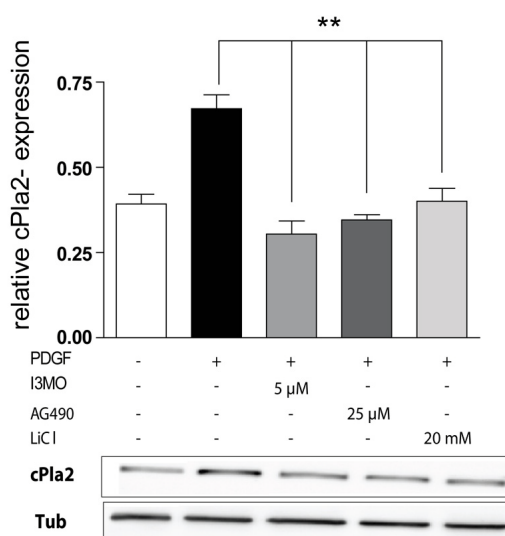


Fig. 15. Influence of I3MO, LiCl and AG490 on PDGF-BB-induced cPLA₂ expression.

Serum-starved VSMC were pretreated with I3MO (5 μM), AG490 (25 μM), LiCl (20 mM), or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 15 h. Cell lysates were prepared and subjected to western blot analysis for cPLA₂ expression. Graphs show means of densitometrically analysed protein/tubulin (Tub) ratios of three independent experiments ± SEM. (**, $p < 0.01$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

In addition, cyclin D1 expression has been described to be promoted by STAT5b and STAT3¹⁷⁵⁻¹⁸⁰ and also its implication in migration has been reported.¹⁸¹⁻¹⁸³ Hence, we first studied whether PDGF-BB-induced cyclin D1 expression after stimulation for 6, 15 and 20 h. We found that induction of cyclin D1 expression was increased after stimulation for 6 h (data not shown). We next examined the effects of I3MO, LiCl and AG490 on cyclin D1 expression after stimulation with 10 ng/ml PDGF-BB for 6 h.

Fig. 16 shows that all three compounds significantly reduced cyclin D1 expression.

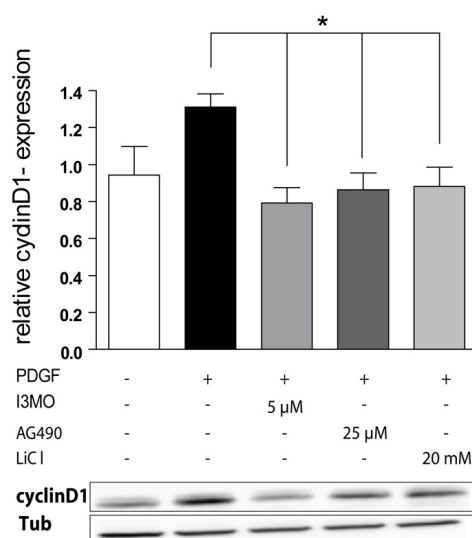


Fig. 16. Influence of I3MO, LiCl and AG490 on PDGF-BB-induced cyclin D1 expression.

Serum-starved VSMC were pretreated with I3MO (5 μ M), AG490 (25 μ M), LiCl (20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 6 h. Cell lysates were prepared and subjected to western blot analysis for cyclin D1 expression. Graphs show means of densitometrically analysed protein/tubulin (Tub) ratios of three independent experiments $\pm$ SEM. (*, $p < 0.05$, one-way ANOVA followed by Dunnett post-test versus PDGF treatment).

These data demonstrate that I3MO is able to interfere with the expression of STAT3/STAT5b-dependent promigratory target genes, cPLA₂ and cyclin D1. Inhibitors of JAK2 and GSK-3 can mimic the I3MO phenotype.

4. Influence of I3MO and LiCl on cytoskeletal rearrangement

After stimulation with chemotactic molecules, cells undergo an early cytoskeletal rearrangement. Protrusions as lamellipodia and filopodia form and actin monomers organize into thick actin filament bundles, named stress fibres.⁴¹ Also in smooth muscle cells, formation of stress fibres has been reported.¹⁸⁴ Furthermore, stimulation with PDGF leads to actin-rich membrane ruffles,¹⁸⁵ which are thought to be active sites of actin reorganization when located at the dorsal surface.¹⁸⁶

To study whether I3MO already affects these early events in cellular migration, we used fungal FITC-labeled phalloidin, which binds to polymerized actin, to visualize actin structures in our cells.¹⁴⁸ In fibroblasts, cells have been seen to organize actin in lamellipodia and filopodia after 30 min. After 1 h, they have been observed to develop stress fibres.¹⁸⁷ Therefore, we examined VSMC treated with vehicle, I3MO and LiCl and stimulated with 10 ng/ml of PDGF-BB for 1 h.

Fig. 17 shows representative photographs of one experiment. We found, that PDGF-BB induces stress fibres and membrane ruffling in VSMC after one hour. I3MO (5 μ M) as well as LiCl (20 mM) seemed to reduce at least stress fibre formation. Although the photographs suggest that I3MO as well as LiCl interact with early signalling cascades, additional and more meaningful studies concerning the cytoskeleton are needed to confirm this observation. This experiment was carried out together with Irene Sroka, PhD.

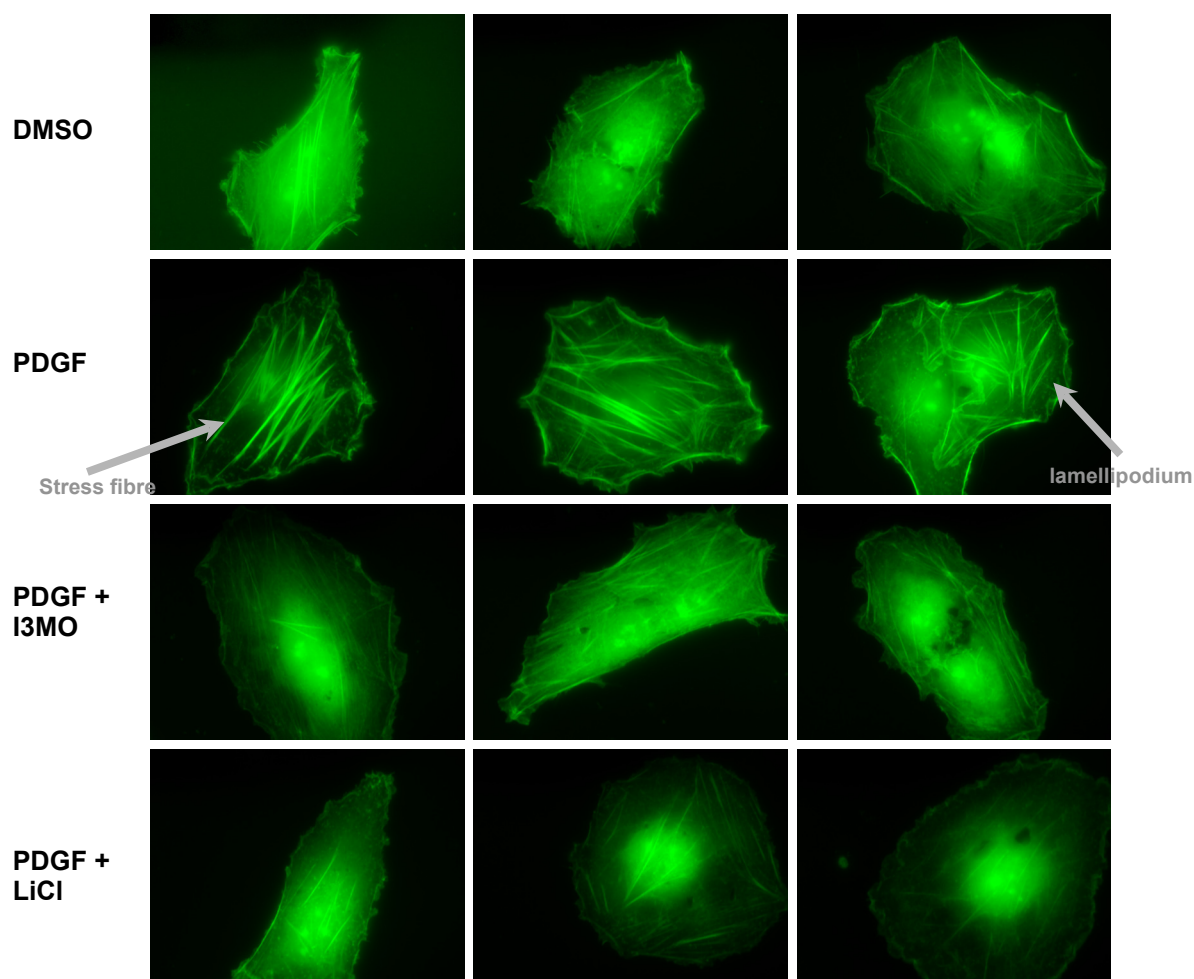


Fig. 17. Influence of I3MO and LiCl on PDGF-BB-induced stress fibre formation.

VSMC were cultivated on collagen-coated coverslips, serum-starved, pretreated with I3MO (5 μ M) LiCl (20 mM) or vehicle (DMSO 1 %) for 30 min and stimulated with PDGF-BB (10 ng/ml) for 1 h. Cells were fixed and stained with FITC-labeled phalloidin. Samples were analysed with fluorescence microscope. Experiment was carried out together with Irene Sroka, PhD.

E. DISCUSSION

E. DISCUSSION

1. Antimigratory activity of I3MO

Before this thesis, an antiproliferative effect of I3MO on VSMC in cell culture has been shown in our lab. Cells were hereby arrested in G0/G1-phase. Additionally, neointima formation was reduced by I3MO in a cuff-induced neointima mouse model.¹⁴⁴ Since proliferation and migration of VSMC are both crucial in the formation of atherosclerosis and restenosis (see chapter B, section 2.2), we here investigated the effect of I3MO on VSMC migration in a wound healing assay. We found that I3MO dose-dependently reduced VSMC migration after stimulation with PDGF-BB (see Fig. 8).

These data are consistent with previous findings of Irene Sroka in a Boyden-chamber model. In this assay, VSMC had to migrate actively through a porous membrane along a PDGF-gradient. Also in this assay, migration of VSMC after PDGF-BB stimulation was inhibited dose-dependently by I3MO.¹⁴⁵ We therefore clearly demonstrated that I3MO inhibits PDGF-BB-induced migration of VSMC.

To further investigate influences of I3MO on migration, we examined its effect on early cytoskeletal rearrangements. At least stress fibre formation seemed to be reduced (Fig. 17). Although the experiment suggests that I3MO interacts also with early cellular events essential for migration, further studies are needed to confirm this observation.

Indirubins have been reported to inhibit cell migration in other cell types^{122,188}, but the finding that I3MO is able to inhibit VSMC migration is novel and adds an important aspect to the potential anti-atherosclerotic and anti-restenotic activity of I3MO.

2. Possible antimigratory targets of I3MO

2.1. Phosphorylation of STATs

We were further interested in the molecular target of I3MO responsible for impaired migration after PDGF-BB stimulation. As already mentioned, phosphorylation of protein kinases, including ERK, p38 and Akt, was not affected by I3MO after stimulation with 20 ng/ml of PDGF-BB.¹⁴⁴ As we used 10 ng/ml of PDGF-BB in our setting (see chapter D, section 1.1.), we repeated the experiment and found that also

in this concentration PDGF-BB increased phosphorylation of protein kinases, including p38 and ERK. I3MO did not abrogate the phosphorylation (Fig. 14).

Furthermore, previous work in our lab showed that I3MO completely inhibited phosphorylation of STAT1/3/5 and partially inhibited phosphorylation of STAT6 after stimulation with 20 ng/ml of PDGF-BB.¹⁴⁴

Among other cellular processes, including proliferation, apoptosis, and differentiation, STATs have been reported to be involved in cell migration.¹⁸⁹ In particular STAT3 and STAT5b have been demonstrated to be essential in cell migration¹⁶⁷⁻¹⁷¹, including in VSMC migration.^{91,93,151,172} In our experiments, using 10 ng/ml of PDGF-BB, phosphorylation of STAT3 and STAT5b was also significantly increased compared to the unstimulated sample, and I3MO abrogated phosphorylation (Fig. 11).

Furthermore, we found a reduced expression of STAT3 and STAT5b target genes, including cPLA₂ and cyclin D1, both known to be involved in migration. cPLA₂ is an enzyme involved in the hydrolysis of membrane glycerophospholipids to free arachidonic acid, a precursor of prostaglandins, leucotrienes and other eicosanoids.¹⁹⁰ Previously, expression of cPLA₂ has been described to be up-regulated after stimulation with PDGF-BB in VSMC and the enhanced expression has been proposed to be JAK2/STAT3-mediated.⁹² Furthermore, cPLA₂ has been found to be involved in VSMC migration after stimulation with PDGF-BB and thrombin.^{93,151} Selective inhibitors of cPLA₂ reduced VSMC motility^{93,151}, whereas arachidonic acid rescued VSMC motility after inhibition of JAK2⁹³ or expression of a dominant negative STAT3.¹⁵¹ Concordant with these studies, we found that expression of cPLA₂ was enhanced after stimulation with PDGF-BB for 15 h and I3MO reduced this effect (Fig. 15).

In addition, we found that I3MO reduced cyclin D1 expression after stimulation with PDGF-BB for 6 h (Fig. 16). Cyclin D1 is a protein centrally involved in the development of human cancers.¹⁹¹ In addition, its implication in cell migration has been reported¹⁸¹⁻¹⁸³, and STAT3 and STAT5b have been described in several studies to promote cyclin D1 expression.¹⁷⁵⁻¹⁸⁰ In VSMC, PDGF-BB has been reported to induce cyclin D1 expression after 8 h and this effect was abolished by dominant negative STAT5b.⁹¹

Taken together, our results demonstrate that I3MO inhibits STAT3 and STAT5b phosphorylation in VSMC stimulated with 10 ng/ml of PDGF-BB and that PDGF-BB induced expression of the promigratory proteins cyclin D1 and cPLA₂. But whether

these activities of I3MO truly account to its observed antimigratory effect, needs to be shown. A clear evidence for the causal connection between inhibition of STAT phosphorylation and inhibition of migration by I3MO is missing. Current investigations in the lab working with a constitutively active form of STAT3 aim to provide the proof for this causality.

2.2. The JAK-signaling pathway

Assuming STATs as effectors of I3MO's antimigratory actions on VSMC, we were further interested in possible upstream activators of STATs as direct targets of I3MO. Although it is well established that STATs are activated by JAKs after cytokine activation, the upstream activators after growth factor stimulation are less clear.⁸⁴

However, JAK2 has been demonstrated to induce STAT phosphorylation after PDGF stimulation in SMC.^{84,92,93,192} JAK2 inhibition has been found to reduce SMC proliferation^{84,192} and migration⁹³ after stimulation with PDGF. Furthermore, JAK2 inhibitors as well as dominant negative mutants of JAK2, have been found to reduce STAT3 phosphorylation^{84,92,93}, -translocation, -DNA-binding activity and -reporter gene expression^{92,93} after stimulation with PDGF in SMC. We therefore compared AG490, a chemical inhibitor of JAK2¹⁵²⁻¹⁵⁴, to I3MO, regarding its intracellular actions.

We showed that AG490 effectively blocked VSMC migration after PDGF-BB stimulation (Fig. 9). Furthermore, concordant with the literature,^{84,92,192} we found that phosphorylation of STAT3 on Y⁷⁰⁵ was significantly reduced after stimulation with 10 ng/ml of PDGF-BB by higher concentrations of AG490 (50 μ M) (Fig. 12). However, AG490 did not abrogate phosphorylation of STAT3 to the same extent as I3MO did. Interestingly, we found that also phosphorylation of STAT5b on Y⁶⁹⁴ was reduced by AG490 even at lower concentrations (Fig. 12). Phosphorylation of other protein kinases, including p38 and ERK, was not inhibited by AG490 after stimulation with 10 ng/ml of PDGF-BB (Fig. 14).

Finally, AG490 also reduced STAT3 and STAT5b promigratory target gene expression, including cPLA₂ and cyclin D1, exactly mimicking effects of I3MO (Fig. 15 and 16).

Although I3MO to our knowledge has never been described as inhibitor of JAK2, but only as an inhibitor of JAK1 *in vitro*¹⁴³, we found marked similarities in the way of cellular actions of AG490 and I3MO. However, in preliminary experiments we had found in our lab that JAK2 phosphorylation was not increased after stimulation with

PDGF-BB in VSMC. This finding argues against JAK2 as upstream mediator of PDGF-induced STAT phosphorylation.¹⁴⁴ Since in the literature JAK2 phosphorylation after PDGF stimulation in VSMC has been reported^{92,93,192}, definitely more experiments are needed to resolve the issue of JAK2 as a possible upstream signaling molecule of STAT3 and STAT5b in our VSMC and to elucidate whether I3MO and AG490 share the same signaling pathway or the same target.

Next to direct inhibition of JAK2 activity, I3MO could also interfere with signaling steps upstream of JAK2 activation. Possible candidate targets for I3MO could then be ROS (reactive oxygen species) or Src. Both, ROS^{193,194} and Src^{64,65} have been shown to promote migration of VSMC. Furthermore, PDGF-induced JAK2 activation has been reported to be ROS-dependent^{192,195,196} and/or Src-dependent^{84,197}.

VSMC contain different sources of ROS, including xanthine oxidases, the mitochondrial respiratory chain, lipoxygenases, nitric oxide synthases and NADPH oxidases (NOXes).¹⁹⁸ In the literature, NOX5 has been shown to be required for JAK2 activation after PDGF stimulation in human airway smooth muscle cells. PDGF-induced phosphorylation of JAK2 was abolished by an unselective inhibitor of all Nox-isoforms¹⁹⁹, Diphenylen iodonium (DPI).¹⁹² Additionally, JAK2 has been reported to be activated by exogenous H₂O₂, a molecule of the ROS-group²⁰⁰, in fibroblasts¹⁹⁵ and VSMC¹⁹⁶. Notably, previous work in our lab has shown that I3MO reduced PDGF-BB-increased ROS-production in VSMC.¹⁴⁴ Furthermore, exogenous H₂O₂ was sufficient to increase STAT3 phosphorylation in VSMC and this effect was abolished by I3MO.¹⁴⁴ Earlier experiments in our lab also showed that PDGF-BB-stimulated VSMC migration as well as STAT3 phosphorylation was reduced by DPI¹⁴⁵.

Furthermore, preliminary experiments in our lab showed that the Src inhibitor SU6656²⁰¹ reduced PDGF-BB-induced VSMC migration¹⁴⁵, and STAT3/STAT5b phosphorylation.¹⁴⁴ The observation that the JAK2 inhibitor AG490 and the Src inhibitor SU6656 had similar effects, is not completely unexpected: in the literature, it is proposed that STAT activation by PDGF requires both, JAKs and Src kinases and that the two kinases may share a common pathway in different cell types,^{202,203} including human airway smooth muscle cells.⁸⁴ As already mentioned, Src has also been presumed to be an upstream activator of JAK2 after PDGF stimulation.^{84,197}

To sum up, all these experiments make it plausible that AG490 and I3MO may affect at least proteins of a common signaling axis. However, defining the exact signaling

steps induced by PDGF-BB in VSMC and affected by I3MO remains an open and interesting question.

2.3. The GSK-3 signaling pathway

GSK-3 is an enzyme related to glycogen metabolism. Furthermore, in the last years it has become clear that the kinase is involved in a number of diseases, including Alzheimer's disease, cancer, and inflammation. In addition, it has been reported to be strongly involved in cell migration. I3MO has been described as a GSK-3 inhibitor in several cell lines (see chapter B, section 6.3.2.).

Furthermore, it has been reported that STAT3 phosphorylation after stimulation with IFN- γ and LPS and STAT5 phosphorylation after stimulation with interferon- α (IFN- α), IFN- γ and Granulocyte-macrophage colony-stimulating factor (GM-CSF) is GSK-3-dependent in different cell lines. Among other GSK-3 inhibitors, I3MO, 6-bromo-I3MO and LiCl were shown to inhibit STAT3 and STAT5 phosphorylation in these cell types.¹⁷³

We therefore investigated the effects of LiCl, a known GSK-3 inhibitor¹⁵⁹⁻¹⁶², on VSMC migration after stimulation with PDGF-BB. We found that LiCl completely reduced VSMC migration and that this effect was not simply due to osmotic changes (Fig. 10). Additionally, LiCl exerted similar effects as I3MO on early cytoskeletal rearrangements after stimulation with PDGF-BB (Fig. 17).

Although we failed to demonstrate that LiCl reduced STAT3 or STAT5b phosphorylation after 10 min (Fig. 12), we showed that phosphorylation of both STATs was reduced after 1 hour (Fig. 13). Phosphorylation of other protein kinases, including p38 and ERK, were not inhibited by LiCl (Fig. 14).

Additionally we showed that STAT3 and STAT5b promigratory target gene expression, including cPLA₂ and cyclin D1, was reduced by LiCl (Fig. 15 and Fig. 16).

Due to the different kinetics of STAT3 and STAT5b inhibition (10 min vs. 1 h) we are prone to exclude that I3MO and LiCl may share one single target and/or one single mode of action. However, we cannot rule out that I3MO may additionally inhibit GSK-3 in VSMC after PDGF-BB stimulation. Further studies would be interesting to elucidate whether I3MO can influence GSK-3 activation in VSMC or whether a constitutively active form of GSK-3 could overcome the antimigratory effect of I3MO.

Lithium's effect on VSMC migration after PDGF-BB stimulation has not been reported so far. We therefore investigated also its influence on VSMC proliferation. Indeed,

Irene Sroka showed that LiCl led to an accumulation of cells in G1-phase of the cell cycle, similar to the effect elicited by I3MO.¹⁴⁵

The finding that a GSK-3 inhibitor inhibits migration and proliferation of VSMC is somehow conflicting: although GSK-3 inhibition has been reported to have antimigratory effects in other cell types^{122,128,164-166}, expression of a constitutively active nonphosphorytable mutant of GSK-3 has been demonstrated to reduce VSMC migration after PDGF-BB stimulation.¹²⁹ Moreover, GSK-3 has been proposed to suppress SMC proliferation and this effect was reversed by PDGF.^{130,131}

We cannot exclude at this stage that the observations made with LiCl in our cells are not due to inhibition of other targets of LiCl, including inositol monophosphate phosphatase (IMP) or inositol polyphosphate-1-phosphatase (IPP), two enzymes often referred as to be inhibited by LiCl.^{61,204,205} Nevertheless, the effect of LiCl was impressive and it would be interesting elucidate the role of LiCl in PDGF-BB-induced proliferation and migration of VSMC.

Summarizing our results, we clearly demonstrated that in addition to proliferation and to neointima formation *in vivo*, I3MO inhibits PDGF-BB-induced migration. Although the direct molecular target remains unclear, these effects of I3MO may offer a therapeutic potential in the treatment of atherosclerosis and restenosis, including the application in drug-eluting stents.

F. SUMMARY

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Previous work in our lab showed that I3MO is able to inhibit PDGF-BB-induced VSMC proliferation. Furthermore, the compound was able to reduce neointima formation *in vivo* in a mouse model (experiments performed in cooperation with Prof. Binder, Medical University of Vienna). Finally, I3MO inhibited phosphorylation of STATs without interfering with other common early signaling events.

Both, migration and proliferation of VSMC are responsible in the early onset of atherosclerosis. We therefore intended to additionally investigate the antimigratory properties of I3MO. The present work showed that I3MO was indeed able to inhibit PDGF-BB-induced migration of VSMC. It was also able to interfere with early cytoskeletal rearrangements. I3MO abolished STAT3 and STAT5b phosphorylation after stimulation with 10 ng/ml of PDGF-BB, thereby indicating that these STATs may be involved in PDGF-BB-induced VSMC migration. We found that I3MO also reduced expression of the STAT3 and STAT5b target genes, cPLA₂ and cyclin D1, which have been described to be involved in migration. To further elucidate the underlying mechanisms of I3MO-effects, we compared cellular actions of I3MO to a chemical inhibitor of JAK2, AG490 and to an inhibitor of GSK-3, LiCl. We found that both AG490 and LiCl inhibited PDGF-BB-induced VSMC migration. AG490 further reduced STAT3 and STAT5b phosphorylation after PDGF-BB stimulation. Both compounds reduced STAT3 and STAT5b target gene expression. Although we found considerable similarities between AG490 and I3MO regarding their intracellular actions, the exact cellular target of I3MO remains unclear.

However, our studies affirm I3MO as a possible agent in the treatment of vasoproliferative diseases, including atherosclerosis and restenosis.

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H. APPENDIX

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1. Abbreviations

A

AhR	Aryl hydrocarbon receptor
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli protein
APS	Ammonium persulfate
Arf6	ADP-ribosylation factor 6
Arp2/3 complex	Actin-related protein 2/3 complex

B

bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin

C

CAK	CDK-activating kinase
CB	Cytoskeleton buffer
CDKs	Cyclin-dependent kinase
cPla ₂	Cytosolic phospholipase A ₂

D

DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPI	Diphenylene iodonium

E

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase

F

FAK	Focal adhesion kinase
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G

GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK-3	Glycogen synthase kinase-3

H

HEPES	Hydroxyethylpiperazineethanesulfonic acid
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HRP	Horseradish peroxidase
I	
I3MO	Indirubin-3'-monoxime
IFN- α	Interferon- α
IFN- γ	Interferon- γ
IGF-1	Insulin-like growth factor- 1
IMPase	Inositol monophosphate phosphatase
IP ₃	Inositol-1,4,5-trisphosphate
IPPase	Inositol polyphosphate-1-phosphatase
J	
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
L	
LCK	Lymphocyte kinase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
M	
MAPK	Mitogen-activated protein kinase
Mc	Monoclonal
MCP-1	Monocyte chemoattractant protein-1
MEK	MAPK/ERK kinase
MES	2-N-morpholino-ethanesulfonic acid
MIF	Macrophage inhibitory factor
MK2	MAPK activated protein kinase 2
MKK	MAPK kinases
MLCK	Myosin light chain kinase
MLCP	MLC phosphatase
MMP	Matrix metalloproteinase
MTOC	Microtubule organizing centre
N	
NO	Nitric oxide
NOX	NADPH oxidase
P	
p38	P38 MAP kinase
PAA	Polyacrylamide
PBS	Phosphate buffered saline
Pc	Polyclonal
PCI	Percutaneous coronary intervention
PDGF	Platelet derived growth factor
PI ₃ K	Phosphatidylinositol 3-kinase

APPENDIX

PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PIP ₃	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PLC- γ	Phospholipase C- γ
PLD	Phospholipase D
PMSF	Phenylmethanesulfonyl fluoride
PTA	Percutaneous transluminal angioplasty
R	
ROCK	Rho-associated, coiled-coil-containing protein kinase
ROS	reactive oxygen species
RTK	Receptor tyrosine kinase
S	
SDS	Sodium dodecyl sulfate
SGK	Serum- and glucocorticoid-induced kinase
SH2	Src homology 2 domain
SHP-2	SH2-containing protein-tyrosine phosphatase
SOCS	Suppressor of cytokine signaling
Src	Src family tyrosine kinase
STAT	Signal transducer and activator of transcription
T	
TBS-T	Tris-buffered saline/Tween 20 buffer
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TNF- β	Tumor necrosis factor- β
TRIS	Trihydroxymethylaminomethane
U	
uPA	Urokinase plasminogen activator
V	
VSMC	Vascular smooth muscle cell
W	
WASP	Wiskott-Aldrich syndrome protein
WAVE	Warpin-homologous protein

2. Alphabetical list of companies

Beckmann Coulter	Fullerton, CA, USA
Biorad laboratories	Hercules, CA, USA
Broad Institute	Cambridge, MA, USA
Calbiochem	La Jolla, CA, USA
Carl Roth	Karlsruhe, Germany
Cell signaling	Danvers, MA, USA
Fujifilm	Tokyo, Japan
GraphPad Software Inc	San Diego, CA, USA
Lonza Group Ltd.	Basel, Switzerland
New England Biolabs	Beverly, MA, USA
Olympus Europe GmbH	Hamburg, Germany
Santa Cruz	Santa Cruz, CA, USA
Sigma Aldrich	St. Louis, MO, USA
TECAN	Mannedorf, Switzerland
Upstate	Charlottesville, VA, USA

3. Curriculum vitae

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