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DISSERTATION

Titel der Dissertation

Effects of Roscovitine and Indirubin-Derivatives
on Platelet Derived Growth Factor Signalling
in Vascular Smooth Muscle Cells

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Doktorin der Naturwissenschaften (Dr. rer. nat.)

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Abstract

Abnormal vascular smooth muscle cell (VSMC) proliferation and migration contribute to vascular narrowing during the pathogenesis of atherosclerosis and restenosis. An ideal compound for treating both diseases should therefore impair both proliferation and migration of VSMC. A major proliferative stimulus and the strongest chemoattractant for VSMC is the Platelet Derived Growth Factor-BB (PDGF-BB). The kinase inhibitor roscovitine (ROSC) shows a high specificity for cyclin dependent kinases (CDKs) which are crucial for cell cycle progression. We therefore wanted to investigate the mode of action of ROSC and promising derivatives in PDGF-BB stimulated VSMC and define the changes in cell signalling. Screening a panel of synthetic ROSC derivatives for a general antiproliferative activity in VSMC we identified LGR1406 which showed significant higher potency than ROSC itself. We showed that LGR1406 and ROSC both inhibit proliferation of VSMC without affecting early signalling pathways downstream of the activated PDGF receptor. LGR1406 is hereby the far more potent compound with an apparent IC_{50} of 3.0 µM in the BrdU assay compared with an IC_{50} of 17 µM of ROSC. A detailed molecular analysis using western blot analysis as well as *in vitro* kinase assays showed that both compounds affect entry of cells into S-phase of the cell cycle, expression of cyclin A and E as well as phosphorylation of pocket proteins presumably by inhibition of CDK activity. However, ROSC only delayed these events whereas LGR1406 permanently blocked cell cycle progression in G1-phase of the cell cycle. We conclude that LGR1406 could be used for further development as e.g. stent coating agent but *in vivo* studies as well as further investigations on its molecular mechanism of action are needed.

Additionally we investigated the influence of Indirubin-3'-monoxime (I3MO) on PDGF-BB-induced migration of VSMC. In our group it had previously been shown that I3MO potently blocks VSMC proliferation in response to PDGF-BB and neointima formation in a mouse model (*in vivo* experiments were performed in cooperation with Prof. Binder, medical university of Vienna). Furthermore we had shown that it suppresses the production of reactive oxygen species (ROS) and the activation of the transcription factor STAT3, without interfering with other common early signalling events. In this study we now show that I3MO dose-dependently inhibits PDGF-BB-induced migration of VSMC in the same concentration range as it limits proliferation. Also we found that I3MO interferes with early cytoskeletal rearrangements involved in cell migration without affecting the small GTPase Rac-1. However, we found that I3MO reduced the expression of STAT3 target genes involved in migration. Our data suggest that the antimigratory effect of I3MO is probably

mediated via the reduction of ROS production in the cell and consequent inhibition of STAT3 phosphorylation. Our studies further substantiate that I3MO is a promising compound for the treatment of vasculoproliferative diseases. But also here are some open questions regarding its molecular mechanism of action.

Zusammenfassung

In der Pathogenese von Atherosklerose und Restenose spielen die abnorme Proliferation (Zellteilung) sowie Migration von glatten Gefäßmuskelzellen (VSMC) eine große Rolle, indem beide Prozesse wesentlich zur Einengung von Blutgefäßen beitragen. Ein idealer Wirkstoff zur Bekämpfung von beiden Krankheitsbildern sollte sowohl die Proliferation als auch die Migration von VSMC verhindern. Ein wichtiger Stimulus für die Zellteilung und zugleich der stärkste Stimulus für die Migration von VSMC ist PDGF-BB (Platelet Derived Growth Factor-BB). Der Kinase Inhibitor Roscovitine (ROSC) zeigt eine erstaunliche Spezifität für Cyklin-abhängige Kinasen (CDKs). Darum wollten wir mit dieser Studie den genauen Mechanismus von ROSC auf PDGF-BB-stimulierte VSMC untersuchen und außerdem neue Derivate der Substanz testen. Wir untersuchten unterschiedliche ROSC Derivate auf ihre wachstumshemmenden Eigenschaften auf VSMC und stießen dabei auf eine vielversprechende Substanz, LGR1406, die eine bedeutend bessere Wirkung zeigte als die Ausgangsstruktur ROSC selbst. Wir fanden heraus, dass beide Substanzen die Proliferation von VSMC inhibieren ohne frühe Signaltransduktionswege im PDGF *Signalling* zu behindern. LGR1406 zeigte allerdings mit einem IC_{50} von 3 µM eine bedeutend bessere Wirkung als ROSC mit einem IC_{50} von 17 µM. Eine detaillierte molekulare Analyse mittels western blot und *in vitro* Kinase *Assays* zeigte dass beide Substanzen den Eintritt der Zelle in die S-phase des Zellzyklus behindern, ebenso die Expression von Cyclin A und E und die Phosphorylierung der Pocket Proteine Rb und p107. Allerdings konnten wir zeigen, dass ROSC selbst diese Vorgänge nur verlangsamt wohingegen LGR1406 die Zellen permanent in der G1 Phase des Zellzyklus arretiert. Als Ursache für die Wirkungen von beiden Substanzen vermuten wir die Inhibierung von CDKs durch ROSC und LGR1406. In weiterer Folge sind noch genauere Untersuchungen zum Wirkmechanismus und Tierstudien nötig, aber wir konnten mit dieser Studie erstmals zeigen, dass ROSC Derivate wie LGR1406 die Proliferation von VSMC verhindern können. Darum könnten solche Substanzen möglicherweise zur Beschichtung von Stents für die Therapie der Restenose weiterentwickelt werden.

Außerdem untersuchten wir den Einfluss von Indirubin-3'-monoxime (I3MO) auf PDGF-BB-induzierte Migration von VSMC. In unserer Gruppe konnte in vorangegangenen Studien gezeigt werden, dass I3MO die Proliferation von VSMC nach PDGF-BB Stimulierung verhindern kann. Ebenso konnte I3MO die Neointima-Bildung in einem Maus-Modell verringern. Zudem konnten wir zeigen, dass I3MO die Produktion von reaktiven Sauerstoffspezies (ROS) und die Aktivierung des Transkriptionsfaktors STAT3 verhindert ohne andere frühe Signaltransduktionswege zu beeinflussen. In der vorliegenden Arbeit wird nun gezeigt, dass I3MO die Migration von VSMC nach PDGF-

BB Stimulierung dosisabhängig reduzieren kann und zwar im gleichen Dosisbereich wie es auch die Proliferation von VSMC hemmt. Außerdem konnten wir zeigen, dass I3MO frühe Umlagerungen des Zytoskeletts die mit der Migration von Zellen in Zusammenhang stehen verhindert und zwar ohne die Aktivierung des kleinen GTP-Proteins Rac-1 zu beeinträchtigen. Andererseits konnten wir eine Reduktion der Expression von STAT3 abhängigen Genen beobachten welche eine Rolle in der Migration spielen. Unsere Ergebnisse führen zu dem Schluss, dass der anti-migratorische Effekt von I3MO möglicherweise vermittelt wird über die Reduktion von ROS und die daraus folgende Inhibierung von STAT3 vermittelt wird. Unsere Resultate untermauern, dass I3MO eine gute Substanz zur Behandlung vaskuloproliferativer Erkrankungen sein könnte. Aber noch sind weitere Studien zum Wirkmechanismus nötig.

1. TABLE OF CONTENTS

2. INTRODUCTION

Atherosclerosis

About 50% of the overall mortality in western societies is related to cardiovascular diseases comprising infarction, stroke, heart failure and restenosis [1]. Atherosclerosis, the main underlying disease of stroke and heart failure is the leading cause of death in the western world [1, 2]. The pathology of atherosclerosis is characterised by multiple processes including endothelial dysfunction, activation of macrophages, changes in levels of distinct cytokines and growth factors, abnormalities in lipoprotein metabolism and matrix alterations, all leading to a chronic inflammatory response in the arterial wall [3, 4]. These alterations in a vessel wall trigger various effects in different cell types and finally lead to lumen loss due to migration and hyperproliferation of vascular smooth muscle cells (VSMC), deposition of lipids and extracellular matrix (ECM) and invading macrophages. The lesions that narrow the vessel lumen are called plaques and in the worst case these can become instable and disrupt. Subsequently plaque contents are exposed to the blood stream resulting in platelet aggregation and clotting which often directly leads to strokes or infarctions. There are various treatment modalities either preventing the progression of atherosclerosis or re-widening diseased arteries. These strategies are ranging from drug administration to surgeries. However, even if an artery has been re-widened its lumen is often reduced again, a process called restenosis. The latter is characterized by the formation of a new inner layer of the vessel wall (neointima). Combating atherosclerosis is a major goal of medical investigations and since it is a complex disease the ultimate solution has not been found until today.

Pathology of atherosclerosis

For a long time the altered lipoprotein metabolism, more precisely the high plasma levels of low-density lipoproteins (LDL), their accumulation within the intima and their modification (e.g. oxidation) has been considered to be the main cause of the disease [2, 5]. However, during the last two decades it has become more and more evident that inflammatory processes play a crucial role, too [5]. The initial cause of atherosclerosis is still controversially discussed, but a disturbed or injured endothelium certainly plays a pivotal role [6, 7]. This so called endothelial dysfunction can be caused for example by shear stress, modified lipoproteins, bacterial antigens or oxidative stress. Furthermore it is out of question that a number of risk factors, such as smoking, hypercholesterolemia, hypertension, obesity and diabetes, contribute to the development of atherosclerosis [2, 5]. It has been reported that these risk factors also reduce the number and functionality of bone marrow derived endothelial progenitor cells. They hereby negatively affect the repair capacity of the endothelium and are causally linked to endothelial dysfunction [7, 8].

However, it seems that there is no exclusive model to explain this complex pathology. It is rather a multifactorial process ultimately leading to an inflammatory state of arterial walls [4]. The consequence of endothelial dysfunction and the altered conditions in the artery in general is deposition of lipids within the arterial wall. The lipid is taken up by invading macrophages observable as fatty streaks characterising early lesions which do not present clinical symptoms [2]. Moreover, it comes to migration and hyperproliferation of VSMC, consequential narrowing the vessel lumen and finally formation of fibrous plaques, also called fibroatheroma [5, 9]. Those are histologically characterised by layers of VSMC embedded in a matrix of collagen fibrils and elastin overlying a core of lipid-laden macrophages, free lipids and potentially necrotic tissue [2]. Furthermore these plaques can develop into so called 'complicated lesions' characterised by enhanced necrosis and calcification. Finally the plaques can eventually become instable and this may result in plaque rupture and thrombotic occlusion of the overlying artery [5]. A clinical symptom that arises from the limited blood flow is chronic stable angina but it has become clear that up to 50% of individuals affected by myocardial infarction have not experienced any kind of premonitory symptoms [5, 10].

Early phase of atherosclerosis

A healthy arterial vessel wall is composed of the adventitia, an outer layer of connective tissue, the media, which is built up of VSMC and the intima, a monolayer of endothelial cells (EC) separated from the media by a basement membrane [10]. The differentiated VSMC are responsible for the contraction and dilation of an artery because they possess a contractile potential due to specialized proteins such as smoothelin or smooth muscle alpha actin (α-SMA). These proteins are part of the unique cytoskeleton of differentiated VSMC that allows for contraction and dilation of the cells and hence the artery. As most known cell types, VSMC can exit from the cell cycle and enter a quiescent state termed G0, which is the normal, differentiated state of most cells in higher organisms. Likewise in healthy vessels VSMC show a low proliferation rate and express a set of cell type specific markers including α-SMA, myosin heavy chain or caldesmon [11, 12]. If exposed to an appropriate signal e.g. growth factors or cytokines, VSMC can exit from this resting state and undergo phenotypic changes. This dedifferentiation is marked by the reinitiation of cell proliferation and by enhanced motility. Furthermore the cells start to produce and secrete ECM. Therefore the new phenotype is often called 'synthetic' phenotype (Fig.1).

It is now widely accepted that the first step in the development of atherosclerosis is endothelial dysfunction [1]. This perturbation of endothelial function is marked among other things by the expression of adhesion molecules such as vascular cell adhesion molecule-

1 (VCAM-1) by EC. This allows leucocytes (e.g. monocytes) to attach to and invade the vessel wall whereas in healthy vessels EC resist leukocyte adhesion (Fig.1A) [10]. Attached leucocytes as well as EC secrete monocyte chemoattractant protein 1 (MCP-1) in response to high levels of LDL. This leads to the recruitment of T-cells further enhancing the inflammatory response [5]. Monocytes that invaded successfully the endothelium and reached the subendothelial space release a panel of cytokines and growth factors. Amongst others these are platelet derived growth factor (PDGF), transforming growth factor α and β (TGFα /β), insulin like growth factor (IGF) and interleukin 1 (IL1) all acting on the endothelium as well as on the underlying VSMC (see section 2.1.3) [13]. The EC thereupon magnify the expression of adhesion molecules and proinflammatory factors like MCP-1. This clearly leads to a positive feedback loop because even more leucocytes are attracted and invade the endothelium.

Also the invading monocytes themselves are influenced by the surrounding cytokines (e.g. M-CSF), proliferate and differentiate into macrophages. In the meantime more and more oxidised lipoproteins aggregate within the intima where they are taken up by macrophages via the scavenger and oxidized LDL receptor pathways. The cells thereupon turn into foam cells, meaning lipid-laden, less mobile macrophages that mark early atherosclerotic lesions [2, 5, 10] (Fig.1B). The macrophages themselves additionally oxidise LDL thereby potentiating the process [5].

The whole pathological process includes structural and functional changes of EC, VSMC and leucocytes all contributing to progression of the disease but our knowledge of in vivo interactions between these cell types is still fragmentary [2].

The role of VSMC in atherosclerosis

The endothelial dysfunction and the resulting inflammatory state of a vessel wall also influences the underlying VSMC. As already mentioned, in healthy vessels VSMC are in a differentiated state, possess contractile properties and show a low proliferation rate. If they are exposed to an appropriate stimulus, VSMC can undergo phenotypic changes and reinitiate cell proliferation. Such stimuli (e.g. PDGF) are found among the factors secreted by EC and leucocytes as a result of endothelial dysfunction. The VSMC react with migration from the media into the intima and hyperproliferation leading to an accumulation of more and more VSMC within the intima. Furthermore they start to secrete cytokines (IL-1, TNFα /β, M-CSF and others), likewise contributing to the proinflammatory cytokine cocktail (Fig.1A). Also VSMC react with the production of large amounts of ECM required for the retention of lipoproteins within the intima (‘synthetic’ phenotype) [3].

Therefore VSMC actively contribute to the progression of atherosclerosis not only by narrowing the vessel lumen through their rising cell mass but also by production of cytokines and by indirectly leading to the accumulation of lipids within the intima. The cytokine production is a major part during early steps of atherosclerosis but the deposition of ECM strongly contributes to the disease as well. At first sight the proliferation of VSMC does not seem to play a major role during the early phase of atherosclerosis but every cell that dedifferentiates and starts to produce cytokines and ECM drives the progression of the disease a little forward. Therefore VSMC contribute to the maintenance of the inflammatory state of the vessel wall from the very beginning [1, 3].

Later on, during the progression of atherosclerosis a huge number of VSMC accumulates and strongly contributes to the narrowing of the vessel. However, the migration and beginning proliferation of the cells during early stages is the basis for this accumulation of VSMC during later stages of atherosclerosis. The first step in VSMC activation is their response to cytokines such as PDGF and it would be beneficial if we could already interfere at this point to prohibit VSMC migration and proliferation and stop the feedback loop [3].

Next to local VSMC from the media, smooth muscle progenitor cells (SMPC) may be recruited from the blood stream and invade the endothelium, further contributing to the accumulation of smooth muscle cells within the intima [3, 14, 15]. In animal models these SMPC substantially contribute to arterial narrowing [15]. However in human atherosclerosis the contribution of SMPC has not been proven so far. There is one report where tissue samples of atherosclerotic lesions in response to organ transplantations (transplant atherosclerosis) were studied and the VSMC found were at least partially derived from SMPC [15]. Therefore it has been suggested that treatment of atherosclerosis should not only interfere with local VSMC growth but should also target mobilization, homing, differentiation and proliferation of circulating SMPC [14].

Irrespective of their origin, VSMC contribute not only to lesion growth by proliferation and production of ECM but also form a fibrous cap covering the lipid core thereby stabilising the plaque and preventing plaque rupture [2]. On the other hand the formation of a fibrous cap also traps the foam cells and leads to their necrosis further complicating the situation [5].

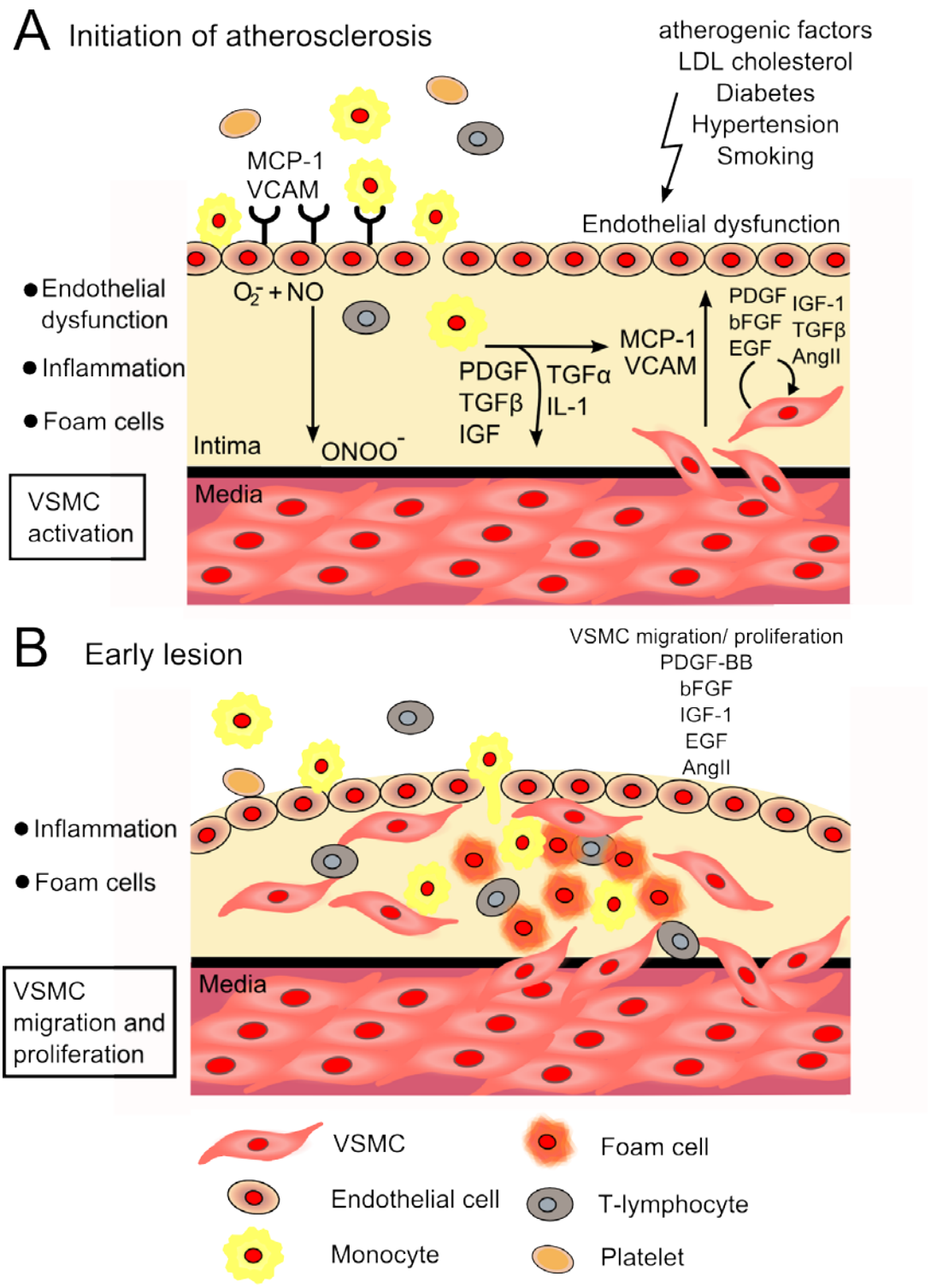

Fig.1. The initiation of atherosclerosis. *The initiation of atherosclerosis is marked by endothelial dysfunction leading to the secretion of proliferative, pro-inflammatory and pro-migratory factors of the endothelium acting on the underlying media composed of VSMC. These factors activate the VSMC and cause their dedifferentiation from a resting state into a proliferative phenotype. (**B**) The VSMC thereupon migrate through the intima and start to hyperproliferate. This process is accompanied by the invasion of monocytes into the vessel wall where they take up LDL cholesterol and turn into foam cells further contributing to the inflammatory process. Figure adopted from Dzau et al. [3]. For abbreviations see section 8.1.*

There is no doubt that interference with VSMC proliferation and migration would help to reduce the number of VSMC and SMPC within the atherosclerotic lesion and therefore progression of the disease. Therapeutical approaches following this direction hence are strongly investigated. Unfortunately, all compounds interfering with VSMC proliferation that have been developed so far show side effects and new antiatherogenic drugs are still in the focus of research (see also section 2.5.2.4).

Summarising, it can be said that once started, the whole process of atherosclerosis becomes self-maintaining by employing a network of positive feedback loops leading to strong inflammation, accumulation of large amounts of lipoproteins, ECM and VSMC, altogether contributing to plaque formation and enlargement. VSMC thereby play a pivotal role in formation, growth and stability of atherosclerotic plaques and therapeutical approaches to interfere with VSMC activation, proliferation and migration are highly welcome to stop the progression of atherosclerosis [13].

Treatment of atherosclerosis

To combat atherosclerosis first of all changes in lifestyle, such as eating a healthy diet and exercising are recommended to eliminate or reduce risk factors. If the patient reached an advanced state of the disease medications or surgical procedures are, however, unavoidable.

Pharmacological treatments

In earlier stages of atherosclerosis, namely before a plaque has been formed, the prescription of different drugs is the preferred treatment modality. One strategy is to reduce the elevated plasma cholesterol levels (hyperlipidemia). Statins, the most commonly used drugs for this purpose, have been quite successfully applied [16]. However, statins have been found to potentially lead to serious toxicities and although rare these complications ask for the development of new anti-hyperlipidemic compounds [16]. Also not every patient suffering from atherosclerosis exhibits hyperlipidemia. Moreover the reduction of cholesterol levels albeit lowering the risk, does not protect from all forms of atherosclerotic vascular disease. Still 70% of patients with hyperlipidemic treatment will get symptomatic disease regardless of this therapy [17].

To reduce the likelihood of platelet aggregation in narrowed arteries so called anti-platelet therapies are used [18]. They all target key elements of the platelet regulatory machinery. Among the several drugs used Aspirin is the most common one, irreversibly targeting the

cyclooxygenase (COX) and therefore platelet aggregation, inflammation and clotting. These therapies are also widely used but are associated with side effects including gastric ulcer, renal failure and bleeding [19].

Another way of treating atherosclerosis is lowering the blood pressure that is mainly regulated by the renin-angiotensin system (RAS). Therefore inhibitors of key elements of this hormone system such as angiotensin-converting enzyme (ACE) inhibitors and calcium channel blockers are commonly prescribed to combat hypertension. Studies using animal models have shown a positive effect of ACE inhibitors on the development of atherosclerosis however large clinical trials were not conclusive [20].

Additionally other medications may be used to control for specific cardiovascular risk factors such as diabetes.

Surgical treatments

If medication fails or the disease has progressed too far, surgeries e.g. angioplasty, endarterectomy, thrombolytic therapy or bypass surgery may be necessary.

An endarterectomy means the incision of the affected artery and removal of the plaque. Thereby the endothelium is injured, too, leading to an inflammation and possibly to a renarrowing (restenosis) of the artery. Depending on the clinical study looked at, the incidence of restenosis after an endarterectomy lies between 6-14% [21].

For a thrombolytic therapy either a thrombolytic substance (e.g. tissue plasminogen activator) is administered intravenously or a thin catheter is inserted into the affected artery to either deliver drugs that dissolve blood clots or to mechanically break up the occlusion. This method has been considered to be rather ineffective for chronic atherosclerotic lesions but is used for the acute treatment of cerebral infarctions or strokes [22, 23].

Bypass surgeries (e.g. coronary artery bypass surgeries) are another type of interventions that have been widely used in the past. Arteries from elsewhere in the patient's body or tubes made of synthetic fabric are used to bypass the narrowed region of the atherosclerotic artery and thereby improve blood flow. However, it is a rather severe surgery and due to the development of percutaneous interventions the number of coronary bypass surgeries was reduced [24].

Today the most common method used is the angioplasty. Depending on the artery affected it is distinguished between coronary angioplasty, peripheral angioplasty and renal artery angioplasty. During the year of 2000 more than one million patients underwent angioplastic procedures worldwide [25].

Angioplasty

The term angioplasty describes a panel of procedures that are employed to mechanically improve perfusion by widening the vessel lumen. The most prevalent method is the percutaneous transluminal angioplasty (PTA) generally accompanied by the implantation of a tube-like mesh structure, a so called stent [26]. It is used for peripheral, cerebrovascular as well as for coronary vessels [27]. First a tightly folded balloon catheter is inserted into the narrowed artery (Fig.2A). Next it is inflated to a fixed size using water pressure and then the catheter is removed again (Fig.2B). If a stent is to be used it is tightly folded, too, and inserted together with the balloon catheter [3](Fig.2A). Unfortunately the widened vessel often undergoes restenosis (Fig.2C).

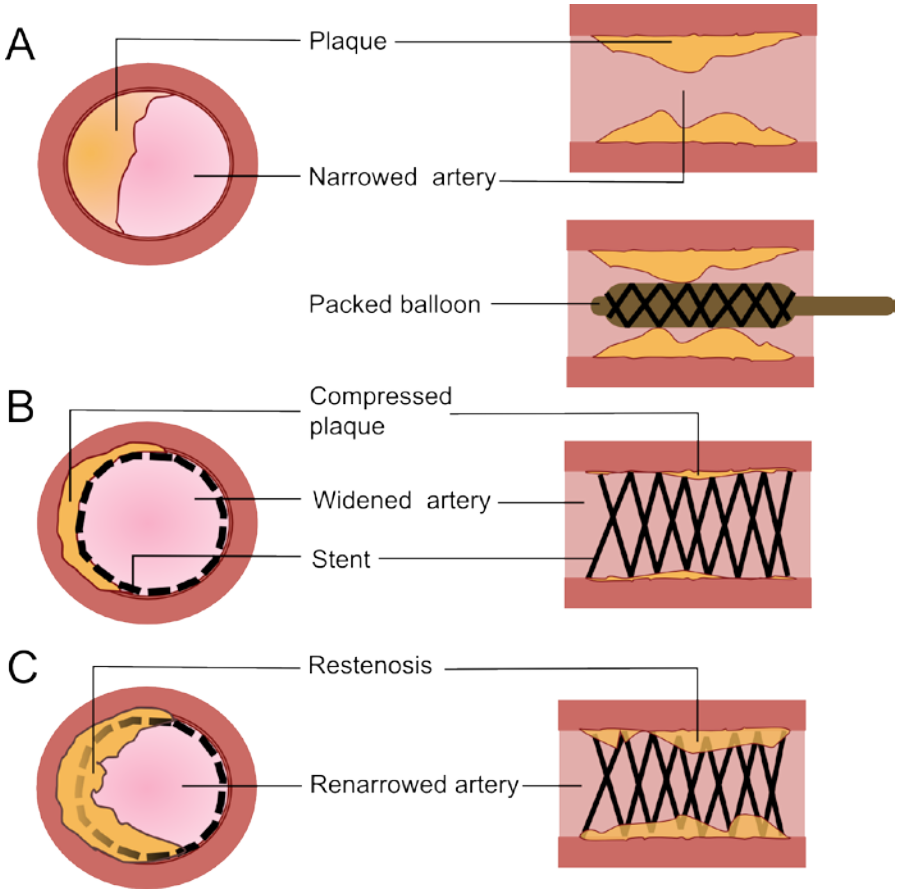

Fig.2. In-stent restenosis. *(**A**) A narrowed artery can be widened again by an angioplastic surgery using a balloon catheter. (**B**)This can be accompanied by the insertion of a stent in order to better maintain the vessel lumen. (**C**) One of the major complications of such an intervention is the renarrowing (restenosis) of the artery due to inflammatory processes followed by hyperproliferation and migration of VSMC.*

Restenosis

Pathology of restenosis

Angioplasty with or without stent placement is not a gentle process but virtually always leads to tissue trauma and complications. A major problem is that the endothelium is injured and at least partially stripped away (endothelial denudation). This may promptly lead to thrombosis or to an inflammatory cascade within the vessel wall [28]. In both cases the artery will be renarrowed either by a blood clot or by hyperproliferating VSMC overgrowing the stent (building a neointima). The latter is called restenosis and is generally defined as a narrowing of the vessel lumen by more than 50% [11, 26](Fig.2C). It should be noted that restenosis is not only a problem after angioplasty but also affects 20% of patients who received a bypass surgery and 6-14% of those who were subjected to an endarterectomy [21]. As it is true for atherosclerosis, also restenosis is a complex disease and its pathophysiology is still incompletely understood [28].

Atherosclerosis and restenosis obviously share a common etiology, namely inflammation of the vessel wall, though the underlying cause is different. Atherosclerosis results most likely from endothelial dysfunction whereas mechanical injury is the initial cause of restenosis. Nonetheless both result in a different pathologic outcome, probably because in atherosclerosis we observe a normal artery whereas restenosis hits an already diseased one [29]. One difference that can be seen is that during atherosclerosis single lipid-rich plaques accumulate in the vessel wall whereas restenosis is characterised by concentric neointimal thickening [28].

Inflammation and restenosis

After angioplasty an elevation of activated leucocytes and distinct inflammatory factors is observed. The strength of the inflammatory response is greater in individuals that had already displayed elevated baseline levels of several inflammatory parameters. Therefore such factors as e.g. C-reactive protein (CRP) or fibrinogen levels can be used as predictive markers for restenosis [29, 30]. This inflammatory process leads to the expression of selectins and other adhesion molecules in EC, mediating the binding and invasion of circulating monocytes to and through the vessel wall [27]. The involvement of monocytes very much resembles the early events in atherosclerosis. Also in restenosis the secretion of MCP-1 and expression of VCAM-1 play a critical role, directing transmigration of monocytes through the endothelium were they play again a pivotal role in maintaining and enhancing the inflammatory state [27].

Moreover the endothelial denudation caused by angioplasty leads to an exposure of the subendothelial matrix to the blood stream and the release of thrombogenic contents of the atheroma, thereby triggering platelet adhesion and activation [11]. Especially thrombin elicits further platelet activation and consequently the secretion of proliferative and pro-migratory factors such as platelet derived growth factor (PDGF) acting on VSMC which in turn undergo phenotypic modulations [28].

The endothelial denudation depicts a major problem and therefore research is not only focused on interference with VSMC growth but also on strategies to induce reendotheliasation of the stented area.

The role of VSMC in restenosis

As already highlighted in previous sections, in healthy vessels VSMC are in a differentiated resting state but if they encounter distinct cytokines or growth factors such as PDGF they can dedifferentiate a so called 'synthetic' phenotype.

Consequently the inflammatory state caused by e.g. an angioplasty initiates VSMC hyperproliferation and migration towards the source of chemotactic stimuli. The VSMC thereby form a neointima within the artery. Once formed the neointima is subjected to so called vascular remodelling, meaning a complex array of events that modulate matrix production and cell proliferation, gradually reducing blood flow [26]. First the neointima is composed mainly of VSMC which subsequently deposit huge amounts of ECM. Later on other cell types such as myofibroblasts and also SMPC accumulate and add to neointimal growth [11].

All together restenosis is marked by an acute and strong inflammation which is then followed by excessive proliferation of VSMC and further on by aberrant production of ECM and finally vascular remodelling ending up in a renarrowed artery [26, 28]. As in the pathology of atherosclerosis, VSMC play a crucial role in restenosis if not an even greater one because the neointima is mainly built up of VSMC. Already within one week after endothelial denudation VSMC loose their typical phenotypic markers and contractile properties and are transformed into a synthetic phenotype producing large amounts of ECM. PDGF-BB is one of the most potent suppressors of the contractile gene expression [11]. Therefore it is a major goal for the treatment of restenosis to interfere with (e.g. PDGF-BB-induced) VSMC signalling.

Stenting

Restenosis usually occurs within half a year after the surgery and concerns about 40-60% of patients who were treated with balloon angioplasty alone, whereas the insertion of a stent reduces the incidence of restenosis to 25% [27, 31]. Therefore restenosis depicts the major drawback of this minimal invasive technique [27]. The insertion of conventional stents is providing a rigid scaffold for the vessel and reduces the immediate renarrowing of the vessel that usually occurs due to elastic recoil but does not limit neointima formation [28]. However, further advancements have been made by the development and use of drug-eluting stents (DES) interfering with VSMC growth and reducing the chance of in-stent restenosis to less than 5% [26, 32]. A major problem of stenting is that not only the angioplasty itself injures the endothelium but also the insertion of a stent injures the vessel wall and strips away endothelial cells (denudation).

Current marketed first-generation DES use drugs, such as rapamycin and paclitaxel targeting an effector of a signal transduction pathway (mTOR) involved in VSMC proliferation and tubulin, respectively (for more detail see chapter 2.5.2.4) [33]. Unfortunately these drugs exert side effects on endothelial cells and evidence is growing that several patients develop late-stent-thrombosis [34]. Therefore research is still focused on finding new drug candidates that interfere with VSMC proliferation and migration. Additionally effort is made to develop different materials for stents themselves, as for example biodegradable matters [35].

The ideal stent-coating agent is still to be found and most probably is not a single compound but rather a mixture of anti-proliferative/ anti-migratory compounds on the one hand and endothelium-protective factors on the other hand in order to achieve re-endothelialisation without neointima formation [26, 34].

PDGF signalling

PDGF is the most potent stimulus of VSMC proliferation and migration. Therefore drugs interfering with PDGF signalling and thereby inhibiting activation of VSMC depict interesting targets for the further development as stent-coating drugs [26, 36].

PDGF receptor activation

PDGF was first detected in platelet-derived granules showing growth-promoting activity for VSMC and fibroblasts [37]. Soon the corresponding receptor has been identified and the importance of PDGF signalling during embryonic development has been emphasized using gene-targeted mice showing lethality at the time around birth [38]. There exist two

receptor subunits, α and β, that form homo- or hetero-dimers upon binding of PDGF to build a functional PDGF receptor (PDGFR) [39]. The ligand PDGF itself exists in four isoforms (A-D) that are only functional if forming homo or heterodimers. Among the PDGF dimers PDGF-BB is the only one binding to all types of receptor dimers, whereas the other dimers show selectivity for one or the other receptor dimer (see Table I). This might be the reason why PDGF-BB has been shown to be the far most potent stimulus for VSMC migration and proliferation [40].

	PDGFRαα	PDGFRαβ	PDGFRββ
PDGF-AA	+	-	-
PDGF-AB	+	+	-
PDGF-BB	+	+	+
PDGF-CC	+	+	-
PDGF-DD	-	-	+

for abbreviations see section 8.1

Table I. Binding specificities of PDGF and PDGFR isotypes

Up to now the five homo and heterodimers shown in Table I are considered to constitute the complete PDGF-family. However, it cannot be excluded that other PDGF dimers exist, such as PDGF-AC or PDGF-AD but so far these have not been found *in vitro* or *in vivo* [39].

Both PDGFR isotypes are receptor tyrosine kinases (RTK) and possess an intrinsic kinase activity within their intracellular domains (Fig.3). Upon ligand-induced receptor dimerisation and accompanying conformational changes the kinase domains come close enough to each other and enable mutual phosphorylation on crucial tyrosine residues (Y857 of PDGFRβ, Y849 of PDGFRα) [41]. This autophosphorylation is leading to further activation of the kinase domains themselves because their substrate clefts get accessible [41]. Therefore the receptor kinase is fully active, phosphorylating a number of target proteins, most prominently the PDGFR itself thereby providing docking sites for SH2 domain-containing proteins.

Early events in PDGFR signalling

Within minutes after receptor activation different signalling molecules are recruited including kinases like Src (Rous sarcoma virus kinase) or PI3K (phosphoinositol 3 kinase), adaptor proteins such as Grb2 (growth factor receptor-bound protein 2) or Nck but also

phosphatases as e.g. the phosphotyrosine phosphatase (PTP) Shp-2 (SH2-containing phosphatase 2).

The recruitment of distinct signalling proteins is defined by the surrounding amino acids of each phospho-tyrosine residue. For example Src kinase is binding specifically to the phosphorylated tyrosines 579 and 581 in the juxtamembrane domain of the receptor (PDGFRβ). Stable interaction of the PDGFR and PI3K requires phosphorylation of the tyrosines 740/751 (PDGFRβ) and Shp-2 specifically binds to phosphotyrosine residues 763 and 1009 (Fig.3).

The signalling cascades are tightly regulated and require changes in localisation and modification of many different proteins. The most important players are kinases and phosphatases that change the phosphorylation status of their target proteins.

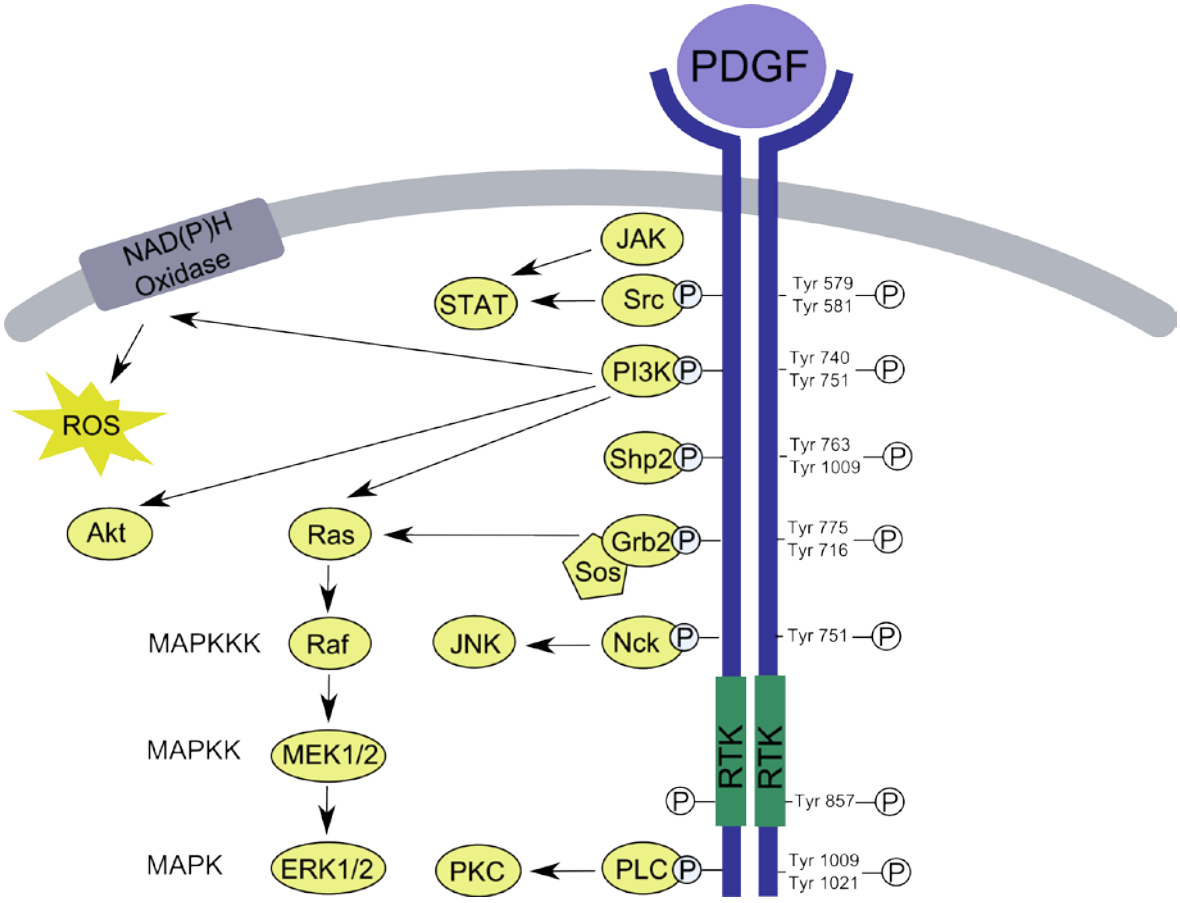

Fig.3. Schematic overview about PDGF receptor signalling. *Receptor dimerisation upon ligand binding leads to autophosphorylation of the intracellular parts of the receptor and subsequently to recruitment of several signalling molecules and hence the activation of a panel of signal transduction pathways. Shown are the positions of tyrosine residues of the PDGFRβ chain. For abbreviations see section 8.1.*

Activation of kinases in early PDGFR signalling

Binding of the Src kinase leads to a relaxation of its intrinsic inhibitory conformation of the kinase domain and thereby to its activation. PI3K as well as phosholipase C (PLC) are both activated by recruitment from the cytosol to the plasma membrane and concomitant co-localisation with their lipid substrates. PLC is additionally activated by phosphorylation through the PDGFR or Src. The PI3K is additionally activated by binding to active Ras. The latter is a small G-protein that accumulates in the proximity because its activating GTP-exchange factor Sos is recruited by several adaptor proteins such as Grb-2 [39]. Ras plays a pivotal role in many signalling cascades and is activated by a panel of different growth factors and cytokines [41]. The recruitment and activation of Ras is also responsible for triggering the mitogen activated protein kinase (MAPK) pathway via MEK and ERK [41].

Activation of phosphatases in early PDGFR signalling

One of the best described phosphatases which is recruited to the activated PDGFR is Shp-2 that has been implicated in the regulation of proliferative as well as migratory responses of VSMC [42]. Next to Shp-2 another important phosphatase for PDGF signalling is PTEN (phosphatase and tensin homolog) dephosphorylating the PI3K [40]. PTPs counteract receptor activation by dephosphorylation of tyrosine residues thereby inhibiting RTK function and diminishing docking sites for signalling molecules. However, the activity of PTPs is almost simultaneously inhibited with the activation of the PDGFR by production of reactive oxygen species (ROS) such as hydrogen peroxide via activated PI3K (Fig.3) [39]. The local increase in hydrogen peroxide concentration leads to the oxidation of an essential cystein residue within the catalytic side of PTPs. So although PTPs are recruited to the activated receptor they can not exert their phosphatase activity immediately but come later into play when hydrogen peroxide concentration decreases for example due to recruitment of peroxiredoxin. Then they are important to shut off the signal in order to prevent persistent receptor activation [41]. Shutting off the receptor signal is as important as the well-controlled activation of it because persistent activation leads to hyperproliferation of VSMC which is associated with atherosclerosis and restenosis.

Signalling cascades downstream of the PDGF receptor

Each of the signalling molecules mentioned above is directly and immediately activated by the PDGFR leading to the activation of complex networks driving many different signalling cascades.

Fig.3 shows a schematic overview about current knowledge of early PDGFR signalling. How exactly these signalling cascades are connected to the cellular events of migration

and proliferation is only incompletely understood. Investigating these events is difficult because it is hard to distinguish the primary PDGF-dependent events from secondary effects resulting from the expression of immediate early genes [41]. Even enzymes that direct immediate effects such as PI3K and PLC are also activated at later timepoints. Moreover genes that are expressed upon PDGF stimulation are often targeted by several pathways simultaneously for example activation of the cyclin D1 promoter requires active STAT3/5 as well as the Ras/ Raf/ MEK/ ERK pathway [43].

Mitogen activated protein kinase (MAPK)-signalling

Among the signalling pathways activated, the ERK-MAPK pathway is probably best understood. As depicted in Fig.3 it finally leads to the phosphorylation and activation of ERK1/2 which is thereupon accumulating in the nucleus and regulating transcription factors as e.g. Elk. Thereby it leads to the expression of target genes involved in cell cycle progression such as cyclin D1. Next to the ERK MAPK, other MAPKs such as p38 or the c-jun N-terminal kinase (JNK) are activated as well. The MAPKs play a crucial role in PDGF-induced proliferation and seem to have less importance in migration. Especially Ras significantly contributes to cell proliferation but there are also Ras-independent pathways involved [38]. JNK is less well studied concerning its role in PDGF-signalling. It can be activated by PI3K under certain circumstances or through the conventional MAPK pathway via MEKK1/4 and MKK but is definitely influenced by the adaptor protein Nck [40]. All MAPKs influence the cellular response through modulation of expression of their target genes [38].

PI3K and PLC-signalling

First of all, PI3K and PLC both phosphorylate lipids within the plasma membrane and thereby provide docking sites for distinct proteins that are then recruited from the cytoplasm to the plasma membrane. Therefore they both play crucial roles in regulating the localisation of downstream signalling molecules.

The PI3K has several responsibilities in PDGF-induced signalling. The activation of PI3K triggers the local production of ROS (reactive oxygen species like hydrogen peroxide) via the activation of Nox (NAD(P)H-oxidase) protein complexes. Thereby the PI3K influences the activity of phosphatases and other redox-sensitive proteins. Moreover, via its role in the modification of membrane lipids the PI3K also mediates the phosphorylation of Akt. Activated Akt in turn induces a number of pro-survival signals. It positively regulates transcription factors that lead to the expression of survival genes and negatively regulates pro-apoptotic factors as e.g. caspases. Next to this, activated PI3K is also involved in

calcium mobilisation together with PLC and activates small G-proteins as Rac and Rho that are involved in cytoskeletal rearrangements and migration. Hence PI3K-signalling is very important for both cell migration and proliferation upon PDGF induction [40].

The recruitment and activation of PLC as mentioned before leads to an increase in cytoplasmic calcium. Additionally it activates certain members of the protein kinase C (PKC) family and influences the activity of Na^+/H^+ channels [38]. It has been demonstrated that PLC-dependent effects mediate cell proliferation as well as migration [40]. Activation of PKC may lead to the degradation of p21 an inhibitor of cell cycle progression thereby providing a link between PLC/ PKC and proliferation [44].

STAT-signalling

Also other signalling molecules such as STAT1/3/5 (Signal Transducers and Activators of Transcription 1/3/5) are recruited and activated upon PDGF stimulation, but those processes are currently not as well described as the signalling events mentioned before [39]. For the activation of STATs it has been shown that Src kinase is involved but there are additional papers claiming that JAKs (janus kinases) play a role, too. STAT transcription factors are activated through multiple extracellular signals and are probably best described in inflammatory processes where they are major players in e.g. interferon signalling. However, STATs have also been implicated in growth factor signalling.

STATs have to be phosphorylated at crucial tyrosine residues in order to form homo- or heterodimers with other STATs and are thereupon translocated into the nucleus where they bind to distinct promoter-sequences called IREs (interferon response elements) [45]. The cellular responses to different stimuli (cytokines or growth factors) often result in a remarkable specificity of STAT activation. Whereas most of the STATs mediate antiproliferative signals in response to inflammatory processes STAT3 stimulates cell proliferation and prevents apoptosis. PDGF has been shown to stimulate the transcription of the proliferative factor c-myc via a Src-STAT3 axis. C-myc is a proto-oncogene involved in early phase of cell cycle progression [38]. Hence it is not surprising that overstimulation of the STAT3 pathway has been shown to cause cellular transformation in mice. STAT3 will be discussed in more detail in a later chapter (section 2.7.2.2).

In interferon-induced signal transduction the activity of STATs is additionally regulated by negative feedback loops employing so called ‘suppressors of cytokine signalling’ (SOCS). Those proteins seem to regulate STATs by direct inhibitory interactions with receptors and signalling proteins as well as targeting associated proteins for degradation. Concerning

growth factor-induced STAT activation, there is not much known regarding shut down of the signal but it is thought that SOCS play a role here, too [46].

Regulation of PDGF signalling

All together there are many signalling cascades involved in the cellular responses to PDGF and it seems that none of them influences any outcome exclusively. Moreover, these signalling cascades are not activated in isolation, but there is also crosstalk between pathways, feedback loops play a role and this is why our knowledge is far from showing a complete picture of all these signalling events [39]. It should be noted that the molecules involved in PDGFR signalling are also involved in many other pathways and can be recruited to many different receptors within a cell. Moreover PDGFR activation leads to cross-activation of the FGF receptor [47]. Changes in subcellular localisation of distinct pools of enzymes and molecules are now thought to significantly contribute to signalling outcome [41]. All together signalling events downstream of the PDGF-receptor show a network-like pattern that is far more complex than shown in Fig.3 [38].

The different receptor dimers elicit similar but not identical cellular responses [38]. Both PDGFRαα and PDGFRββ mediate proliferation but their influence on migration is different. Although both lead to partially similar cytoskeletal rearrangements PDGFRαα rather inhibits whereas PDGFRββ clearly enhances chemotaxis. PDGFRαβ also enhances chemotaxis but is in general less well investigated [38].

It is out of question that PDGF signalling is tightly regulated by feedback control mechanisms in a healthy cell and that deregulation is associated with disease such as atherosclerosis because PDGF is the most potent stimulus for VSMC growth and migration [40]. Therefore interference with PDGF signalling is an important goal for the treatment of atherosclerosis and restenosis.

PDGF signalling in vascular disease

Since PDGF-BB is the far most potent stimulus for both, proliferation and migration of VSMC, it is not surprising that strong data exist linking PDGF activity to human atherosclerosis [48]. For example elevated levels of PDGF A and PDGF B have been found in tissue samples obtained from endarterectomy surgeries. Moreover several signalling molecules downstream of the PDGFR have been found to be hyperactive in human atherosclerotic plaques [49, 50]. Additionally the selective inhibition of distinct PDGF-induced signalling cascades has been shown to inhibit neointima formation in animal models [51].

PDGF-BB stimulated VSMC are therefore used to investigate the underlying molecular mechanisms of hyperproliferation and migration.

Cell Cycle

The mammalian cell cycle is divided into several phases and is a complex process involving numerous regulatory proteins. Its tight regulation is essential and many of the proteins involved are proto-oncogenes connected to the development of cancer or other proliferative disorders such as atherosclerosis and restenosis.

Cell cycle phases

Morphologically the cell cycle can be divided into the interphase, when chromosomes are decondensed and mitosis (M-phase) when chromosomes are highly condensed and therefore visible using an optical microscope. During M-phase the cell divides and gives rise to two genetically identical daughter cells. During interphase the cell grows, carries out its life function and prepares for cell division if the appropriate stimuli as e.g. growth factors are present. The interphase itself is subdivided into different phases (Fig.4) [52].

Cells that are not cycling but still possess the potential of cell division entered a state of quiescence termed G0. If the appropriate stimuli are present the cell exits from G0 and enters the first ‘gap’ phase (G1), where it prepares for doubling the genome. A lot of enzymes necessary for DNA synthesis are produced now. Here the cell also checks whether the conditions are favourable for cell division or not, whether the cell is healthy enough or not and may still stop the cell cycle. In very late G1-phase the cell passes the so called restriction point and enters S-phase (synthesis phase). From now on the cell will complete cell division even if it is deprived from growth stimuli. During S-phase DNA synthesis takes place and this phase is generally low in protein synthesis except of histone production. During late S-phase there is another checkpoint where the cell controls for DNA damage and decides whether to proceed with cell division or to undergo apoptosis. It is followed by a second gap-phase (G2) where the cell prepares for cell division. The G2-phase is the last part of the interphase and is followed by the M-phase [52].

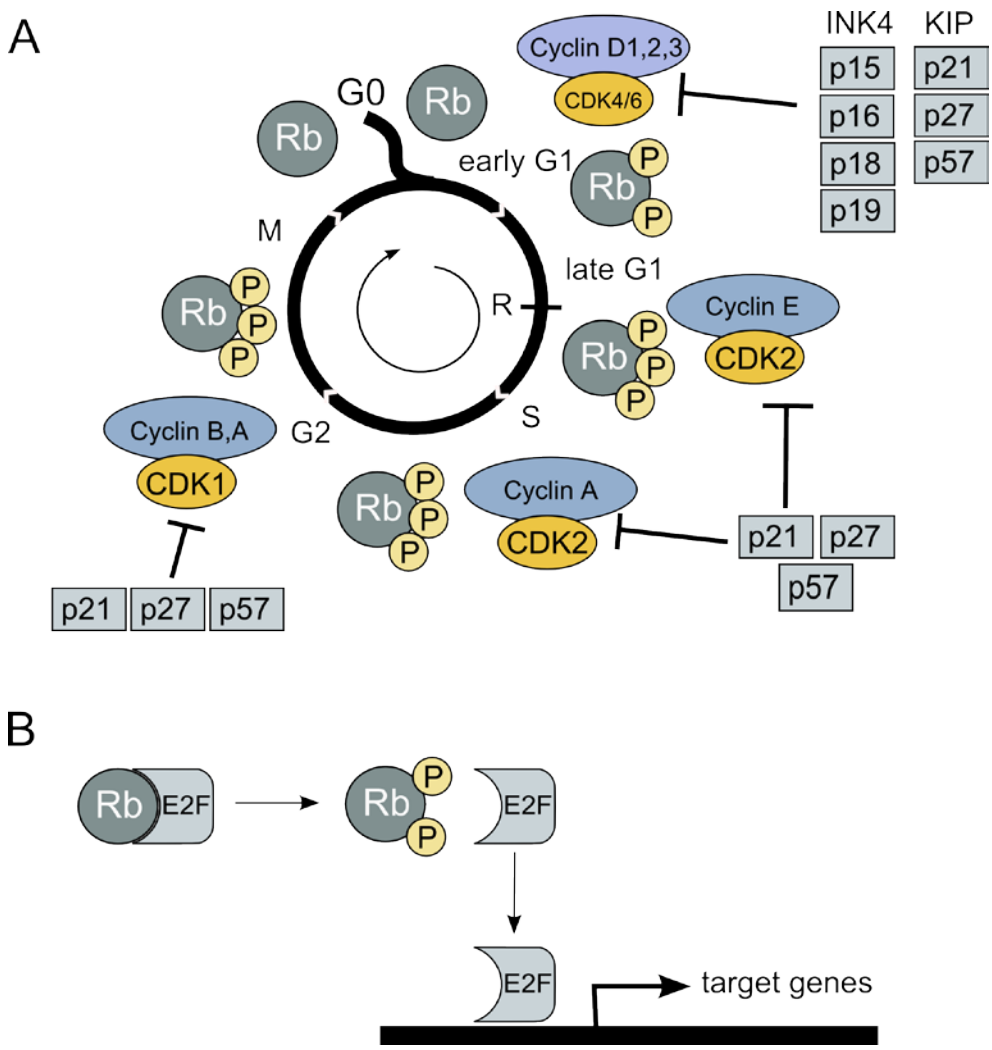

Fig.4. Cell cycle. *(**A**) The cell cycle is divided into 4 phases, two 'gap' phases (G1 and G2), the 'synthesis' phase (S) when DNA is replicated and the mitosis phase (M) when cell division takes place. An important protein that is tightly regulated through phosphorylation during cell cycle progression is the Rb protein. (**B**) Upon phosphorylation it releases E2F transcription factors and therefore allows expression of proteins crucial for cell cycle progression. For abbreviations see section 8.1. (figure adopted from [53])*

Cyclin-dependent kinases (CDKs)

The family of CDKs currently consists of 13 members and most of them play an essential role in cell cycle regulation. However, not all of them share the same responsibilities within a cell and some of them do not participate in cell cycle regulation at all.

Progression through the cell cycle is mainly driven by CDKs that form complexes with cyclins and are thereby activated. Additionally CDK activity is influenced positively and negatively by threonine and tyrosine phosphorylation [41]. CDKs themselves are rather ubiquitously expressed throughout the cell cycle. Their activity is regulated by the limited expression of distinct cyclins during each phase of the cell cycle.

CDK1/2/3/4/6

Entry into, progression through and exit from G1 phase are controlled by D-type cyclins (D1, D2, D3) complexing with CDK4/ 6 and later on E-type cyclins (E1, E2) complexing with CDK2. CDK3 is only poorly investigated but is suggested to complex with cyclin C and to stimulate G0/G1 transition [54]. During S-phase, cyclin A and later cyclin B are expressed and control for the activity of CDK2 and CDK1, respectively (Fig.4A) [52]. The expression levels of cyclin D and E have been considered for a long time to be the rate-limiting step in G1-S-phase transition [55]. There are many reports supporting this notion but data from gene targeted mice showed that progression through cell cycle can take place without these cyclins (discussed below). In cultured cells overexpression of D-type cyclins shortens G1 phase and the inhibition of CDK4 either by pharmacological inhibitors or by overexpression of CDK inhibitor proteins (CKI) lead to an arrest in G1 phase. Additionally cyclin D1 overexpression can drive cell cycle progression even when cells are arrested in response to DNA damage. Also enhanced cyclin E expression can shorten G1 phase and the absence of cyclin E stops cell cycle progression [55].

However, gene-targeted mice for different cyclins and CDKs showed that there must be ways of compensation for all of those proteins because none of the knockouts was completely deficient in cell replication. Cyclin D1, D2, D3, cyclin E1, E2 as well as CDK4 single knock out mice were all viable. Most surprisingly also a CDK2 knock out is viable and apart from male and female sterility free of pathologies [56]. This finding was really astonishing because CDK2 activity has been considered to be indispensable for cell cycle progression [55]. Double or triple knock outs of D-type cyclins were in part embryonic lethal or lead to death within the first weeks after birth. Cyclin E1/E2 as well as CDK4 and 6 double knock outs were embryonic lethal, too. But still all of these knock out mice developed until midgestation what requires cell division [55]. Therefore there must be more than cyclins D, E and CDK2/4/6 to drive cell cycle progression through G1 phase. Recently it has been shown that in fact only CDK1 is essential for cell cycle progression of most cell types because even the combined knock out for CDK2/3/4/6 was still allowing organogenesis and development to midgestation [57].

Interestingly, simple eukaryotes such as yeast possess only a CDK1 homologue and get along with a single CDK [58]. So nowadays the model presented in Fig.4 is in general valid for most mammalian cell types with the notion that some cell types may have different requirements for single CDKs, and that CDK1 can compensate for all other CDKs in most cells [57, 59].

Other CDKs

Besides CDK1/2/3/4/6 there are more CDKs known but none of them has been shown to be directly involved in cell cycle regulation until know except of CDK7.

Today 13 CDKs are known. However, until 2000 nine of them had been identified only. Therefore it is very likely that the list will further grow in the future. CDK10-13 are not very well described in the literature because they are known only for a short time. Also CDK8 and 9 are poorly investigated [60].

CDK7 and CDK9 play a role in RNA polymerase II activation and are therefore regulating transcription in general [61, 62]. Hence they both are indirectly involved in cell cycle regulation because gene transcription is necessary for cell cycle progression. CDK7 is additionally involved in DNA repair whereas CDK9 acts as transcriptional cofactor and is possibly playing a role in AIDS progression as well as differentiation processes in distinct tissues [61, 62]. Moreover CDK9 is overexpressed in neuroblastoma [62]. As already mentioned CDK7 is also involved in cell cycle regulation. It binds to cyclin H thereby forming the CDK-activating kinase (CAK) and phosphorylates CDK2 and CDK1 [63].

CDK5 is an atypical CDK that is not ubiquitously expressed. It is found mainly in the neuronal system where it does not influence cell cycle but rather differentiation, cell morphology and motility [64]. It is most abundant in post-mitotic neurons where its activity is obligatory for migration and differentiation processes within the developing brain. CDK5 has been best characterised in influencing the cytoarchitecture of the central nervous system. It is activated by non-cyclin proteins p25, p35 and p39. Recently it has been found in some other cell types as well and roles for CDK5 have been proposed e.g. in myogenesis and haematopoietic cell differentiation [65].

Pocket proteins and E2F transcription factors

A central role in driving cell cycle progression is played by so called pocket proteins, namely Retinoblastoma protein (Rb), p107 and p130 which are residing in the nucleus. These are the main targets of active CDKs and if hyperphosphorylated they dissociate from members of the E2F family of transcription factors (E2F1/2/3a/3b/4/5/6/7 and E2F8). The pocket protein- E2F complexes are further influenced by CDK inhibitor proteins such as members of the INK or KIP family of proteins. Those bind to and stabilise the complexes thereby inhibiting transcription (Fig.4A) [66].

The pocket protein-E2F complexes already reside in the nucleus bound to the E2F promoters and dissociation of the pocket proteins in general enables target gene transcription and therefore leads to cell cycle progression. The pocket proteins not only influence E2Fs by binding to them but also recruit chromatin-modifying enzymes such as histone deacetylases or methyltransferases and are therefore thought to 'actively' repress the promoters [67]. Among the E2F target genes are cyclins themselves and proteins involved in DNA synthesis such as DNA polymerase II (Fig.4B) [66, 67]. Together the E2F target genes are required for ordered progression through the cell cycle not just at the G1/S transition but throughout the cell cycle [67].

During G0 phase the pocket proteins are only minimally (or hypo-) phosphorylated, therefore bound to E2Fs. During cell cycle progression the pocket proteins are subsequently more and more phosphorylated (hyperphosphorylated) by CDKs. It has been found that in serum deprived cells almost all E2F responsive promoters are occupied by E2F4/ p107 or E2F4/ p130 complexes [66]. The cyclin E promoter is so far the only E2F responsive promoter known that is strongly bound by Rb. Therefore the expression of the late G1-phase cyclin E is dependent on the phosphorylation of the retinoblastoma protein whereas most of the other E2F target genes require phosphorylation of p107 and p130 [66].

It should be noted that during the last years it has become clear that the pocket proteins can also positively regulate gene expression if bound to distinct members of the E2F family. The nine members of the E2F family identified so far are therefore grouped into activator E2Fs and repressor E2Fs. E2F1/2/ and 3a are activators of transcription and are only low expressed in quiescent cells but highly expressed during late G1 and found in complexes with Rb. On the other hand E2F3b/4/5/6/7/8 are repressors of transcription and found in complexes with p107 and p130 [66, 68]. The activating E2Fs are themselves target genes of E2Fs. Therefore during G1 phase when the hyperphosphorylation of pocket proteins leads to expression of E2F target genes one result is the transcription of the activator E2Fs 1/ 2/ 3 [67]. Despite binding different E2Fs another difference between the single pocket proteins is their level of expression. Whereas Rb is expressed in proliferating and quiescent cells, p107 is most prominent in proliferating cells only and p130 is expressed mainly in arrested cells [66].

Despite the obvious differences between the pocket proteins it has been shown that they can in part compensate for each other although p107 and p130 are more closely related to each other than to Rb [66]. Therefore it is not clear whether it is really helpful or even

possible to distinguish strictly between Rb or p107/p130 dependent genes. Cobrinik concluded in 2005 that much remains to be learned about pocket proteins and their function in cell cycle regulation in development and disease [66].

Summarising it can be said that although the model presented in Fig.4 is very likely valid for most of the mammalian cells there are many levels of redundancies between the single players. Different CDKs as well as single pocket proteins can compensate for each other and there are additional enzymes exerting CDK independent activities on cell cycle progression.

VSMC cell cycle regulation and disease

As it is true for most tightly regulated biological processes defects in cell cycle control have severe consequences such as cancer, fibrotic disorders or cardiovascular diseases like atherosclerosis and restenosis.

As described in previous chapters atherosclerotic lesions are very heterogeneous at the cellular level, containing not only VSMC but also endothelial cells, macrophages and other inflammatory cells, each of which participate in lesion formation. Injury or dysfunction of the endothelium is the earliest event but VSMC proliferation occurs rapidly afterwards and plays a central role in lesion formation and growth. The same is true for restenosis where mainly VSMC build up the neointima. Interfering with VSMC proliferation is therefore considered as a major strategy to combat vasculoproliferative disorders.

In general VSMC cell cycle is controlled as discussed in previous chapters and usually in a healthy artery the cells do not proliferate at all. But some aspects of mammalian cell cycle seem to be cell type specific and there is still much to be learned about VSMC. There is much effort made to better understand VSMC cell cycle and find unique targets that would allow a specific inhibition of VSMC proliferation without targeting other cell types [3].

Regulation of cell cycle progression of VSMC

Early signalling events in cell cycle progression of VSMC

As highlighted in previous chapters VSMC proliferate in response to several stimuli as e.g. growth factors such as PDGF and many of these factors activate the same or similar signalling cascades. Amongst these are MAPK, PI3K or JAK-STAT pathways (see section 2.3).

Using pharmacological inhibitors, dominant negative or constitutively active enzymes it has been shown that Ras, PI3K, PLC, ERK and Src trigger G1/S-phase transition of VSMC [69-74]**.** Goncharova et al. showed that PI3K signalling was necessary and sufficient for VSMC proliferation and migration and suggest a pivotal role of PI3K in vascular remodelling [71]. This was confirmed by Liu et al. who found that PI3K as well as PLC were necessary for PDGF-induced proliferation [73]. Another group found that interfering with Ras activation also inhibits smooth muscle cell proliferation and discuss the possible use of Ras signalling inhibitors to control smooth muscle proliferation after vascular injury [70]. The inhibition of JNK, p38 or ERK with either dominant negative mutants or pharmacological inhibitors has proven the importance of MAPK in VSMC proliferation. However, the effect was much stronger for ERK compared with the other MAPKs [74, 75]. Also Akt activation has been shown to play an essential role in VSMC proliferation, for example via the activation of the mTOR/p70 S6 kinase pathway [33].

Regulation of cell cycle progression of VSMC at the transcriptional level

As in most mammalian cell types studied so far E2F transcription factors play a pivotal role in VSMC cell cycle. Next to E2Fs also other partly cell type specific, transcription factors are essential during cell cycle progression, too. This is also true for VSMC. In quiescent VSMC specific transcription factors are expressed (GAX and GATA-6) that stimulate the expression of the CDK inhibitor p21. Upon PDGF stimulation these factors are downregulated, hence p21 levels drop and CDKs are activated [3].

Next to signalling proteins also other molecules influence VSMC cell cycle. For example, the addition of nitric oxide (NO) leads to a blockade of the cyclin A promoter and moreover to an enhanced expression of p21 [3].

Inhibition of VSMC cell cycle progression as treatment modality for vasculoproliferative diseases

The inhibition of VSMC proliferation is a major goal in preventing atherosclerosis or restenosis after angioplasty. This has been tried by many different approaches targeting various stages of cell cycle progression. However, the therapeutic application of all these strategies has so far been limited to local treatments and hence to restenosis. Systemic administration of drugs or other therapeutical strategies would be problematic as they target every cell they encounter thereby eliciting severe side effects. Moreover the activity of the liver would lead to drug metabolism and clearance before the agents even reach

their target site. For restenosis treatment the local delivery of the drugs can be achieved by directly coating stents. A main problem remains that the adherence of endothelial cells to stent surfaces under flow conditions is particularly poor, thereby hindering re-endothelialisation [35].

Brachytherapy

One idea was to apply either β or γ radiation locally to the stented area (called brachytherapy) but there are major concerns about this method because it leads to DNA damage of all surrounding cells and additionally may cause cell death rather than cell cycle arrest [3, 76]. Also DNA damage could lead to cellular transformation and cancer. Therefore research is focused on finding more specific methods to hinder VSMC proliferation.

Targets for anti-restenotic therapies

Because PDGF is the most potent proliferative stimulus for VSMC, first it has been tried to directly inhibit the PDGFR using PDGFR antibodies or gene silencing (via RNA interference strategies). All attempts failed most likely because such a multifactorial pathology can not be treated by interfering with a single mitogenic factor. Therefore, the focus of interest has shifted towards targeting directly the VSMC cell cycle where all mitogenic stimuli come together [3]. Simultaneously, cytotoxic effects should be avoided because this again would elicit an inflammatory process rather than contribute to healing of the vessel.

Gene therapy

Another strategy envisages gene therapies such as e.g. antisense RNA to silence genes essential for VSMC proliferation [77]. This is a field of intensive investigations because the last decade has seen immense advances in gene therapy strategies and gene vector technology. Hence researchers aim to treat a diverse number of diseases with such strategies. For vascular pathologies gene therapies are expected to exert local and transient actions with minimal side-effects [78]. Possible targets range from classical cell cycle targets such as Rb and p21 to signalling molecules such as caldesmon [77, 79]. Another important issue for the development of gene therapies is the mode of vector delivery. Because the liver takes up very efficiently systemically administered vectors, cardiovascular gene therapeutic strategies are limited to local delivery. This is mostly achieved by directly coating the inserted stent with the vector [77]. In a pig model stable expression of green fluorescent protein (GFP) in the vasculature has been shown over a 4-week period using a fibronectin-coated stent platform [80].

Pharmacological inhibitors – drug eluting stents

The most used strategy to inhibit VSMC proliferation after angioplasty is the use of pharmacological inhibitors and the delivery via drug eluting stents (DES). There are a number of DES on the market but they all target only 3 different proteins, namely mTOR, calcineurin and tubulin.

The Akt target mTOR, a serine/ threonine kinase is involved in cell cycle regulation at different levels. It integrates both intracellular and extracellular signals and regulates a variety of essential biological processes, such as cell proliferation and survival. Therefore it is not surprising that the knock out is embryonic lethal. Interestingly it exerts different physiological functions when bound to different proteins. The best-studied mTOR downstream effectors are S6K1 (p70 ribosomal protein S6 kinase 1) and 4EBP1 (eIF4E binding protein 1). Their phosphorylation by mTOR releases them from inhibitory factors and subsequently leads to enhanced cell growth by enhancing protein translation. Cells overexpressing mTOR often are larger in size (hypertrophic) and gain proliferation advantages. Besides the involvement in atherosclerosis mTOR has been shown to be dysregulated in other human disorders, including obesity and diabetes as well as in many tumors [81]. In the 1970s rapamycin (traded as Sirolimus) was isolated from *Streptomyces* h*ygroscopicus* and later on it was shown to potently inhibit mTOR [82]. In VSMC rapamycin potently inhibits G1/ S-phase transition [34]. Additionally rapamycin shows immunosuppressive activity and therefore it was used to treat restenosis. Since then several derivatives have been placed on the market [34].

Calcineurin, a protein phosphatase, induces NFAT transcription factors (nuclear factor of activated T cell) and is crucially involved in T-cell activation [83]. Therefore the potent calcineurin inhibitor tacrolimus (FK506) is primarily an immunosuppressive compound but moreover also limits VSMC proliferation [84]. Tacrolimus and its derivatives in use (Pimecrolimus) complex with intracellular factors (immunophilins) and then bind to and thereby inhibit calcineurin [83]. Among the NFAT target genes are mainly proinflammatory cytokines such as IL-4 and IL-5 [85]. However, it was also demonstrated that the calcineurin pathway in VSMC is required for the maintenance of a normal VSMC phenotype [86]. Therefore the use of tacrolimus could implicate dedifferentiation of VSMC.

Finally stents coated with a stabiliser of microtubules, Paclitaxel (taxol), are in use. Microtubule-paclitaxel complexes can not disassemble anymore and normal cellular functions such as disassembly of the mitotic spindle apparatus are blocked. Therefore paclitaxel leads to a mitotic arrest in VSMC [87]. An advantage of this compound is its

hydrophobicity that allows it to be fixed to tissue elements, thus most likely leading to prolonged tissue residence [88]. However, arresting cells in M-phase of the cell cycle often leads to apoptosis and excessive apoptosis in the stented area is not beneficial. Therefore compounds that stop early cell cycle progression are preferable.

All currently marketed DES show side effects on endothelial cells and evidence is growing that several patients develop late-stent-thrombosis [34]. Therefore it is still searched for new drug candidates that interfere with VSMC proliferation and migration. As highlighted in section 2.2.4 the ideal stent-coating agent is most probably not a single compound but rather a mixture of anti-proliferative/ antimigratory compounds and endothelium-protective factors. Future DES should allow re- endothelialisation without neointima formation [26, 34].

Cell Migration

Basics of cell migration

Cell migration is an important process during development and life of multicellular organisms. It is essential for embryonic development for tissue formation and remodelling but also for immune responses and wound healing. Cell migration requires complex and orchestrated rearrangements within the cell but also precise sensing of, and orientation within the environment [89]. Inappropriate cellular migration is involved in several diseases including chronic inflammatory disorders, tumour formation and metastasis and atherosclerosis as well as restenosis [90].

Directed migration requires a gradient of either a chemoattractant or a repellent and a cell that is able to sense the gradient and then actively move towards or against it. The process therefore needs first of all extracellular signals and receptor mechanisms (Fig.5A). Upon sensing the cell needs to be asymmetrically polarized, to detach from its surrounding and form protrusions towards direction of migration (Fig.5B). This requires fundamental reorganisation within the intracellular cytoskeleton as well as breakdown of extracellular associations. Subsequently the front part of the cell (leading edge) is attached to the substrate and the cell is contracted leading to the movement of the whole cell body. Simultaneously the rear part detaches from the substrate (Fig.5C, D). Migration can therefore be seen as a cyclic process of detachment, formation of protrusions, contraction and re-attachment [89, 91]. However in fast migrating cells such as neutrophils it is not possible to observe the distinct stages separately and they do not develop strong cell-matrix interactions during migration but it rather looks like they were gliding over the substrate (amoeboid motility). Also it should be noted that migration *in vitro* obviously

differs from migration *in vivo* where it is much more directed with cells forming longer and more stable prolongations [89].

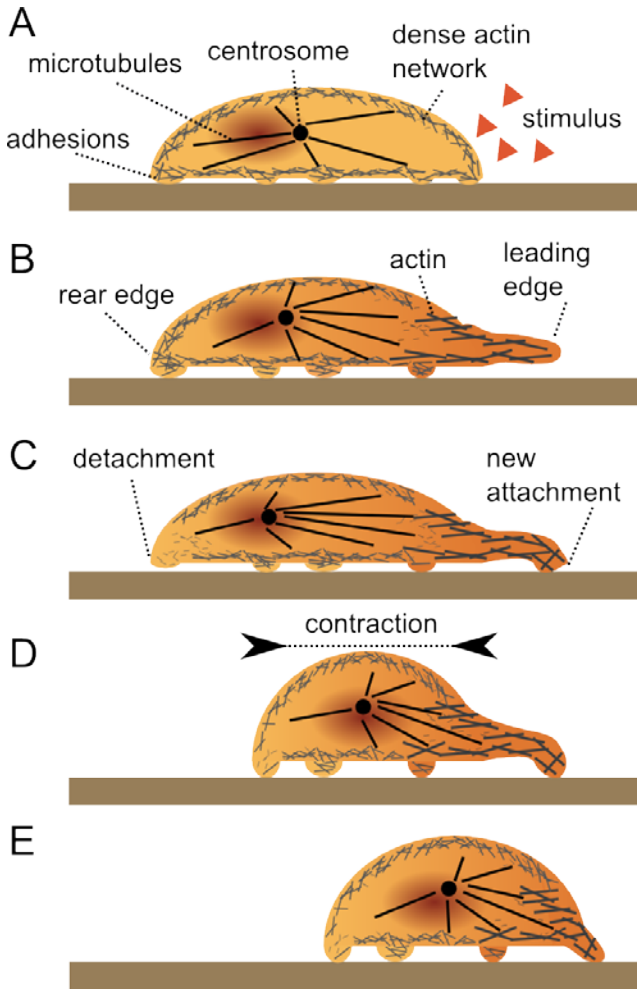

Fig.5. Cell migration. *(**A**) Upon sensing of an extracellular signal the cell becomes polarised. (**B**)Thereupon the cytoskeleton is rearranged and protrusions are formed. (**C**) The cell attaches at the leading edge to the substrate and the rear section of the cell detaches. (**D**) Next the cell contracts and the cell body is moved forward. (**E**) The cycle is repeated until the cell reached its final destination.*

Cytoarchitecture

The central role in migration is played by the cytoskeleton that is made up of three types of filaments: microfilaments (or actin filaments), intermediate filaments and microtubules. The reorganisation of the cytoskeleton is conducted by a whole machinery of enzymes positioning, breaking down and building up these complex networks.

Micro- or actin filaments are composed of two intertwined actin chains approximately 8 nm in diameter. They are most concentrated beneath the cellular membrane and responsible for resisting tension and keeping the cell's shape (Fig.5A). Together with myosin they are important for muscular contraction and cytoplasmic streaming. The term intermediate filaments (about 10 nm in diameter) denominates a group of chemical diverse structures namely vimentin, keratin, lamin and neurofilaments. They all play similar roles in the cell as they function in the maintenance of cell-shape by organizing the internal three-

dimensional structure of a cell and anchoring organelles. Lamin for example builds up the nuclear envelope. Finally the cytoskeleton is formed also by microtubules that are hollow cylinders commonly built of 13 protofilaments which are in turn polymers of α-tubulin and β-tubulin. They are about 25 nm in diameter and can reach up to 25 µm in length. The microtubules are anchoring and transporting membranous organelles, are responsible for intracellular vesicle traffic and also confer stability to the cell. They are positioned and organised by MTOCs (microtubule-organisation centres) such as the centrosome and are located within the inner parts of the cell (Fig.5A) [92]. Among the three elements of the cytoskeleton only actin and intermediate filaments become associated with cell–cell and cell–matrix junctions [93].

Migration step by step

The cytoskeleton

The actin cytoskeleton is the main cellular engine that connects complex signal cascades downstream of receptors such as the PDGFR with cell migration [94]. The positioning as well as assembly and disassembly of single actin filaments and functional higher-order networks is regulated by a panel of actin-binding proteins (ABP) such as integrins or vinculin. Up to now more than 70 families of ABPs have been identified, comprising more than 250 different proteins [95]. These ABPs are essential for migration because actin filaments on their own would be ineffective and their usefulness depends on linkage between each other and to other cell components such as microtubules or the cell membrane [96].

An important feature of actin filaments but also of microtubules is their intrinsic polarity that results from head-to-tail association of different protein subunits. This is the molecular basis that allows for polarisation of the cell which is mainly built up by rearranging of actin filaments and maintained by microtubules and also needs extensive crosstalk between the two systems [97]. Moreover both types of filaments are dynamic structures that can either polymerise or depolymerise and the net growth is depending on the concentration of free subunits. All three characteristics of actin filaments and microtubules their polarity, the dynamic structure and the interaction with each other are the basis of cell migration [97].

Polarisation

When a cell is exposed to a distinct migratory stimulus the cell either initiates directed movements or simply changes from a resting into a more motile or chemokinetic phenotype. A directed migratory response requires a gradient of the stimulus that can be either soluble (chemotactic stimulus) or be found in the substrate itself as for example matrix components or surface markers on surrounding cells (haptotactic stimulus) [89]. The corresponding receptors themselves are usually equally distributed over the cell surface. Therefore the signalling response needs to convert the extracellular signal into intracellular polarity. For this reason the receptors are generally more or less loosely associated with or located in the proximity of the cytoskeleton. Such hotspots of signalling often comprise integrins and several different types of membrane bound receptors and are called focal adhesions because here cells are also anchored to the extracellular matrix [91].

Cellular adhesions

Adhesions to the substrate at the front part stabilise protrusions and serve as traction points for movement of the whole cell body [89]. They are built up or broken down in response to extracellular cues but the molecular mechanisms underlying the decision of where and when to assemble or disassemble such adhesions remain unclear so far. Integrins and their interaction with the actin cytoskeleton play a role as well as Rho GTPases or the extracellular cleavage of adhesion components by proteases but we are far from understanding these complex events [91].

Signalling cascades

Since most of the receptors for chemo- or haptotactic stimuli are recruiting the PI3K one of the first results of receptor activation is the local conversion of membrane phosphatidylinositol to phosphatidylinositol-3-phosphat (PIP_3). This asymmetric accumulation of PIP_3 in turn leads to the local activation of members of the Rho family of small GTPases (Rac, RhoA, cdc42) and consequently to the polymerisation of actin. The localisation of small GTPases is a key event in polarisation and cells lacking cdc42 are unable to respond to chemotactic stimuli anymore. The process of actin polymerisation begins with a nucleation event where either complexes made of Arp2/3 (actin-related protein 2/3) or of formins (mDia1/2) determine the starting point [91]. Nucleation is favoured by PIP_2 because it frees actin from the inhibitory capping protein gelsolin [98]. Arp complexes are regulated by WASP, a molecule downstream of Rac and cdc42, and bind to existing actin filaments where they promote the growth of a branch at an angle of 70°. The formin complexes in contrast are regulated by cdc42 and RhoA and bind to actin

filaments promoting their linear extension [91]. The single actin monomeric subunits (G-actin) assemble into long filaments (F-actin) and two of those each form the actin filament. The addition of each monomer is ATP dependent and makes migration a costly process for a cell. The assembly of actin filaments is controlled by different capping enzymes that bind to growing filaments and either stabilise or destabilise them. Additionally these enzymes control the availability of activated actin monomers. The polymerisation of actin and its interaction with myosin (actomyosin-based contraction) pushes the cell forward into the direction of migration [89]. These contraction events are again regulated by small GTPases. Cdc42 as well as RhoA promote contraction while Rac mediates relaxation [91].

Many signalling cascades are involved in regulating cell migration and it is not possible to mention all of them. It is out of question that most of them employ small GTPases and influence the cytoskeleton in one way or the other.

Cytoskeletal rearrangements

After establishing the cell polarity by actin reorganisation the microtubule network plays a pivotal role in maintaining it. Again small GTPases are essential for positioning the MTOCs that in turn control the placement and movement of organelles e.g. the nucleus [91]. Microtubules are anchored with their minus-end to the MTOCs and constantly growing and shrinking at the plus-end until they are captured and stabilised at target destinations. During migration, this search-and-capture of microtubules leads to reorganisation of the microtubule network because in a polarised cell the microtubules are selectively stabilised at the cell cortex of the leading edge. The molecular mechanism underlying the capture of microtubules by cortical molecules is not clear. However, not surprisingly the small GTPases cdc42 and Rac are discussed to be involved. Interestingly, it has been shown that microtubules often seem to be guided through the cell and grow right in the direction of adhesion sites. Since this is also true in cells lacking intermediate filaments, it is most likely the actin cytoskeleton that is assisting the microtubule guidance. This is again underlining the importance of crosstalk and interaction between these two cytoskeletal systems. As it is true for the actin filaments, also the polymerisation and depolymerisation of microtubules is controlled by a huge number of different proteins, so called MAPs (microtubule-associated proteins) [99].

While the cell is moving forward it is necessary that the cytoskeleton is not only rearranged at the leading but also at the rear edge of a cell (Fig.5C). Therefore adhesions need to be broken down, actin filaments have to be depolymerised and cross-links need to be loosened [91]. Also the rear edge has to be contracted and moved forward which is

conducted again by actin and myosin and controlled by RhoA [99]. Parts of the membrane, rich in adhesion molecules, are endocytosed. Moreover, some of the connections are simply cleaved by e.g. metalloproteinases (MMPs) and the binding affinity of some adhesion molecules is reduced by modifications such as dephosphorylation. Again small GTPases but also calcium dependent enzymes such as calcineurin are important for rear edge retraction [91].

Finally it should also be noted that during migration a lot of intracellular trafficking is required. Old components are released from the rear edge and are transported to the leading edge wherefore the cytoskeleton is used as 'rail track'. Migration is a very complex process challenging the whole cell and a huge number of proteins are involved in its tight regulation [89].

VSMC Migration and the role of PDGF

Because migration is a pivotal factor in the pathology of atherosclerosis and restenosis the migration of VSMC is intensively investigated. But VSMC migration is not only a hallmark of disease but is also necessary during vascular development. A lot of signalling cascades and effectors of VSMC migration have been identified during the last years but still a lot of open questions remain [36].

As it is true for other cell types, the central role in VSMC migration plays the cytoskeleton and cellular polarisation in response to distinct stimuli. Furthermore, next to reorganisation of the actin cytoskeleton and the activation of actomyosin motors migrating VSMC also remodel their microtubule network. Therefore it is not surprising that the tubulin stabiliser paclitaxel that is used for stent coating (see 2.5.2.4) also inhibits VSMC migration [100]. However detailed studies addressing signalling cascades and microtubules in VSMC migration are missing [98].

Migratory stimuli for VSMC

A panel of growth factors (PDGF, bFGF, IGF-1, VEGF) and cytokines (IL-1, IL-6, TNFα) as well as single components of the extracellular matrix (collagen I, IV, VIII, fibronectin, laminin and others) which are released at the sites of lesions have been linked to VSMC migration. Up to now more than 25 different stimuli for VSMC have been identified [98]. In a healthy vessel wall VSMC are nonmigratory not only because of the lack of promigratory stimuli but also because of the presence of antimigratory signals. These include soluble factors as cytokines or the presence of stable focal adhesions with the surrounding, low

amount of metalloproteinases or higher levels of inhibitors of the latter (tissue inhibitors of metalloproteinases, TIMPs).

VSMC do not only respond to soluble factors but can also move along a gradient of varying adhesiveness due to different components of the ECM (haptotaxis). This is mostly mediated by integrins and associated signalling events [98]. Moreover physical factors such as shear stress can influence VSMC migration. Among the various chemotactic factors, PDGF-BB has been shown to strongly influence neointima formation and is the most potent chemoattractant for VSMC *in vitro*. Moreover gene targeted mice for PDGF or PDGFR do not develop normal blood vessels. However, the exact signalling cascades mediating the migratory response to PDGF-BB or other chemotactic factors in VSMC are not completely understood [36]. The migration of VSMC *in vivo* is determined by complex processes through multiple interacting signal transduction pathways and needs the integration of both soluble signals and changes in matrix attachment. The experimental approaches of this study were focused on PDGF-BB signalling.

Signalling cascades involved in PDGF-BB-induced VSMC migration

Like other moving cells VSMC build different kinds of protrusions during migration, very thin and long ones called filopodia and broader ones called lamellipodia. This requires the reorganisation of the cytoskeleton as already highlighted in a previous chapter. The subsequent polymerisation of the actin cytoskeleton is controlled by ABPs that are largely dependent on the small GTPases Rac, RhoA and cdc42 as well as on calcium release or the accumulation of PIP_2. PDGF-BB binding to PDGFRβ leads to the activation of the three GTPases. Moreover, PLC is activated and hence it comes to PIP_2 accumulation and furthermore calcium release as depicted in Fig.6. Thereupon nucleation events take place mediated by formins and Arp2/3 complexes and new actin filaments are formed (as described in 2.6.1).

Small GTPases in PDGF-BB-induced VSMC migration

There are many reports on Rac-1 in VSMC and its role for migration in response to different stimuli including PDGF-BB. Both Rac and Cdc42 activate members of the PAK kinase family (p21 activated protein kinase) (Fig.6). Depending on cofactors and context, PAKs can either stabilise or destabilise actin as well as microtubules. However, in VSMC the expression of dominant negative PAK potently inhibits migration and PAKs seem to be essential for the formation of lamellipodia [98]. Moreover Rac and cdc42 activate WAVE and WASP, respectively thereby causing nucleation and hence the formation of new actin

filaments. Cdc42 has been shown to be important for the formation of filopodia [101]. RhoA has been shown to activate ROCK1/2 in VSMC and the inhibition of ROCK also leads to impaired migration. ROCK itself influences on the one hand directly the activity of myosin motors and it is found to be important for the formation of stress fibres and focal adhesions on the other hand (Fig.6). ROCK activates a kinase that is also found in VSMC that counteracts actin depolymerisation [101].

STAT3 signalling in PDGF-BB-induced VSMC migration

STAT3 is another immediate early protein that has been implicated in VSMC migration upon PDGF-BB as well as thrombin stimulation [102, 103]. It has been clearly shown to be phosphorylated upon PDGF stimulation but concerning the kinase responsible for this event the data available are contradictory. A number of groups demonstrated an involvement of JAK using siRNA as well as chemical inhibitors and also in a cell free system the activation of STAT3 by PDGF could be blocked by JAK2 blocking antibodies [103-107]. In addition to JAK, Src contributes to STAT3 activation and accordingly STAT3 activity has been reported to be elevated in cells expressing activated Src and abrogated in cells expressing dominant-negative Src [108, 109]. It is also possible that both types of kinases, Src family members as well as JAKs play a role or even act together in phosphorylating STAT3.

It is out of question that inhibition of STAT3 activation or downregulation of STAT3 itself clearly showed a strong inhibitory effect on VSMC proliferation. This is not surprising because STAT3 binds to and activates the cyclin D1 as well as the c-myc promoter and is responsible for the expression of several more proliferative proteins [103, 105]. However, the connection between STAT3 and migration in VSMC is less clear. It has been shown that the cytosolic phospholipase A, a target gene of STAT3 is essential for migration because it produces lipid second messengers that mediate migration [105].

STAT3 has also been shown to be important for the migration of cancer cells and fibroblasts [110-112]. For fibroblasts it is speculated that STAT3 influences RhoA driven stress fibre formation [112]. In studies using carcinoma cells it was suggested that STAT3 target genes such as VASP are responsible for its promigratory actions [111].

All together the data available support an important role of STAT3 in VSMC migration, but more investigations are needed to clarify how exactly it is involved. A final interesting point is that the activation of different STATs by PDGF was found to be significantly inhibited by antioxidants indicating that ROS production contributes to STAT activation in response to

PDGF [113]. Considering the contribution of oxidative stress in the pathology of vascular diseases this should be kept in mind.

Other signalling cascades in PDGF-BB-induced VSMC migration

Calcium release via the PLC/PKC axis (Fig.6) plays a role in VSMC migration, too. It leads to the activation of myosin motors and therefore to contraction and moreover stabilises actin filaments. Accordingly in migrating VSMC oscillating calcium levels have been reported [98].

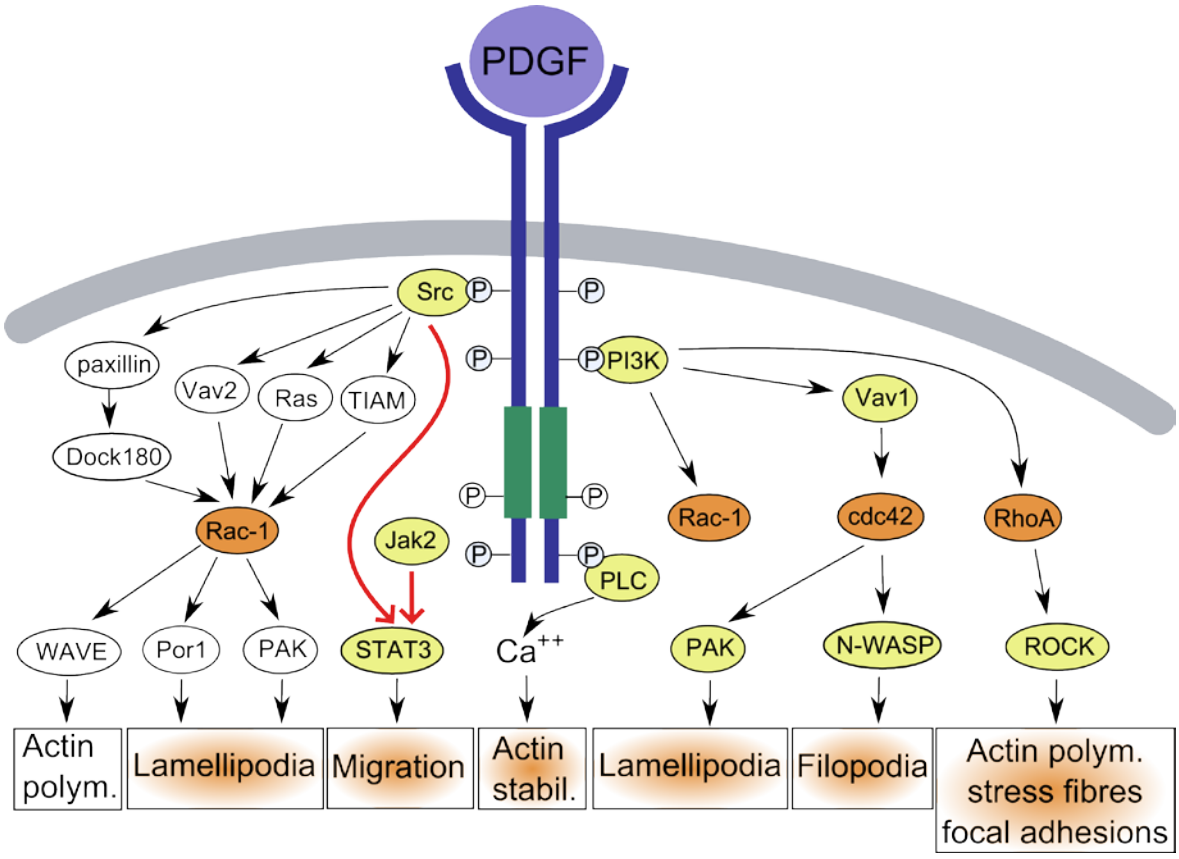

Fig.6. PDGF signalling and migration. *Upon PDGF binding to its receptor a number of signalling cascades are activated. The key regulators of early migratory signalling are the small GTPases Rac, cdc42 and RhoA. They in turn activate effector proteins that lead to the formation of protrusions such as lamellipodia and filopodia or intracellular structures like stress fibres. Furthermore PLC as well as Src kinase are activated leading to calcium release and STAT3 activation, respectively. Also JAK2 has been implicated in STAT3 activation. For abbreviations see section 8.1.*

Moreover Src kinases, the PI3K as well as MAPK have been shown to act on VSMC migration. Many of the migratory stimuli known for VSMC lead to MAPK activation. However, inhibition of single components of the pathways had not such strong effects on migration as inhibition of other signalling pathways. ERK as well as p38 inhibition have

been shown to stop VSMC migration but other reports demonstrated the opposite [74, 101, 114]. It is suggested that ERK activates myosin motors but since many factors do so, a lack of ERK would most likely be compensated. Whether PI3K inhibition interferes with VSMC migration is depending on the stimulus used. Where effects have been observed it has been attributed to impaired microtubule remodelling. PDGF-BB induced migration is not sensitive to PI3K inhibition. Different members of the Src kinase family are expressed in VSMC (Src, Fyn, Yes and Lyn) and have been implicated in migration of the cells. Inhibition of Src impairs VSMC migration most likely at multiple levels because it is involved in FAK activation (see below), Rac activation and provides a link to STAT as well as ERK MAPK signalling [101].

Finally it should be noted that PDGF not only acts immediately on migration but also leads to the expression of other migratory stimuli (such as EGF or FGF-2) and proteins that are later on involved in migration such as the cytosolic phospholipase A [98].

Focal adhesions in VSMC migration

The building and breakdown of focal contacts with the ECM are important during migration. These contacts are less rigid than stable adhesions of nonmigrating cells and are also called nascent adhesions. In VSMC the components of complexes mediating the local adhesion to the substrate contain the focal adhesion kinase (FAK), vinculin, paxillin, tensin, Src and p130. The FAK is a central molecule in these complexes serving as docking site or platform for many different partners but also actively contributing to signalling through its kinase activity. The FAK itself is regulated through multiple tyrosine phosphorylations and it has been found to be upregulated in intimal hyperplasia. PDGF-BB treatment of VSMC *in vitro* leads to a robust activation of FAK especially if the cells are grown on fibronectin [115]. Although the importance of FAK and associated complexes in VSMC migration has been known for some time, the spatial and temporal regulation of its assembly is still not clear. In other cell types it has been demonstrated that formation of nascent contacts requires ligation and subsequent clustering of integrins, phosphorylation of distinct proteins and an intact cortical actin network. Once formed the local contacts serve as traction points for contraction accomplished by actomyosin motors. In VSMC all components necessary for these processes are present but the details of how they are regulated in health and disease are not known [98]. However this is a field of active research, and it has been shown recently that cells growing on different amounts of ECM display different activity of myosin motors and altered traction forces [116].

Roscovitine and derivatives

The history and development of roscovitine

In the early nineties Laurent Mejier and his group screened different purines for protein kinase inhibitors using enzyme-based assays. They reported a widely used plant hormone (cytokinin) isopentenyladenine to be a CDK inhibitor. Furthermore they showed a growth-limiting activity on starfish oocytes (Fig.7) [117]. However it turned out to be a rather unspecific general kinase inhibitor and moreover the IC_{50} for CDK1 was with 45 µM quite high (Table II) [118]. Further screening of available isopentenyladenine analogues for CDK inhibition identified a commercially available antagonist of a plant cytokinin glucosyltransferase later on called olomoucine (Fig.7). It showed first a much lower IC_{50} for CDK1 (7 µM) and second an unexpected selectivity for CDK1/2/5/7/9 without affecting CDK4/6 but still inhibited some other families of protein kinases as well, e.g. MAPKs (Table II) [117]. A follow-up structure-activity study revealed that 2,6,9-trisubstituted purines show the highest activity and selectivity for CDKs [119]. Soon afterwards the first crystal structures of CDK2 with purine inhibitors, namely with isopentenyladenine and olomoucine were published, showing that both bind within the ATP binding pocket [120]. All these findings were especially interesting because they clearly contradicted the dogma that it is not possible to achieve selectivity for protein kinases with inhibitors that compete for ATP binding due to the conserved structure of ATP binding pockets in general [117]. Olomoucine, however, was found to also bind and inhibit the MAPK ERK. It was co-crystallized with this kinase but in an enzyme-based kinase assay it showed a much higher IC_{50} value for ERK (30 µM) than for CDKs (3-10 µM, see Table II) [117, 121, 122]. Further screening of 2,6,9-trisubstituted purines then led to the development of several other more effective analogues of olomoucine. After extensive testings investigators focused on roscovitine (ROSC) that was first introduced in 1996 and has been characterised as even more selective inhibitor of CDK 1,2 and 5 (Fig.7, Table II) [123, 124].

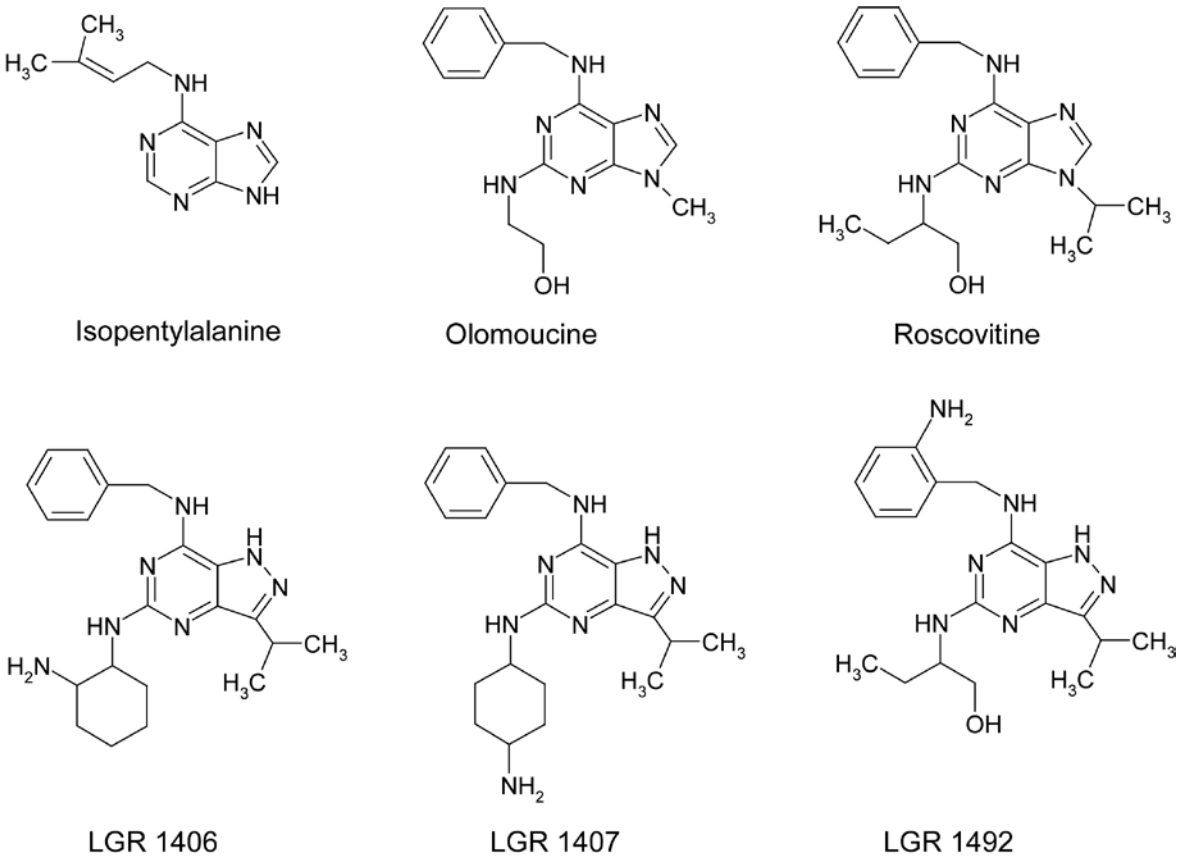

Fig.7. Chemical structures of Isopentylalanine, Roscovitine, Olomoucine and the ROSC-derivatives LGR1406, LGR1406 and LGR1492. *Olomoucine (2-hydroxyethylamino-6-benzylamino-9-methylpurine) as well as ROSC (2-(R)-(1-ethyl-2-hydroxyethylamino)-6-Benzylamino-9-isopropyl-purine) and its derivatives LGR1406 (N-5-(2-Aminocyclohexyl)-N-7-benzyl-3-isopropyl-1(2)H-pyrazolo[4,3-d]pyrimidine-5,7-di-amine)), LGR1407 (N-5-(4-Aminocyclohexyl)-N-7-benzyl-3-isopropyl-1(2)H-pyrazolo[4,3-d]pyrimidine-5,7-di-amine)), LGR1492 (N-5-(1-ethyl-2-hydroxyethylamino)-N-7-(1-amino-2-benzyl)-3-isopropyl-1(2)H-pyrazolo [4,3-d] pyrimidine-5,7-di-amine)) carry a benzylamino-group at the C6 atom of the purine-ring or purine-like ring structure. Olomoucine differs structurally from ROSC and LGR1406 by carrying a methylgroup instead of an isopropyl group at the C9 atom of the purinering. ROSC and its derivatives first differ in the ring structure itself. LGR1406, LGR1407 and LGR1492 have a pyrazolo-4,3-pyrimidine-5,7-di-amine ring as structural basis instead of a purine-ring. Furthermore LGR1406 and LGR1407 differ structurally from ROSC at their side chains at the C2 position where ROSC shows an ethyl and a hydroxymethyl group whereas the derivatives show a 2'-amino-cyclohexane ring.*

Selectivity of roscovitine

Out of 150 kinases tested ROSC inhibits almost exclusively CDKs [117]. However, ROSC, like olomoucine, is not solely acting on CDKs but also inhibited ERK in enzyme-based

assays. Moreover it was co-crystallized not only with CDK2 but also with a kinase involved in vitamin B6 metabolism (pyridoxalkinase, PDXK) [125, 126]. Additionally it should be noted that for some kinase assays published, ROSC was used in 10 µM as maximal concentration. Therefore it still might be that it also inhibits some other kinases with higher IC_{50}s [127].

Enzyme IC50 [µM]

kinase	Roscovitine *, **	Olomoucine °	Isopentenyladenine °
CDK1/ cyclin A	not tested *, **	50	no tested
CDK1/ cyclin B	23 *, 0.65 **	7	45
CDK1/ cyclin E	not tested *, **	10	not tested
CDK2/ cyclin A	1.6 *, 0.7**	7	50
CDK2/ cyclin E	1.1 *, 0.7 **	7	not tested
CDK4/ cyclin D	75 *, >100 **	>1000	200
CDK5/ p35	not tested*, 0.16**	3	80
CDK6/ cyclin D	51*, >100 **	>250	>100
ERK1 (p42)	not tested*, 34**	not tested	not tested
ERK2 (p44)	not tested*, 14**	30	95
PKA	not tested*, >1000**	>2000	50
PKG	not tested*, >1000**	>2000	50
AMPK	not tested *, **	>250	16

* Proqinase data, Bach et al, 2005 [126]
** Knockaert et al, 2000 [125]
° Vesely et al, 1996 [119]

for abbreviations see section 8.1

Table II. IC_{50} values obtained from enzyme-based kinase assays on roscovitine, olomoucine and isopentenyladenine [119, 127, 128]

Today's and future use of roscovitine

Today ROSC is widely used in cell culture experiments to inhibit CDKs but also for cell synchronisation during cloning procedures for e.g. cattles, horses and pigs [129-131]. As blocking CDK activity seems to be a promising target to combat pathological cell proliferation, it is not surprising that research focused on the development as an anti-cancer drug. ROSC is marketed as Seliciclib or CYC202 and is in phase II clinical trials against breast cancer [132]. However, ROSC shows a rather high IC_{50} value concerning its antiproliferative activity (about 25 µM depending on cell type) and therefore several

groups work on the development of better analogues. Recently some publications introduced new ROSC-derivatives, exerting even stronger anti-cancer effects [133, 134].

Literature on roscovitine

ROSC has been studied in a panel of different cell types but most of the studies focus on cancer cell lines. When searching the Pubmed data base in March 2009 for original research articles using ROSC (or Seliciclib, CYC202) within the title about 60 out of 150 articles deal with cancer. In 33 publications other cell types were investigated and another 20 describe mechanistic studies or provide data about metabolism of ROSC (Table III). The remaining articles deal with inhibition of viral replication (nine) or use ROSC for ameliorating cloning strategies (30). Although some publications on non-cancer cells are available, the number of different cell types investigated is limited because most of these papers deal with either proliferative/ inflammatory renal diseases (nine) or neuronal cells (15). The last nine articles out of this group cover fibroblasts, keratinocytes, macrophages and tobacco cells. A single publication investigated the influence of ROSC on VSMC. It should be noted that ROSC is mentioned in a huge number of publications (a pubmed search for ROSC gives out 625 articles) where it is used as 'specific' inhibitor of CDKs in control experiments.

topic	type of studies	no. of publications
cancer	*in vivo, in vitro* and clinical studies	60
cloning	*in vivo, in vitro* studies	30
mechanistics/ signalling	*in vitro, in silico* studies, crystallography	12
renal disease	*in vivo, in vitro* studies	9
viral replication	*in vivo, in vitro* studies	8
neuronal system	*in vivo, in vitro* studies	8
metabolism and pharmacokinetics	*in vivo, in vitro* studies	8
ROSC analogues	*in vitro* studies	4
others	*in vivo, in vitro* studies	12
		151

Table III. Pubmed search for roscovitine/ Seliciclib or CYC202 mentioned within the title, March 2009

Roscovitine and cancer

In cancer cells ROSC either leads to apoptosis or elicits G2/ M arrest [132]. Depending on the cell type and experimental conditions the IC_{50}s of ROSC for growth inhibition vary from 8-40 µM. The majority of publications report an IC_{50} of about 20 µM for growth inhibition but in many cells this concentration already leads to apoptosis. Therefore the right

concentration has to be determined for each cell type. In human colon cancer cells ROSC treatment led to a moderate increase in G2/M phase, decreased both retinoblastoma protein phosphorylation and total retinoblastoma protein but increased the phosphorylation of ERK1/2. However, the latter did not contribute to the cell cycle effects of the drug. Furthermore a clear reduction in cyclins D1, A, and B1 mRNA and a reduced expression of cyclin D1 that was independent of the p38 and PI3K pathways were observed. Also a loss of RNA polymerase II phosphorylation as well as total RNA polymerase II protein was reported suggesting that ROSC was inhibiting transcription, possibly via inhibition of CDK7 and CDK9 complexes [135]. In human breast cancer cells ROSC leads to a G2 arrests and concomitantly induces apoptosis. The induction of apoptosis was explained by the stabilization and activation of p53 protein through phosphorylation at Ser-46 which in turn initiates caspase-independent apoptosis [136]. This is especially interesting because a lot of breast cancer cell lines lack caspase-3 [137]. But other studies suggested CDK5 to be the molecular target of ROSC-triggered apoptosis of highly invasive breast cancer cells [138, 139]. On the other hand an upregulation of p53 was not only found in ROSC-treated breast cancer cells but also in cervix cancer cells (HeLa cell line) [140]. All together it is so far not clear how exactly ROSC elicits apoptosis and most likely this has to be investigated separately for each cell type. The effects on cell cycle distribution, however, can easily be explained by CDK inhibition but it is not clear why ROSC preferentially leads to G2/M and not to G1 arrest as it most potently inhibits CDK2 not CDK1 (Table II, Fig.4).

Roscovitine and other cell types

As already mentioned the influence of ROSC has not only been studied in cancer cells but also in other cell types. Jhou et al. found that ROSC limits growth and diminishes nitric oxide production as well as cytokine gene expression in lipopolysaccharide-stimulated macrophages. They suggest that ROSC-induced cell cycle arrest is capable of reducing pro-inflammatory responses via down-regulation of NF-kappaB [141]. In normal human fibroblasts ROSC induced a dose-dependent arrest in G1 phase accompanied by a significant reduction of hyperphosphorylated retinoblastoma protein and CDK2 activity whereas levels of cyclin D1, and cyclin E were not affected [142]. ROSC also inhibited proliferation of human epidermal keratinocytes in a dose-dependent manner and at higher concentrations it induced apoptosis evidenced by caspase-3 activation [143]. Moreover one group showed antiproliferative effects of ROSC on VSMC and claimed that ERK inhibition is responsible but there are some major concerns about this paper that will be discussed later on (section 5.1) [144]. An additional publication used ROSC with VSMC but did not focus on mechanistic questions and just used it as control [145]. It can be concluded that the

few data available on non-cancer cells show that ROSC also inhibits proliferation of normal cells and suggest that this is also mainly due to CDK inhibition.

Besides its growth limiting activity a couple of publications showed that ROSC additionally exerts effects on calcium and potassium channels [146]. For example Ganapathi et al. showed that ROSC inhibits distinct potassium channels that act as a delayed rectifier in cardiac myocytes [147]. Moreover it has been reported that ROSC impairs L-type calcium channel activity and has in this regard been suggested for the treatment of cardiac arrhythmias and hypertension [148]. But ROSC not only affects cardiac channels but additionally slows down deactivation of neuronal calcium channels and therefore could cause side effects [149].

Roscovitine derivatives

ROSC shows a rather high IC_{50} for growth inhibition and there are concerns about side targets as e.g. ERK MAPK, therefore more effective and specific derivatives should be developed. As already mentioned recently new ROSC-derivatives have been introduced. In cooperation with Prof. Strnad (Olomouc, Czech Republic) new ROSC-derivatives have been synthesized. These differ from ROSC in the purine structure itself as well as regarding their side chains. The purine rings of ROSC have been replaced by a pyrazolo-pyrimidine structure (for depicted structures see Fig.7). In addition some of the derivatives possess an aminocyclohexyl-group instead of an ethyl-2-hydroxyethylamino group.

In conclusion ROSC has been extensively studied in cancer cells and there are also a lot of data available on its metabolism and pharmacokinetics. Because of its growth-limiting activity ROSC could be a promising compound for stent coating. However, its effects seem to be cell type specific and conclusive studies on its action on VSMC are missing.

Indirubine-3’-monoxime

Source and traditional use

Indirubin-3'-monoxime (I3MO) is a derivative of indirubin which is an active component from a recipe of traditional chinese medicine called Danggui Longhui Wan used for the treatment of various diseases in particular chronic myelogenous leukaemia but also as antiinflammatory drug [150]. The red-coloured indirubin is found in indigo naturalis (Qing Dai), a powder obtained from the leaves of several plants of various genera including Indigofera tinctoria (Fabaceae) or Isatis indigotica (Brassicaceae) [151]. However, the major constituent

of indigo naturalis is not indirubin (3,2'-bisindole) but its dark blue 2,2'-isomer indigo (Fig.8).

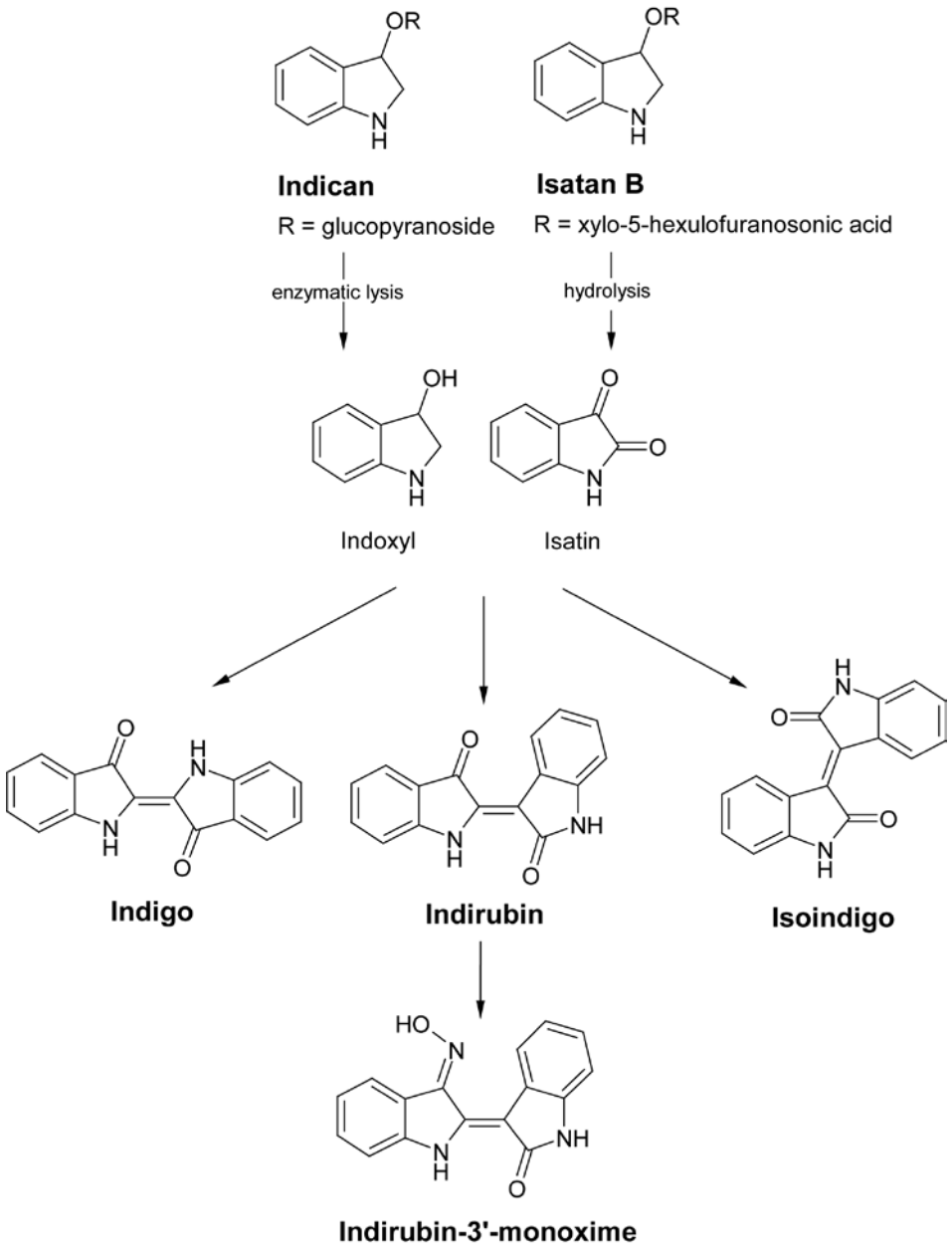

Fig.8. Chemical structures of indirubin-3'-monoxime, indirubin, indigo and its precursor molecules indican and isatan B. *The precursor molecules indican and isatan are enzymatically cleaved or undergo hydrolysis and the resulting substances indoxyl and isatin dimerize to form indigo, isoindigo and indirubin, respectively. Indirubin-3'-monoxime is a common synthetic derivative of indirubin* ***(figure is adopted from [157])***

Although it is only a minor by-product, the biological actions of Qing Dai have been attributed to indirubin. Both compounds are not found in fresh leaves but are converted by enzymatic or acidic lysis and consequent dimerisation of colourless precursor substances (indican, isatan B, Fig.8) [152, 153]. Interestingly indigo as well as indirubin have been also

found in various molluscs and bacteria and in human urine where it seems to be a by-product of tryptophan catabolism [154]. As indirubin itself displays a low aqueous solubility and absorption and furthermore leads to gastrointestinal toxicity, a huge number of derivatives have been introduced during the last years. These include halogenated and alkylated indirubins or indirubin-N'-glycosides [155, 156]. Among those derivatives indirubin-3'-monoxime (I3MO) is the best characterised and most widely used and is one of the few commercially available derivatives (Fig.8)[154].

Cellular effects of I3MO

Indirubin is known to exert anti-cancer function by either inducing G2/M-arrest or apoptosis. The latter was shown for a variety of different types of human cancer cell lines such as cervical cancer, leukaemia, laryngeal carcinoma, hepatoma and colon cancer cells [150, 158]. But also anti-apoptotic effects have been demonstrated in cerebellar granule neurons [159]. Furthermore indirubin inhibited inflammatory reactions of murine splenocytes and in a mouse model of delayed-type hypersensitivity [160]. Moreover I3MO has been reported to be effective against replication of wild-type and drug-resistant strains of HIV-1 [161]. All together there have been a number of biological activities contributed to indirubin (and derivatives) many of which seem to be cell type specific [162].

Molecular targets of I3MO

The molecular mechanism of action is, however, not completely understood so far. Also it seems that indirubin has probably more than one single target in a cell or that the targets differ between cell types and/or species. In *in vitro* kinase assays it has been shown to inhibit GSK-3, CDK1/5, AMPK, LCK and SGK and it is commercially marketed as GSK3 and CDK inhibitor [163-165]. Also in cell based assays it has been demonstrated that I3MO inhibits CDKs [166], glycogen synthase kinase-3β (GSK-3β) [166], JNK [159], Src [167] and FGFR1 autophosphorylation [168] and furthermore suppresses activation of nuclear factor-kappa B (NF-κB) [169] as well as STAT3 [167]. But next to these inhibitory effects I3MO activates the co-transcriptional factor aryl hydrocarbon receptor (AhR) [170] as well as p38 MAPK [168]. Moreover the induction of apoptosis in cancer cells has been shown to be mediated through the activation of caspase-3 and caspase-7 [171].

Cell cycle arrest observed upon indirubin treatment can easily be contributed to CDK inhibition but some investigators state different mechanisms. For example Zhen et al. claim that inhibition of myeloid leukaemia cell proliferation by I3MO is solely due to inhibition of FGFR1 autophosphorylation [168]. Sethi et al. on the other hand suggest that anti-cancer and anti-inflammatory activities of indirubin may be at least in part mediated

through the suppression of the NF-κB activation because they observed inhibition of activation of IκBα kinase in several cancer cell lines [169]. The antiinflammatory activity of I3MO could also be explained by the binding to AhR. AhR plays a role in proliferation and differentiation during development, is involved in the recognition and response to antigenic substances and can down-regulate inflammatory responses [170]. The anti-apoptotic effects on cerebellar granule neurons have been linked exclusively to JNK inhibition because other inhibitors of CDKs and GSK-3β were ineffective. In macrophages, however, I3MO had not the same effect as well established JNK inhibitors [159, 172].

Concerning the inhibition of cancer cell growth by I3MO also STAT3 was suggested to play an important role at least in breast and prostate cancer cells [167]. This idea is further supported by the fact that STAT3 is constitutively active in many tumor cells and is activated by the oncogenic kinase Src [173]. Nam et al. demonstrated that different indirubin derivatives inhibited Src activity and the expression of anti-apoptotic target genes of STAT3 [173].

Summarising, I3MO exerts various effects on different cell types and directly or indirectly affects a number of proteins. But although several targets have been identified in *in vitro* assays the direct link between cellular effects and molecular targets is often missing.

Aims of the study

Characterisation of Roscovitine derivatives for anti-proliferative effects in VSMC

Within the framework of the EU project 'Pro-Kinase-Research' we were looking for new anti-atherogenic compounds. We focused on CDKs and other protein kinases as potential drug targets. Preliminary screens had revealed that several ROSC-derivatives could limit the growth of rat aortic VSMC. As discussed in chapter 2.8 ROSC is known to act as a CDK inhibitor [123], but most of the publications only show *in vitro* data and there are only two publications mentioning effects on VSMC [144, 145]. None of them solved the mechanism of action of ROSC satisfyingly. We therefore wanted to investigate the mode of action of Roscovitine and promising derivatives in PDGF-stimulated VSMC and define the changes in cell signaling. We planed to examine the effect of Roscovitine and its derivatives on mitogen activated kinases, cell cycle parameters and the effect on VSMC migration towards PDGF. As discussed in section 2.2.1 an ideal antiatherogenic compound should impair both proliferation and migration of VSMC.

Influence of Indirubine-3'-monoxime on VSMC migration

In her PhD Thesis Andrea Schwaiberger [154] showed that I3MO potently inhibited PDGF-BB-induced VSMC cell cycle progression (Fig.9). More precisely she showed that I3MO reversibly inhibited PDGF-induced VSMC cell cycle progression in a concentration-dependent manner, leading to a G0/G1-phase arrest. Accordingly, a reduction in Rb hyperphosphorylation was found. However, effects on cyclin D1 levels were inconclusive. Furthermore it was demonstrated that I3MO impaired PDGFR autophosphorylation on a residue essential for tyrosine kinase activity and specifically inhibited the activation of STAT1/3/5/6 without affecting other common early signalling pathways (p38, ERK, Akt) (Fig.9). The work further focused on STAT3 and showed that I3MO inhibited the activation and subsequent nuclear accumulation of this transcription factor. Due to these results it was suggested that the effect on STAT3 may be at least partially responsible for the antiproliferative activity of I3MO. Additionally, Andrea Schwaiberger showed that the effect on STAT3 was stimulus-independent since I3MO prevented phosphorylation of STAT3 in response to IFN-γ as well as H_2O_2. Moreover, an antioxidative effect of I3MO was demonstrated because it attenuated the PDGF-induced generation of ROS indicating an impact on redox-regulated signal transduction in VSMC (Fig.9). Furthermore it was shown that I3MO attenuated neointimal hyperplasia in an experimental animal model (mouse

femoral cuff-model, in cooperation with Prof. Dr. Binder, Medical University, Vienna). The studies indicated that I3MO is a promising compound for e.g. stent coating but left some open questions regarding its molecular target(s). As both proliferation and migration contribute to neointima-formation we not only wanted to further investigate the underlying molecular mechanism of the antiproliferative effect of I3MO but also its potential to influence PDGF-BB-induced migration of VSMC.

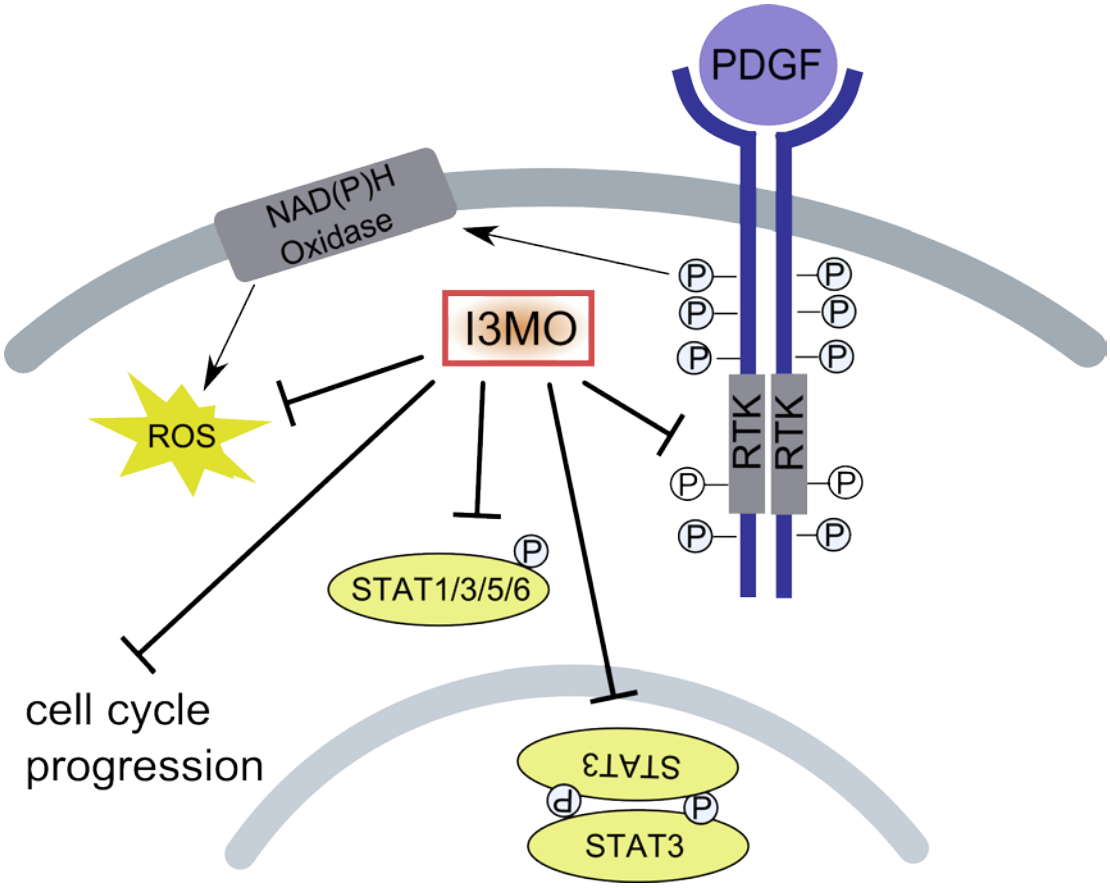

Fig.9. Molecular actions of I3MO in the context of PDGF-induced signal transduction and proliferation as shown by Andrea Schwaiberger, PhD thesis 2008 (figure is adopted from [154])
I3MO slightly inhibits PDGF-BB-induced autophosphorylation of the PDGFRβ and strongly impairs the ROS generation caused by PDGF-BB stimulation. The treatment of VSMC with I3MO completely abrogates the phosphorylation of STAT1/3/5/6 upon PDGF-BB stimulation as well as the translocation of STAT3 to the nucleus. For abbreviations see section 8.1.

3. MATERIALS AND METHODS

Materials

Unless noted otherwise all cell culture reagents and media were obtained from Lonza Group Ltd. (Basel, Switzerland) and all other reagents from Carl Roth (Karlsruhe, Germany). Trypsin, collagenase II and HAM's F-12 medium were purchased from Invitrogen (Carlsbad, CA, USA) and DMSO from Fluka (Buchs, Switzerland). Roscovitine was obtained from Calbiochem (La Jolla, CA, USA) and recombinant human PDGF-BB from Bachem (Weil am Rhein, Germany). Laurent Mejier (CNRS, Roscoff, France) kindly provided indirubine-3'-monoxime and Miroslav Strnad (Laboratory of Growth Regulators, Olomouc, Czech Republic) kindly provided the Roscovitine derivatives.

Protein inhibitors	**Sold as inhibitor of**	**Conc. used**	**Provider**
R-Roscovitine	CDK1/2/5	0.3 – 40 µM	CAL
Roscovitine derivatives	not marketed	0.3 – 100 µM	STRNAD
DPI	flavoproteins	3 µM	SIGMA
AG490	JAK2	10 – 50 µM	CAL
SU6656	Src	2 µM	CAL
Indirubine-3'-monoxime	CDKs, GSK3	1 – 7 µM	MEIJER
LiCl	GSK3	10 – 20 mM	ROTH
Other inhibitors	**Mode of action**	**Conc. used**	**Provider**
Mitomycin C	DNA cross linker S-phase inhibitor	2.5 nM – 25 µM	CAL
Staurosporine	Inducer of apoptosis	25 – 50 nM	CAL

CAL	Calbiochem (La Jolla, CA, USA)
STRNAD	Miroslav Strnad (Lab of Growth Regulators, Olomouc, Czech Republic)
SIGMA	Sigma Aldrich (MO, USA)
MEJIER	Laurent Mejier (CNRS, Roscoff, France)
ROTH	Carl Roth (Karlsruhe, Germany)

for additional abbreviations see section 8.1

Table IV. Chemical inhibitors used for the study.

Cell Culture

Buffers and solutions

PBS-Phosphate buffered saline:

123 mM NaCl, 12.3 mM Na2HPO4, 3.2 mM KH2PO4, pH 7.4

PBS+:

137 mM NaCl, 9.6 mM Na2HPO4, 1.5 mM KH2PO4, 0.5 mM MgCl2 x 6 ddH_2O, 0.5 mM CaCl2 x 2 ddH_2O pH 7.4

Trypsin/ EDTA:

0.05% Trypsin, 0.02% EDTA

Standard medium:

DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% calf serum

Starvation medium:

DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1% calf serum

Freezing medium:

DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 30% calf serum, 10% DMSO

Digestions buffer:

HAM's F12 medium supplemented with 253 U/ml collagenase II, 10 mM HEPES, 0.28 mM ascorbic acid, 0.1% BSA

Isolation of rat aortic VSMC

VSMC employed in this study originate from two independent isolations from rat thoracic aortas of 3 sibling Sprague-Dawley rats each. Aortas were washed in PBS+, detached from surrounding fat tissue, cut open and incubated with digestion buffer for 15 min at 37°C. Aortas were thoroughly cleaned from surrounding tissue, washed with digestion buffer, cut into small pieces and incubated for 3 h in digestion buffer at 37°C. After centrifugation for 10 min at 230xg the supernatant was discarded and cells were incubated with standard medium in a humidified incubator at 37°C, in the presence of 5% CO_2. Purity of VSMC cultures was checked by immunostaining of α-smooth muscle actin with a FITC-labled antibody. Early passages (3-6) were frozen in liquid nitrogen.

Smooth muscle alpha actin staining

Cells were seeded at a density of 0,2 x 10^6/ well on to coverslips in 12-well plates for 24 h and washed once with ice-cold PBS. Cells were fixed with 1 ml of 1:1 acetone/ MeOH for

15 min on ice and washed twice with ice-cold PBS. Coverslips were incubated with 20 µl of antibody solution (SMA-antibody 1:250 in PBS supplemented with 1% of BSA) for 60 min in a dark and humified chamber at 4 °C. Afterwards the coverslips were washed twice with PBS and mounted on a glass slide using mounting medium (Sigma Aldrich, MO, USA). After drying overnight in the dark the specimens were analysed using a fluorescence microscope (Olympus BX51, Olympus Europe GmbH, Hamburg, Germany) and cell imaging software (cell^F software, Europe GmbH, Hamburg, Germany).

Culture of VSMC

VSMC were cultured in a humidified incubator at 37°C, 5 CO_2 using standard medium. For passaging the cells were washed once with PBS and incubated with trypsin/ EDTA for 3-5 min at 37 °C. The reaction was stopped using standard medium and cells were centrifuged for 4 min at 1400 rpm (373 x g). Cells were counted with a trypan-blue staining based cell viability analyser (Vicell^{TM} XR, Beckman Coulter, Fullerton, CA, USA), seeded at the indicated density and after 24 h serum-starved for another 24 h prior to all experiments. Passages 6-14 were used.

Storage of VSMC

Cells were frozen in aliquots of 1 x 10^6 cells each in liquid nitrogen using freezing medium and the following freezing protocol. Cells were kept at 4 °C for 20 min, afterwards at -20 °C for 24 h and at -80 °C for 2-3 days and were then kept in liquid nitrogen. For thawing cells were put into 15 ml of standard medium, centrifuged for 4 min at 1400 rpm (373 g) and put again into fresh standard medium.

Protein extracts

Buffers and solutions

Lysis buffer:

50 mM HEPES, 50 mM NaCl, 50 mM NaF, 10 mM $Na_4P_2O_7x10H_2O$, 5 mM EDTA, pH 7.5 prior to usage complemented with 1% Triton X, 1x complete^{TM} and 1 mM PMSF (0.1 M stock diluted in isopropanol)

3xSDS sample buffer:

0.1875 M Tris HCl pH 6.8, 0.2 M SDS, 3% Glycerol, 0.2 mM Bromophenol blue with 15% 2-Mercaptoethanol

Protein isolation

VSMC were seeded into 60 mm dishes (1 x 10^6 cells/dish), serum-starved, pretreated with test drugs or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (20 ng/ml). Cells were washed with ice-cold PBS, incubated with lysis buffer for 10 min at 4°C and centrifuged for 10 min, 16,000xg at 4 °C. Supernatants were mixed with 3xSDS sample buffer and heated to 95 °C for 5 min.

Measurement of protein concentration (Bradford)

The assay is based on the shift of the absorbance maximum of Commassie Brilliant Blue G-250 from 470 nm to 595 nm when binding to basic or aromatic amino acids. Therefore the absorbance at 595 nm is a measure for protein concentration (after Bradford [174]). Protein concentration was determined using Rotiquant© reagent according to manufacturer's instructions (Carl Roth, Karlsruhe, Germany). Briefly Rotiquant reagent was diluted 1:4.75 with ddH2O and aliquots of protein samples were diluted 1:10. In a 96 well plate 190 µl of diluted Rotiquant were mixed with 10 µl of diluted protein sample and optical density at 595 nm was determined using a 96-well plate reader (TECAN Sunrise™). The measurements were performed in triplicate and for quantification a linear calibration curve was determined using different concentrations of BSA (50 – 500 µg/ml).

Rac-1 activity assay

Rac-1 belongs to the protein family of small GTPases and is therefore active when bound to GTP. Only GTP-bound Rac-1 is able to bind to the p21-binding domain (PBD) found in p21-activated protein kinase (PAK). The assay is based on binding of active Rac-1 to PAK-PBD domains coupled to agarose beads. We used a commercially available kit including PAK-PBD-agarose beads, lysis buffer and a monoclonal antibody against Rac-1 (Cell Biolabs, San Diego, CA, USA).

VSMC were seeded into 60 mm dishes (1 x 10^6 cells/dish), serum-starved, pretreated with test drugs or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (20 ng/ml) for 3 min. Samples were processed in the 4 °C room. Cells were washed with ice-cold PBS, incubated with lysis buffer for 1 min at 4°C and immediately added to 20 µl of PAK-PBD-agarose beads. The samples were incubated on a rotator at 4 °C for 45 min. Samples were centrifuged for 10 min, 16,000xg at 4 °C and washed three times with lysis buffer. Supernatants were discarded and the beads were mixed with 40 µl of 3xSDS sample buffer and heated to 95 °C for 5 min. Samples were subjected to a standard western blotting procedure (see section 3.5.).

SDS page and western blotting

Buffers and solutions

Stacking gel:

5% final conc. of PAA (stock solution: 30% polyacrylamid, 0.8% bisacrylamid), 125 mM Tris HCl pH 6.8, 0.1% SDS, freshly prepared and just prior to usage complemented with 0.1% TEMED and 0.05% APS.

Resolution gel:

7,5-12% final conc. of PAA (stock solution: 30% polyacrylamid, 0.8% bisacrylamid), 375 mM Tris HCl pH 8.8, 0.1% SDS, freshly prepared and just prior to usage complemented with 0.1% TEMED and 0.05% APS.

Electrophoresis buffer (10x):

250 mM Tris base, 1,92 M Glycine, 35 mM SDS

Blotting buffer (5x):

125 mM Tris base, 0,97 M Glycine

prior to usage diluted 1:5 with ddH_2O and complemented with 20% Methanol

TBS-T (10x):

25 mM Tris base, 190 mM NaCl, 0,1% Tween-20 (add after pH adjustment), pH 8.0

Home made ECL:

0.1 M Tris base pH 8.5, 1.25 mM luminol (250 mM stock solution in DMSO), 0.2 mM p-coumaric acid (91 mM stock solution in DMSO) freshly prepared and just prior to usage complemented with 0.009% H2O2 (30% stock)

Antibodies

Target	Source	Dilution used	Provider
α-smooth muscle actin, FITC labled	Mouse, mc	1:250	SIGMA
α-tubulin	Mouse, mc	1:2500	SC
phospho Akt Ser473	Rabbit, pc	1:1000	NEB
c-myc	Rabbit, pc	1:1000	NEB
Cyclin A	Rabbit, pc	1:1000	SC
Cyclin D1	Mouse, mc	1:1000	NEB
Cyclin E	Rabbit, pc	1:1000	SC
Cytosolic Phospholipase A2 (cPLA2)	Rabbit, pc	1:500	NEB
phospho Erk1/2 Tyr202/204	Rabbit, pc	1:1000	NEB
phospho JNK	Rabbit, pc	1:5000	PROM
phospho p38	Rabbit, pc	1:1000	NEB
p107	Rabbit, pc	1:1000	SC
Rac-1	Mouse, mc	1:2500	CELLBIO
Phospho-Retinoblastoma protein Ser807/811	Rabbit, pc	1:1000	NEB
Phospho-STAT3	Rabbit, pc	1:1000	NEB

NEB	New England Biolabs (Beverly, MA, USA)
SC	Santa Crz (Santa Cruz, CA, USA)
SIGMA	Sigma Aldrich (St. Louis, MO, USA)
PROM	Promega (Mannheim, Germany)
CELLBIO	Cell Biol abs (San Diego, CA, USA)

pc, polyclonal; mc, monoclonal; for additional abbreviations see section 8.1

Table V. Antibodies used for the study.

Procedure

Stacking and resolution gels were poured into gel chambers (Biorad Laboratories, Hercules, CA, USA) and after loading samples and prestained protein marker (Precision Plus Protein Standard, Laboratories, Hercules, CA, USA) put into electrophoresis chambers filled with electrophoresis buffer (Mini-Protean^{TM}, Biorad Laboratories, Hercules, CA, USA). SDS page was run at constant voltage of 80 V for 20 min and at 120 V until the

loading dye front reached the bottom of the chamber. For blotting, gels were put onto Immun-Blot™ PVDF membranes (Biorad Laboratories, Hercules, CA, USA) covered with filter paper (Whatman plc, Kent, UK) and placed into gel holder cassettes (Mini-Trans-Blot™, Biorad Laboratories, Hercules, CA, USA). The cassettes were put into cooled blotting chambers and run at constant current of 110 mA/ gel for 120 min. Membranes were washed three times with TBS-T for 10 min, blocked with 5% milk powder in TBS-T for 60 min and incubated with primary antibody-solutions (in 2% BSA in TBS-T) overnight at 4 °C. Membranes were washed three times with TBS-T for 10 min, incubated with secondary antibody-solutions (in TBS-T) and washed again three times with TBS-T for 5 min. For detection, home made ECL was prepared freshly and added to the membranes which were incubated for 3-5 min prior to signal detection with a luminescent image analyser (LAS-3000™, Fujifilm, Tokyo, Japan).

Viability measurement – trypan blue

VSMC were seeded into 6 well plates (0.5 x 10^6 cells/ well), serum-starved, pretreated with test compounds or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (20 ng/ml). After 24-48 h cells were trypsinized with 300 µl trypsin/ EDTA and the reaction was stopped using 1 ml of standard medium. Cells were centrifuged and the pellet was dissolved in 600 µl of standard medium. Cell viability and number were evaluated with a trypan-blue staining based cell viability analyser (Vicell™ XR, Beckman Coulter, Fullerton, CA, USA). Measurement parameters were the following: min. diameter 5 µM; max. diameter 35 µM; cell sharpness: 75; cell brightness: 85; viability spot brightness: 70%; viability spot area: 5%, at least 300 cells have been counted for each measurement.

Propidium iodide staining and flow cytometry

Cell cycle distribution of VSMC was determined after propidium iodide (PI) staining according to Riccardi and Nicoletti [175]. The assay is based on the different amount of DNA during each cell cycle phase and the binding of propidium iodide to double stranded DNA. When excited by laser light (488 nm), propidium iodide can be detected with 562-588 nm filters. Therefore the intensity of fluorescence at 588 nm is a measure for DNA concentration and doubles from G1 to G2 phase ().

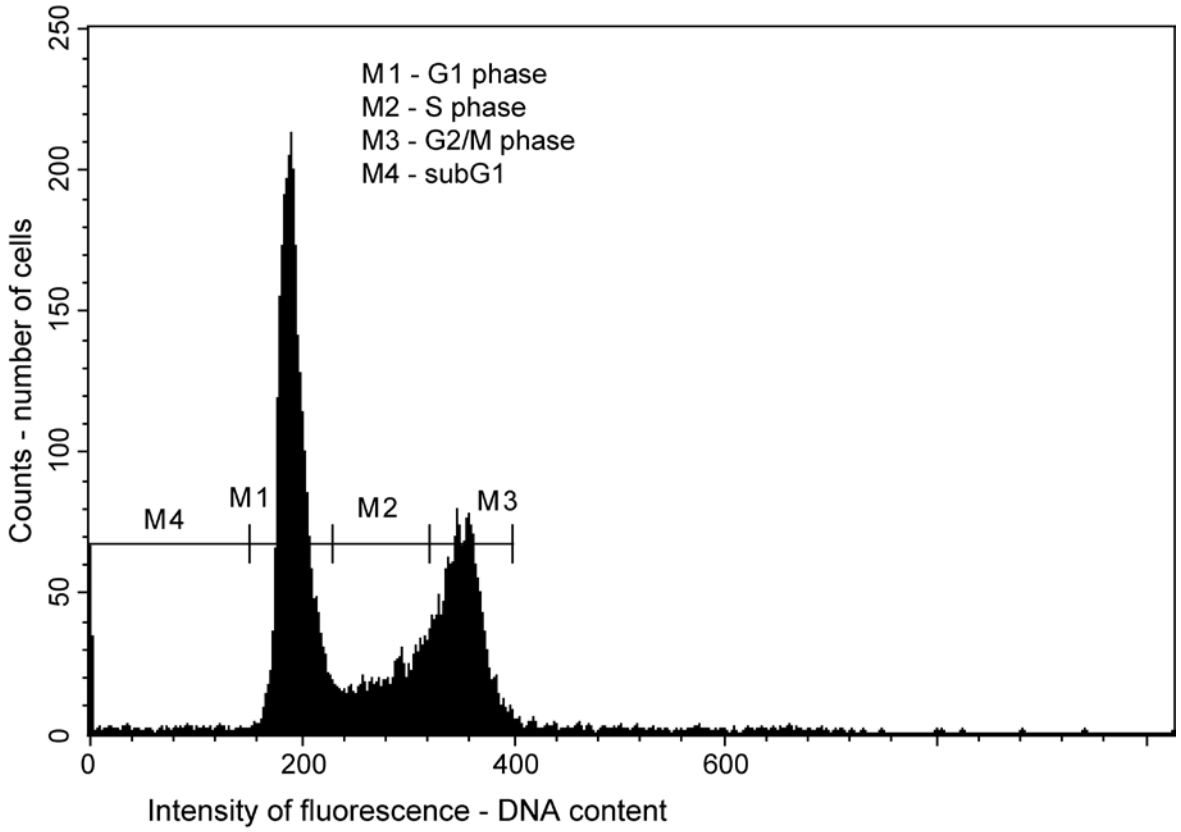

***Fig.10. Propidium staining**. The flow cytometer determines the fluorescence intensity of 10,000 single cells. The histogram shows an example of cycling VSMC. Since the fluorescence intensity is proportional to the amount of DNA in a single cell it can be concluded that cells within the 'M3' gate have the double amount of DNA compared to cells within the 'M1' gate. Therefore 'M1' reflects cells in G1 phase with a single copy of the genome, 'M3' depicts diploid cells in G2/M phase and 'M2' includes the cells in S-phase with an intermediate DNA content. Cells that have less than a single copy of the genome are found in 'M4' (also called sub-G1) and are generally thought to reflect dead or dying cells because they obviously lost DNA material.*

VSMC were seeded into 12 well plates (1 x 10^5 cells/ well), serum-starved, pretreated with test compounds or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (20 ng/ml). After 8-28 h cells were trypsinized, fixed with cold HFS buffer (0.1% (w/v), sodium citrate 0.1% (v/v) Triton X-100 in PBS) containing 50 µg/ml PI and incubated for 2 h at 4 °C. 10,000 cells were analysed by flow cytometry on a FACSCalibur™ (BD Bioscience Pharmingen, San Diego, CA, USA).

FACS buffer:

139 mM NaCl, 20 mM Na2HPO4, 2 mM KH2PO4, 3.8 mM KCl, 10 mM LiCl, 3.1 mM NaN3, 1 mM Na2EDTA, pH 7.4

Crystal violet staining

The assay is based on binding of crystal violet to DNA, therefore staining cell nuclei. The optical density can be measured at 595 nm. Therefore the absorbance at 595 nm is a measure for cell density in the well.

VSMC were seeded into 96 well plates (2 x 10^4 cells/ well), serum-starved, pretreated with test compounds or vehicle (DMSO 1%) for 30 min prior to stimulation with PDGF-BB (20 ng/ml). After 48 h cells were extensively washed with PBS, immediately fixed and stained with crystal violet staining solution (0.5% (m/v) crystal violet in 20% MeOH) for 15 min at room temperature. Plates were washed, dried and the dye was re-solubilised in ethanol/sodium citrate solution (50% (v/v) sodium citrate in ethanol). Optical density was determined using a 96-well plate reader (TECAN Sunrise™, Mannedorf, Switzerland).

5-Bromo-2-deoxyuridine (BrdU) assay

As a measurement of DNA synthesis BrdU incorporation was determined using a cell proliferation kit (Roche Diagnostics, Basel, Switzerland). The assay is based on incorporation of the thymidine-analogue 5-bromo-2-deoxyuridine (BrdU) during DNA synthesis. BrdU can be detected using a monoclonal antibody coupled to horse radish peroxidase (HRP) which oxidises luminol and thereby emits blue light. The luminescence is a measure for the amount of incorporated BrdU and therefore DNA synthesis.

VSMC were seeded into black 96 well plates (2 x 10^4 cells/ well), serum-starved for two days (with two PBS-washing steps and change of starvation medium every day), pretreated with test compounds or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (20 ng/ml). After 4 h BrdU (10 µM) was added and cells were further cultivated for 20 h. Afterwards cells were fixed and stained with an enzyme-coupled anti-BrdU antibody according to the manufacturer's instructions. Relative light units (RLU) were determined using a 96-well plate reader (TECAN GENios Pro™, Mannedorf, Switzerland).

In vitro kinase assay

The kinase assays were performed by Friedrich Totzke and Michael Kubbutat (ProQinase GmbH, Freiburg, Germany).

The kinases were expressed as human recombinant GST or His-tagged fusion proteins in Sf6 insect cells and purified using GSH-agarose columns (Sigma Aldrich) or NiNTH-agarose columns (Qiagen AG, Hilden, Germany). In vitro kinase assays were done in 96 well flash plates™ (Perkin Elmer/NEN, Boston, MA, USA) in 50 µl reaction volumes (60

mM HEPES-NaOH pH 7.3, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Natriumorthovanadate, 1.2 mM DTT, 50 µg/ml PEG20000, 1 µM [γ-^{33}P] ATP (~ 6.5 x 10^5 cpm/well), 10% compound solution). LGR1406 dissolved in 10% DMSO was added in serial dilutions from 1 nM to 10 mM. For substrates and kinases used see Table VI. The plates were incubated at 30°C for 80 min and the reaction was stopped with 50 µl of 2% (v/v) H_3PO_4. Plates were aspirated, washed two times with 200 µl ddH_2O and incorporation of ^{33}Pi was determined with a microplate scintillation counter (microbeta, Wallac). Assays were done in duplicates and performed with a Beckman Coulter/Segian robotic system. The residual activities for each concentration and the IC_{50} values were calculated using Quattro Workflow V2.0.2.2. (Quattro Research GmbH, Munich, Germany).

kinase	conc. kinase	substrate	conc. substrate
CDK1/ cyclin B	100 ng/50 µl	Rb - CTF	1000 ng/50 µl
CDK2/ cyclin A	200 ng/50 µl	H1	250 ng/50 µl
CDK2/ cyclin E	200 ng/50 µl	Rb - CTF	500 ng/50 µl
CDK4/ cyclin D1	50 ng/50 µl	Rb - CTF	1000 ng/50 µl
CDK6/ cyclin D1	200 ng/50 µl	Rb - CTF	1000 ng/50 µl

Abbreviations: CDK cyclin dependent kinase; H1 Histone H1
Rb-CTF Retinoblastoma protein C-terminal fragment

Table VI. Substrates and kinases used for Prokinase kinase assays

Migration assays

Transwell migration assay

For this assay cells were seeded into cell culture inserts containing a membrane with 8 µM pore size (BD Bioscience Pharmingen, San Diego, CA, USA) which were placed into medium-filled 24 well plates. We used collagen I coated cell culture inserts to enhance and fasten the attachment of cells to the membrane. The pore size of 8 µM prohibits dropping of the VSMC (average diameter 13 µM) to the bottom well. Therefore cells have to actively migrate from the insert through the membrane to reach the bottom well. Cells were stained using the fluorescent dye calcein, also known as fluorescein. In living cells the precursor molecule calcein AM (Calbiochem, La Jolla, CA, USA) is converted by an intracellular esterase to calcein which displays fluorescent properties with an excitation and emission wavelength of 495/ 515nm. The assay was modified from De Gendt et al [176]. Prior to usage cell culture inserts were coated with 150 µl of a 150 µg/ml rat collagen I solution in 0.02 N acetic acid (BD Bioscience Pharmingen, San Diego, CA, USA) overnight at 4 °C, thoroughly washed with PBS and dried under sterile conditions. VSMC

were seeded into 100 cm^2 cell culture flask (4 x 10^6 cells), serum starved and stained with 8 µM calcein AM for 20 min at 37 °C and henceforward protected from light. Cells were trypsinized, the reaction was stopped with 2% BSA in starvation medium and after centrifugation for 4 min at 1400 rpm (373 g) resuspended in starvation medium. Cells were counted and seeded into cell culture inserts (2 x 10^5 cells per insert), pretreated with test compounds or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (10 ng/ml) for 5 h. The inserts were then placed into a PBS-containing 24 well plate for washing and further on into a 24 well plate with 150 µl of trypsin/ EDTA per well for detachment of cells. Aliquots of 50 µl of cell-solutions were pipetted into black 96 well plates and fluorescence was measured in a 96 well plate reader (TECAN GENios Pro™, Mannedorf, Switzerland) using the following parameters: excitation wavelength: 492 nm; emission wavelength: 535 nm; gain: 30.

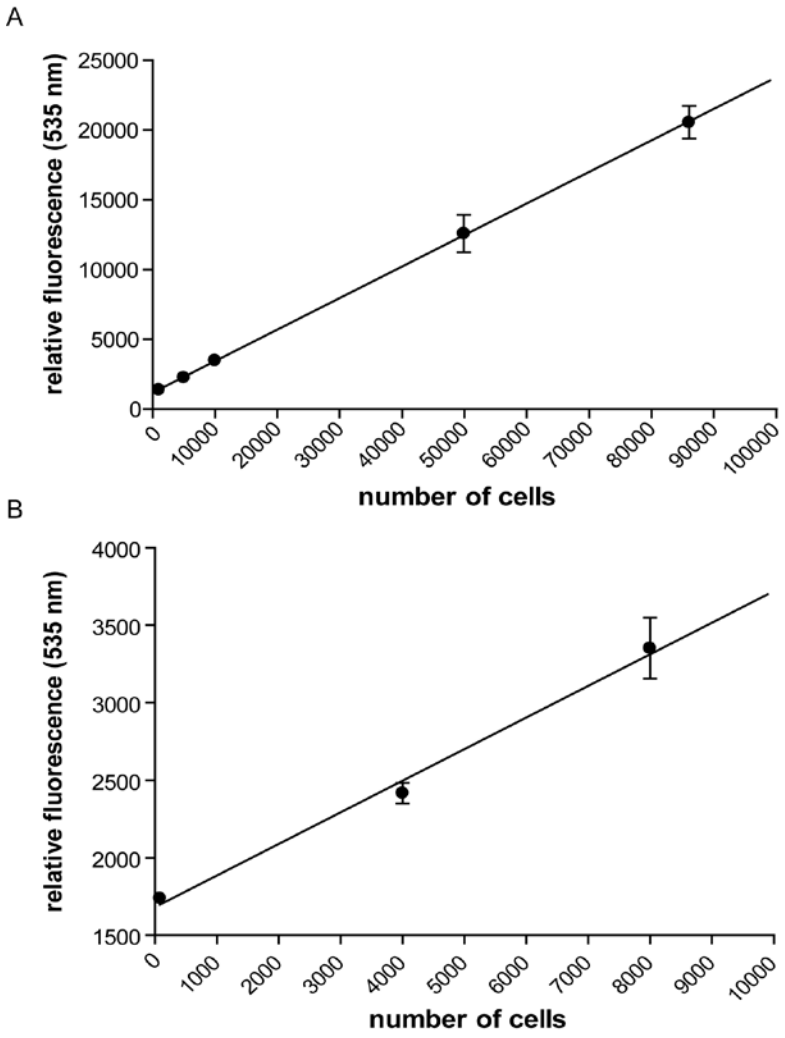

***Fig.11. Calcein AM staining**. Serum-starved VSMC were stained for 30 min with 8 µM calcein AM, counted and indicated numbers of cells were seeded into 96 well plates. Fluorescence was measured after 8 h as described in the methods section. (**A**) 1000-100,000 cells (**B**) 80-8000 cells. Shown are means ± SD and linear regression lines.*

To ensure that calcein AM staining is linearly correlated to cell number and was upright throughout the assay until the measurement, distinct amount of cells were seeded in parallel into a black 96-well plate and a linear calibration curve was determined (Fig.11). Calcein AM staining gives a linear correlation with cell numbers from 1000 to 100,000 cells as well as for smaller cell numbers (Fig.11B).

Wound healing assay

For the wound healing or scratch assay cells were grown to a confluent monolayer, scratched and the area of the scratch was determined before and after PDGF-BB-stimulation. To exclude that proliferation contributes to scratch closure the assay was stopped after 20 h because the VSMC used show a generation time of 26-28 h. Additionally the known S-phase inhibitor mitomycin C was used as control (Fig.33). VSMC were seeded in 6-well plates (0.8×10^6 cells/ well). To ensure that at 0 h and 20 h the very same area of each scratch is captured perpendicular lines were drawn at the bottom side of the plates. When confluence was reached after 24 h, cells were serum-starved for 24 h, scratched with a 1000-µl pipette tip and after washing with PBS further serum-starved for 24 h. Cells were pretreated with test compounds or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (10 ng/ml) for 20 h. The scratch area was captured with a light microscope just before PDGF-treatment and 20 h later. The cell re-colonization rate was evaluated by measuring the cell free area of each scratch, using Cell Profiler software (www.cellprofiler.org, Broad Institute, Cambridge, MA, USA [94]).

Adhesion assay

Prior to usage 96 well plates were coated with 150 µl of a 150 µg/ml rat collagen I solution in 0.02 N acetic acid (BD Bioscience Pharmingen, San Diego, CA, USA) overnight at 4 °C, thoroughly washed with PBS and dried under sterile conditions. VSMC were trypsinized and seeded into 96 well plates (5×10^4 cells/ well), pretreated with test compounds or vehicle (DMSO 1%) for 30 min and stimulated with PDGF-BB (10 ng/ml). After 3 h cells were washed with PBS, immediately fixed and stained with crystal violet staining solution (0.5% (m/v) crystal violet in 20% MeOH) for 15 min at room temperature. Plates were washed, dried and the dye was re-solubilised in ethanol/sodium citrate solution (50% (v/v) sodium citrate in ethanol). Optical density was determined using a 96-well plate reader (TECAN Sunrise™, Mannedorf, Switzerland).

Phalloidin staining

Phalloidin belongs to the group of phallotoxins and specifically binds at the interface between F-actin subunits. It binds to actin filaments more tightly than to monomers and is widely used to stain the actin cytoskeleton of cells. For this assay we used fluorescent-labled phalloidin to visualise the changes in the actin cytoskeleton of VSMC after PDGF-treatment.

Coverslips were coated with 350 µg/ml rat collagen I solution in 0.02 N acetic acid (BD Bioscience Pharmingen, San Diego, CA, USA) for 2 h at room temperature. Coverslips were carefully washed twice with PBS, dried under sterile conditions and stored for up to 3 days at 4 °C. Cells were seeded at a density of 0.2 x 10^6 cells/ well on to collagen I coated coverslips in 12-well plates for 24 h. Cells were then serum-starved, pretreated with test compounds or vehicle (DMSO 1%) for 30 min and subsequently stimulated with PDGF-BB (10 ng/ml) for 1-2 h. Samples were briefly rinsed with PBS and extracted with a prewarmed solution of 0,25% Triton X-100, 50% glutaraldehyde in CB buffer (pH ~ 6.5-6.8) for 1min. Cells were postfixed with 1% glutaraldehyde in CB at room temperature for 15 min, rinsed and washed with PBS for a few minutes. Coverslips were incubated with 20 µl of FITC-labled Phalloidin solution (FITC-Phalloidin 1:150 in PBS) for 30 min in a dark and humified chamber at room temperature. Samples were protected from light from this step onwards. The coverslips were washed three times with PBS for 5 min and mounted on a glass slide using mounting medium (Sigma, MO, USA). After drying overnight in the dark the specimens were analysed using a fluorescence microscope (Olympus BX51, Olympus Europe GmbH, Hamburg, Germany) and cell imaging software (cell^F software, Europe GmbH, Hamburg, Germany).

Cytoskeleton Buffer (CB):

150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, 5 mM Glucose, 10 mM MES (2-(N-Morpholino)-ethansulfonsäure), pH 6.1 with NaOH

Technical equipment and software

Cell viability analyser and cell counter Vi-Cell™ XR (Beckman Coulter, Fullerton, CA, USA)

Luminescent image analyser LAS-3000™ (Fujifilm, Tokyo, Japan)

96-well plate reader TECAN Sunrise™ (TECAN, Mannedorf, Switzerland)

96-well plate reader TECAN GENios Pro™ (TECAN, Mannedorf, Switzerland)

Fluorescence microscope Olympus BX51 (Olympus Europe GmbH, Hamburg, Germany)

Analysing software: cell^F software, Europe GmbH, Hamburg, Germany).

Light microscope Olympus CKX31 (Olympus Europe GmbH, Hamburg, Germany)

Camera: Olympus Live View Digital SLR Camera E-330 (Olympus Europe GmbH, Hamburg, Germany)

Analysing software: Cellprofiler software (www.cellprofiler.org)

Flow cytometer FACSCalibur™ (BD Bioscience Pharmingen, San Diego, CA, USA)

Analysing software: CellQuest™ Pro software (BD Bioscience Pharmingen, San Diego, CA, USA)

Statistic software program GraphPad PRISM™, version 4.03 (GraphPad Software Inc, San Diego, CA; USA)

Cell Profiler software (www.cellprofiler.org, Broad Institute, Cambridge, MA, USA)

Statistics

All statistical analyses were calculated using GraphPad PRISM™, version 4.03 (GraphPad Software Inc, San Diego, CA; USA). Data are expressed as means of at least three independent experiments ± standard deviation (SD) or standard error (SEM). One-way or two-way ANOVAs and Student's t-tests were used for statistical analysis of the results, as indicated in the figure legends. A *P*-value < 0.05 was considered to be statistically significant.

4. RESULTS

Isolation and characterisation of Vascular Smooth Muscle Cells

The VSMC used for this study were isolated twice independently from rat thoracic aortas of Sprague Dawley rats as described in the materials and methods chapter. To confirm the identity of VSMC and to exclude contamination with e.g. fibroblasts, cells were stained with a fluorescent labled α-smooth muscle actin antibody (Fig.12), a specific marker for smooth muscle cells in general, predominantly found in vascular smooth muscle cells [177]. Every experiment was performed with both batches of VSMC and we could not detect any differences between cells of each batch or different passages used.

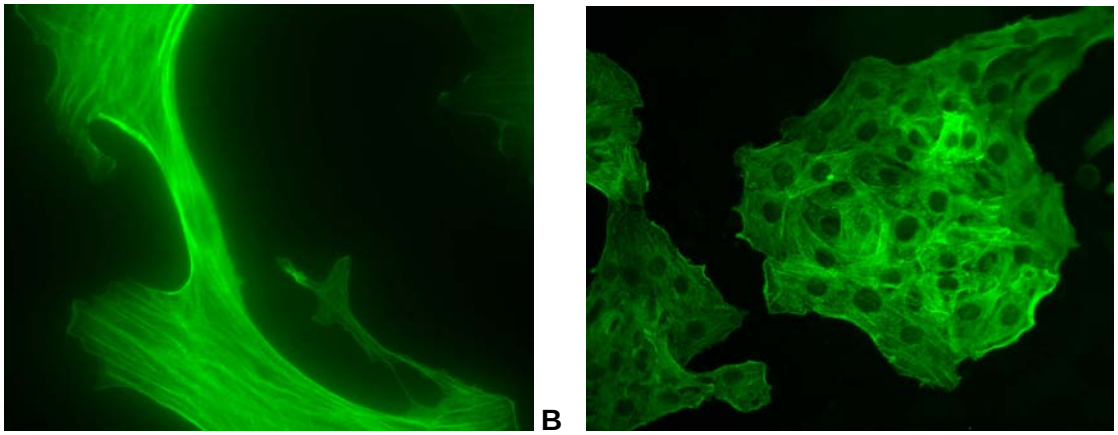

Fig.12. *VSMC of passage 3 stained with FITC-conjugated α-smooth muscle actin antibody. A 2000x magnification, B 400x magnification*

Roscovitine and Roscovitine-derivatives

Screening of Roscovitine-derivatives for anti-proliferative activity in VSMC

The aim of our study was to identify out of a panel of ROSC-derivatives a potent inhibitor of VSMC proliferation and to characterise its molecular mechanism of action in comparison to ROSC itself.

Crystal violet staining

To identify compounds exerting antiproliferative activity against VSMC we screened a panel of ROSC-derivatives using a simple crystal violet staining assay and calf serum (CS) as stimulus. Fig.13 shows that LGR1406, LGR1407, LGR1430 and LGR1492 dose-dependently limited VSMC proliferation. LGR1406 and LGR1492 were giving the best results by significantly inhibiting VSMC proliferation already at a concentration of 10 µM.

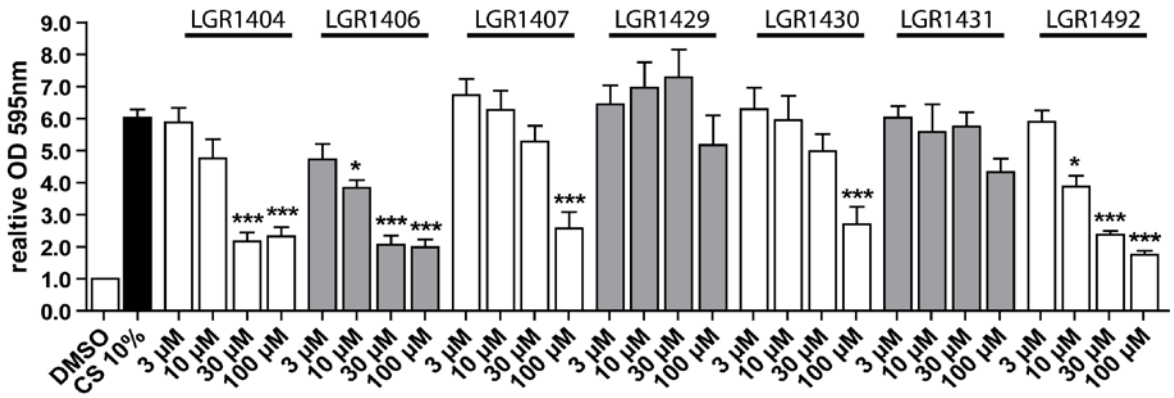

Fig.13. Crystal violet staining after 24 h of serum-stimulation. *Serum-starved VSMC were pretreated with rising concentrations (3 – 100 µM) of various ROSC-derivatives and subsequently stimulated with calf serum (10%). After 24 h cells were stained with crystal violet. The means of three independent experiments ± SEM are shown. * P<0.05, *** P<0.001 (one-way ANOVA followed by a Bonferroni post-test versus CS-treatment)*

BrdU incorporation

We next tested the four most promising substances LGR1406, LGR1407, LGR1430 and LGR1492 for their ability to interfere with DNA synthesis. Since CS includes multiple growth factors and cytokines, we wanted to use a single stimulus for further experiments. PDGF-BB is the most potent proliferative and migratory stimulus for VSMC. PDGF-BB stimulated VSMC are therefore often used to investigate the underlying molecular mechanisms of hyperproliferation as well as migration of VSMC. Furthermore it is widely accepted to use this system to screen for new antiatherogenic compounds. To synchronize the cells and to exclude influences of calf serum components that are present in the standard cultivation medium we serum-starved the cells prior to all experiments for 24 h.

As seen in Fig.14, 20 ng/ml of PDGF-BB effectively stimulated DNA synthesis after 24 h. All substances inhibited DNA synthesis at concentrations of 3 – 10 µM (Fig.14). As in the crystal violet staining assay LGR1406 showed the best result. LGR1430 showed the weakest inhibition at 3 µM compared to the other compounds and we therefore excluded this compound from further testings.

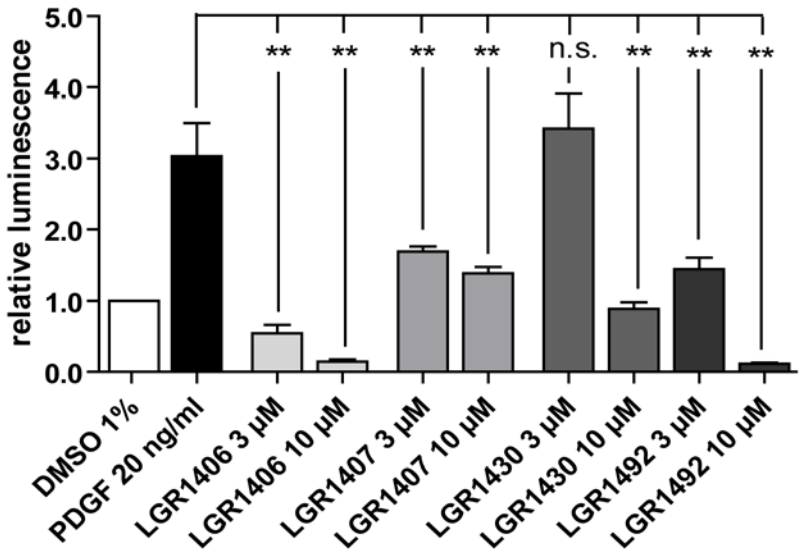

Fig.14. BrdU incorporation after 24 h of PDGF-BB-stimulation. *Serum-starved VSMC were pretreated with two concentrations (3, 10 µM) of ROSC-derivatives LGR1406, LGR1407, LGR1430 and LGR1492 and subsequently stimulated with PDGF-BB (20 ng/ml) in the presence of BrdU. After 24 h the incorporation of BrdU as a measure of DNA-synthesis was determined using an enzyme-coupled anti-BrdU antibody. The means of three independent experiments ± SEM are shown. n.s. not significant, ** P<0.01 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment)*

Cell cycle progression

As we observed a strong inhibition of PDGF-BB-induced DNA synthesis when treating VSMC with either LGR1406, LGR1407 or LGR1492, we further investigated whether the compounds could interfere with progression through G1 or S-phase of the cell cycle. Since VSMC usually show a generation time of about 26 h we treated the cells for 20 h with PDGF-BB and stained them with propidium iodide (PI) to measure the DNA content of single cells. Subsequent flow cytometric analysis of PDGF-BB-treated control cells (black bar) indeed revealed a higher percentage of cells in S- as well as in G2/M-phase compared to cells cultured without stimulus (white bar, Fig.15B). Furthermore we found that only LGR1406 potently stopped cell cycle progression at both concentrations tested (5, 10 µM). Most probably the cells were arrested in G1 phase, however, it should be noted that it is difficult to distinguish between a G1 phase arrest and a very early S phase arrest, with the method used. Treating VSMC with LGR1406 increased the percentage of cells in G1-phase from 68% of PDGF-BB-treated control cells to over 85% and therefore even higher as the value of starved control cells without stimulus (81%).

Although for the BrdU assay the differences between LGR1406 and LGR1492 were not striking (Fig.14) the propidium iodide-based analysis of the cell cycle revealed huge differences. LGR1406 potently arrests cells (presumably in G1-phase) already at a concentration of 5 µM whereas LGR1492 does not show such an effect even at 10 µM.

LGR1492 did not reduce the number of cells in S-phase but at 10 µM slightly reduced the amount of cells in G2/M-phase (from 16% for PDGF-BB treated control cells to 11%). Also LGR1407 led to a slight accumulation of cells in G1 phase when used at 20 µM (75% compared to 68% for PDGF-BB treated control cells) but this was not significant and the level of starved control cells (81%) was never reached (Fig.15). At 20 µM LGR1407 significantly reduced the number of cells in G2/M-phase (from 16% for PDGF-BB treated control cells to 7%), however, the amount of cells in S-phase was not changed (~ 13%). As both, LGR1407 and LGR1492 reduced the number of cells in G2/M-phase without affecting significantly the percentage of cells in G1- or S-phase they may just slow down cell cycle progression without inducing a cell cycle arrest. We therefore concluded that neither LGR1407 nor LGR1492 could stop cell cycle progression as potently as LGR1406.

In all experiments addressing VSMC proliferation LGR1406 consistently showed the highest antiproliferative potency in a concentration range far below that published for the parental compound ROSC (about 15-30 µM, depending on cell type). Therefore we decided to continue our investigations using LGR1406, the most promising ROSC-derivative we tested (for structure see Fig.7).

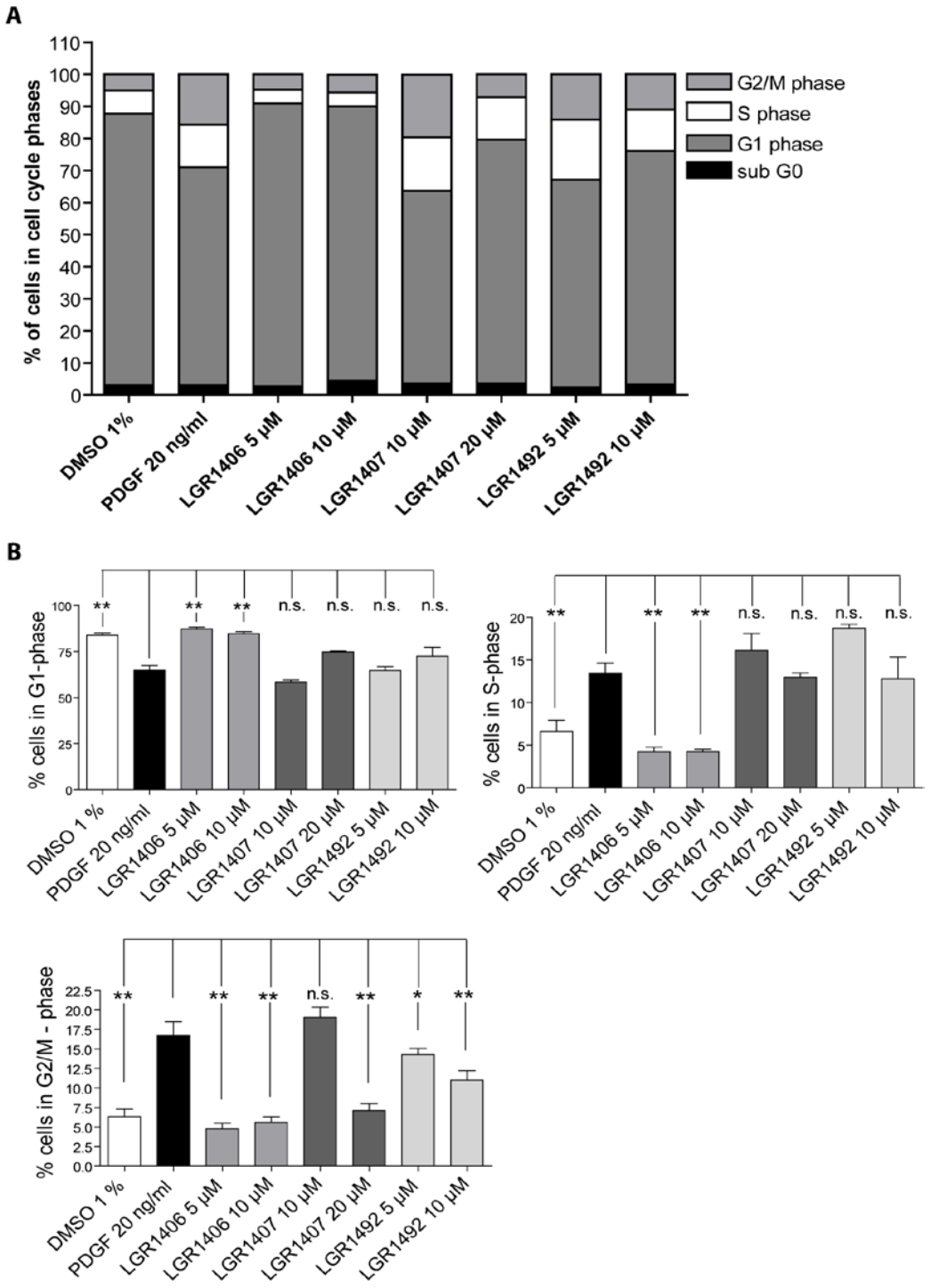

Fig.15. Cell cycle distribution after 20 h of PDGF-BB-stimulation. *Serum-starved VSMC were pretreated with different concentrations (5 – 20 µM) of ROSC-derivatives LGR1406, LGR1407 or LGR1492 and subsequently stimulated with PDGF-BB (20 ng/ml) for 20 h. After 20 h the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI-stained nuclei. (**A**) Means of three independent experiments. Overview of percentage of cells in each cell cycle phase (**B**) The single cell cycle phases are shown separately. Means ± SEM are shown. n.s. not significant, * P<0.05, ** P<0.01 (one-way ANOVA followed by a Dunnet post-test versus PDGF-treatment)*

Influence of LGR1406 and Roscovitine on VSMC cell cycle progression and cytotoxicity

Using different assays we showed that LGR1406 potently inhibits VSMC proliferation at a concentration of 5 µM. In further studies we now wanted to investigate in more detail the influence of LGR1406 and ROSC on PDGF-BB-induced cellular responses and signalling in VSMC.

BrdU incorporation

We, therefore, compared the influence of both compounds on PDGF-BB (20 ng/ml)-induced DNA-synthesis using again a BrdU incorporation assay. DNA-synthesis was dose-dependently reduced by both compounds with LGR1406 exerting clearly a stronger antiproliferative activity on VSMC (IC_{50}: 3 µM) than ROSC (IC_{50}: 17 µM) (Fig.16). The IC_{50} for ROSC is fitting well to values found in the literature (about 15-30 µM, depending on cell type).

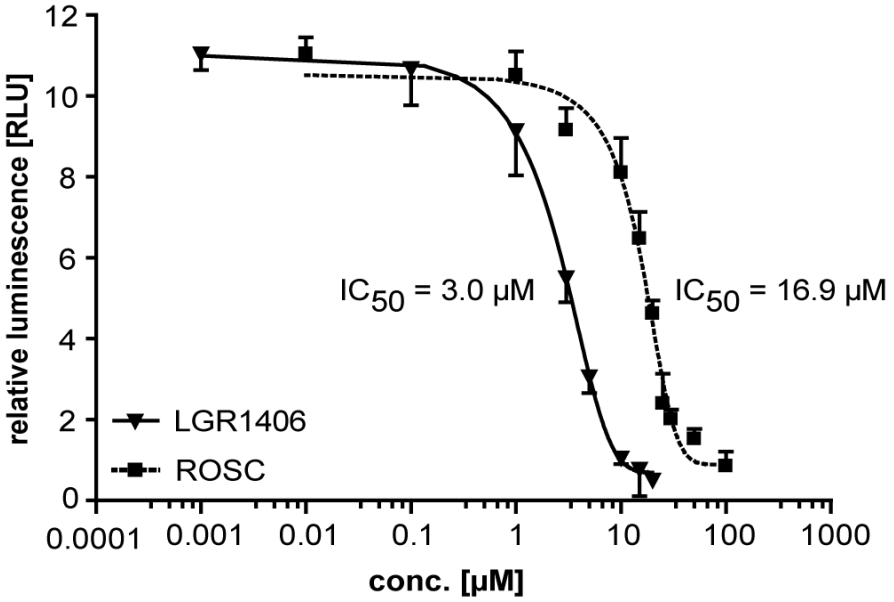

Fig.16. BrdU incorporation after 24 h of PDGF-BB-stimulation. *Serum-starved VSMC were pretreated with rising concentrations of LGR1406 (0.1–20 µM), ROSC (1–100 µM) and subsequently stimulated with PDGF-BB (20 ng/ml) in the presence of BrdU. After 24 h the incorporation of BrdU as a measure of DNA synthesis was determined using an enzyme-coupled anti-BrdU antibody. IC_{50} values were calculated by sigmoidal dose-response curve fitting (variable slope, GraphPad Prism). The means of three independent experiments ± SEM are shown.*

Cytotoxicity

A reduced BrdU-incorporation does not necessarily mean that cells stop in G1 or early S-phase but could also indicate enhanced cell death because cells that undergo apoptosis or necrosis most likely do not synthesize DNA. To evaluate whether the reduction in BrdU-incorporation (DNA synthesis) was due to diminished cell proliferation or enhanced cell death we next monitored changes in cell morphology over time compared to staurosporine as positive control [178]. To reflect the situation of the BrdU assay, we serum-starved the cells for 24 h, preincubated for 30 min with the compounds or vehicle and stimulated cells with PDGF-BB (20 ng/ml). Cells treated with up to 5 µM LGR1406 and 20 µM ROSC, respectively, did not show any signs of apoptosis (Fig.17). In contrast, 24 or 48 h treatment with staurosporine (25 nM) clearly induced detachment and disintegration of cells.

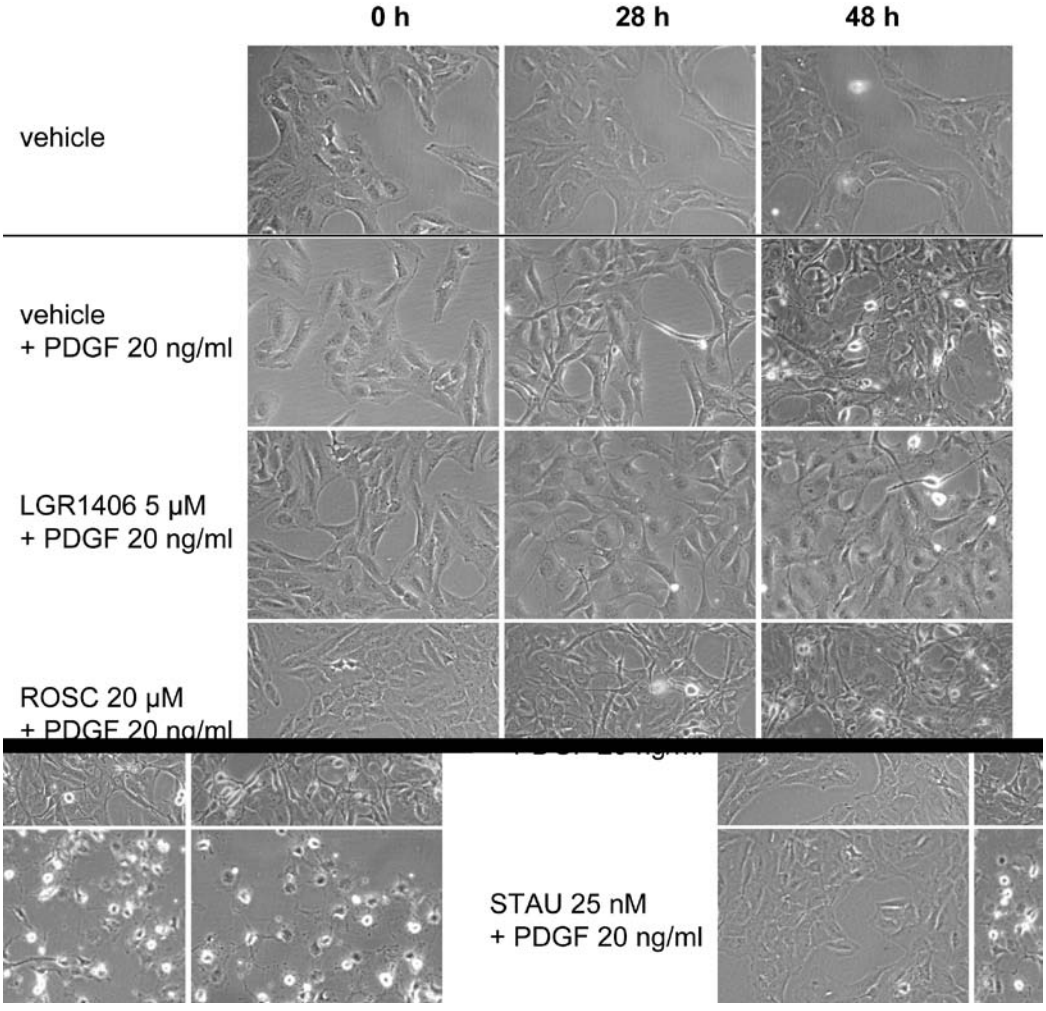

Fig.17. Microscopic pictures of LGR1406 or ROSC-treated VSMC*. Serum-starved VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for 48 h. After 0 h, 28 h and 48 h photographs were taken (magnification 200 x).*

Consistently in contrast to staurosporine, the cell number of LGR1406-treated (3-10 µM) and ROSC-treated (15-25 µM) cells after 48 h did not fall below control as shown by crystal violet staining (Fig.18). Additionally trypan-blue staining of LGR1406 (3-10 µM)- and ROSC-treated (15-25 µM) cells showed that more than 91% of VSMC were viable after up to 48 h of treatment similar to vehicle-treated control cells (viability without PDGF: 95%, viability with PDGF-BB 20 ng/ml: 91%). In contrast only 63% of the cells treated with 10 µM of mitomycin C (MitoC) were viable. Like staurosporine, MitoC is a well-described agent causing apoptosis in VSMC when used in concentrations from 1-30 µM [179] (Fig.19).

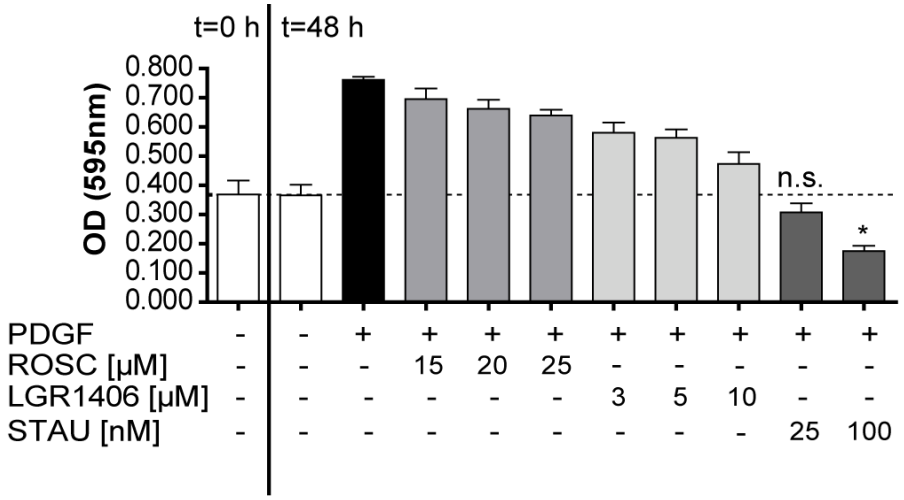

***Fig.18. Effect of LGR1406 and ROSC on cell number**. Serum-starved VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for 48 h. After 48 h attached cells were stained with crystal violet. Solubilised dye was spectroscopically quantified at OD 595 nm. Bars represent compiled data out of 3 independent experiments, mean ± SEM. n.s., not significant, * P<0.05 (one way ANOVA followed by Dunnett post-test versus t0 control).*

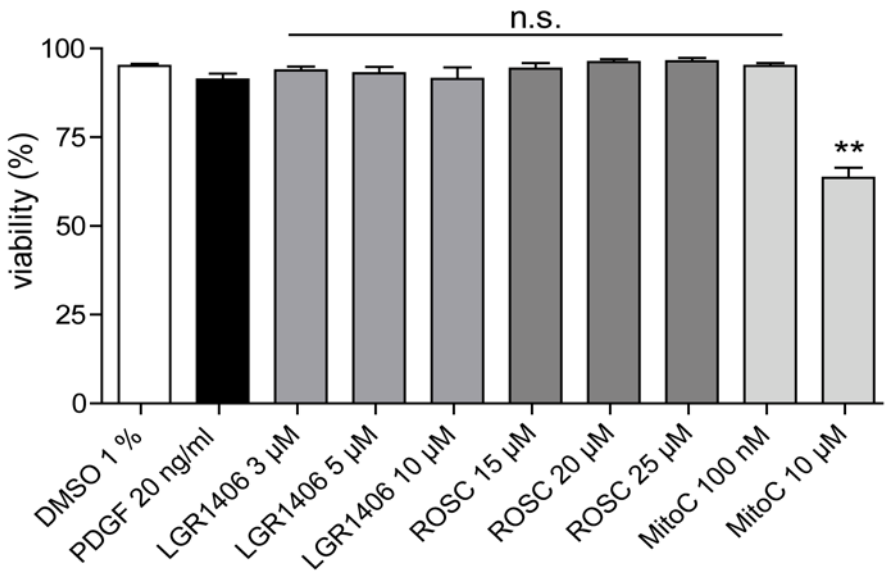

***Fig.19. Trypan blue staining assay**. Serum-starved VSMC were pretreated for 30 min with LGR1406 (3-10 µM), ROSC (15-25 µM), MitoC (10 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for 48 h. After 48 h cells were trypsinized and viability was determined using a cell viability analyser based on trypan blue staining (Vi-Cell™ XR, Beckman Coulter, Fullerton, CA, USA). Bars represent compiled data out of 3 independent experiments, mean ± SEM. n.s., not significant, * P<0.05 (one way ANOVA followed by Dunnett post-test versus t0 control).*

Because of these initial experiments we further on used 5 µM of LGR1406 and 20 µM of ROSC, a widely used concentration of ROSC also in other cell types [123, 135, 143].

Cell cycle progression

Earlier we showed that LGR1406-treatment clearly led to an arrest of cells (presumably in G1-phase) after 20 h of PDGF-BB stimulation (Fig.15). This was in agreement with the observation that LGR1406 limits DNA synthesis (Fig.14, Fig.16). ROSC also inhibited DNA synthesis, although not as potently as LGR1406 (Fig.16). Therefore we addressed the question whether ROSC also leads to a G1-arrest.

When studies on cell cycle progression are to be performed it is better to do timerows in order to clearly distinguish between cell cycle arrests and changes in speed of cell cycle progression. Only if cells do not progress through the cell cycle for a longer period of time it is appropriate to speak of a cell cycle arrest. Therefore we did not analyse a single timepoint but performed detailed studies on cell cycle progression using propidium iodide staining and subsequent flow cytometric analyses.

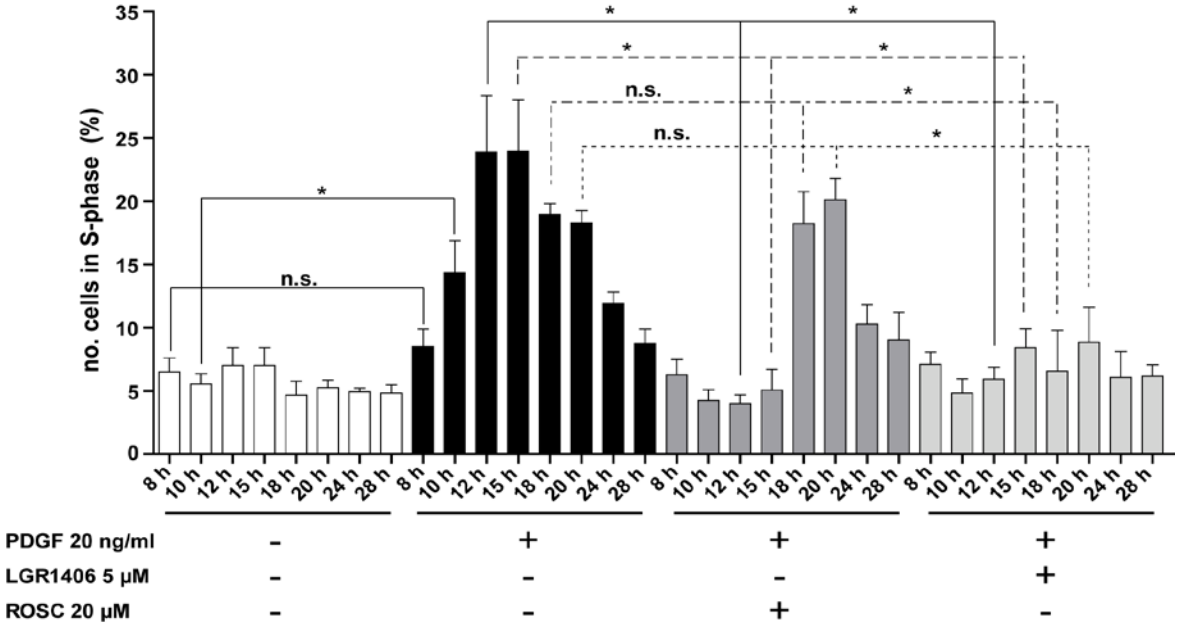

Fig.20. Influence of LGR1406 and ROSC on PDGF-induced S-phase entry of VSMC*. Serum-starved VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) and cultured in the presence or absence of PDGF (20 ng /ml). After the indicated periods of time the percentage of cells in S-phase was determined by flow cytometric analysis of PI-stained nuclei. Columns show the mean of three independent experiments ± SEM. n.s., not significant, *P<0.05 (two-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment). Representative histograms for depicted timepoints are shown in Fig.21.*

Fig.20 and Fig.21 show the time-dependent entry into S-phase of the cell cycle of serum-starved VSMC upon PDGF-BB stimulation in the absence or presence of LGR1406 and ROSC (8-28 h). In unstimulated, vehicle-treated cells no increase of cells in S-phase was observed over time (Fig.20, white bars). This can also be seen in the corresponding histograms (left column, Fig.21). PDGF-BB-stimulated cells showed a significant increase of cells in S-phase as early as 10 h after addition of the stimulus, indicating that the cells pass late G1-phase after about 6-8 h and undergo S-phase transition after 8-10 h (Fig.20, black bars). The increase of cells in S-phase is also nicely seen in the corresponding histograms (second left column, Fig.21). The percentage of cells in S-phase peaked after 12-15 h (~ 23%) and was thereupon reduced reaching again the basal level after 28 h. The increase in cells in S-phase and later on in G2/M-phase was consequently accompanied by a decrease of cells in G1-phase (Fig.22 A). Cells reached G2/M phase about 18 h after PDGF-BB stimulation. The amount of cells in G2/M phase peaked after about 20 h and had not completely reached the basal level after 28 h. In average the VSMC used for our experiments underwent G1/ S-phase transition after about 10 h of

PDGF-BB-stimulation, finished the S-phase after about 22-24 h and completed the cell division after about 28 h (Fig.22 B).

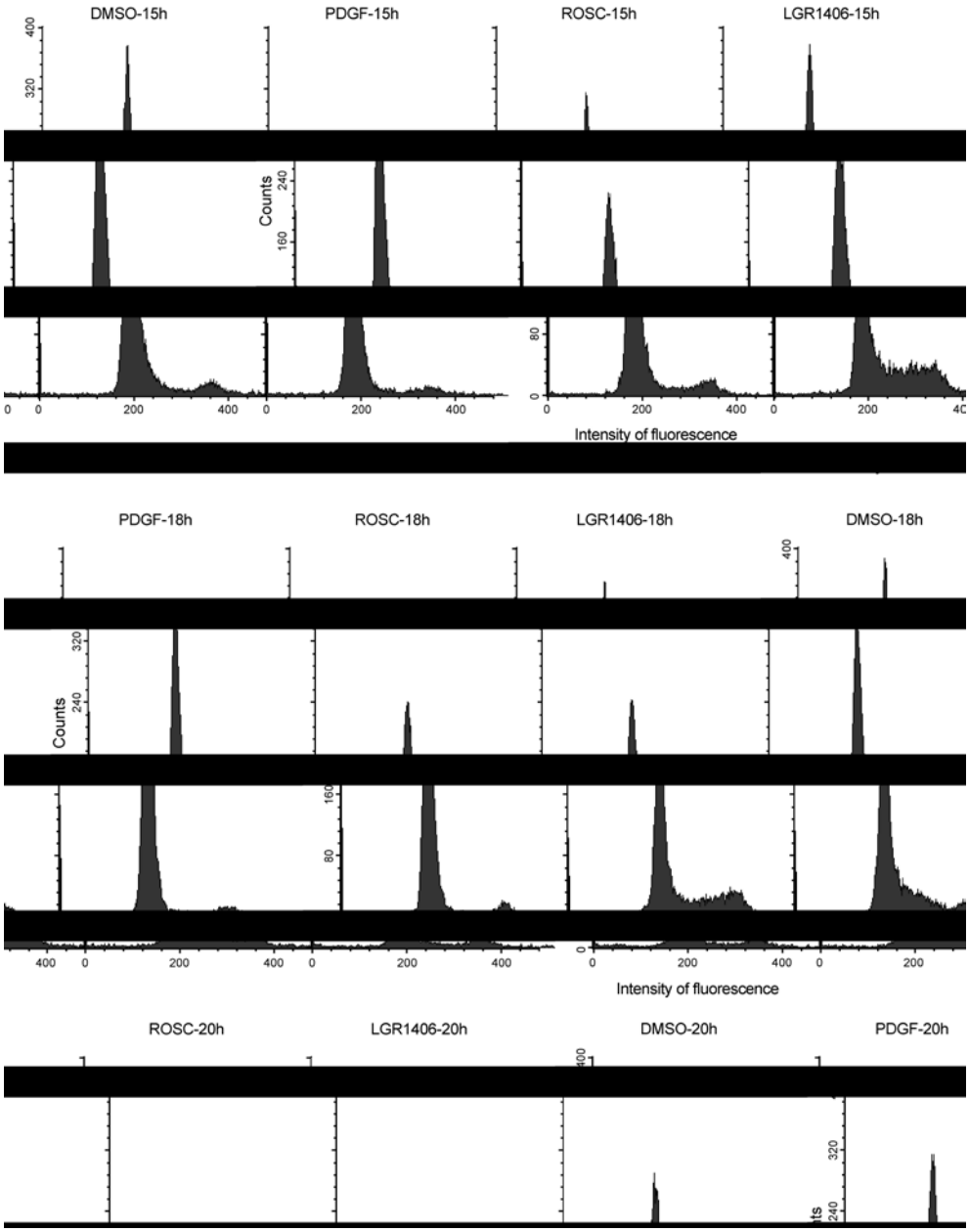

Fig.21. *Corresponding histograms to the data shown in Fig.19 for 15- 20 h of PDGF-BB-treatment. The intensity of fluorescence is proportional to the DNA content of single cells.*

Treatment with 5 µM LGR1406 completely abrogated the PDGF-BB-induced S-phase entry whereas 20 µM ROSC only slightly decreased but delayed cell cycle progression by about 8-10 h (Fig.20, grey bars). Consistently, ROSC-treated cells also entered G2/M phase approximately 8 h later than control cells (Fig.22). In the corresponding histograms

already after 15 h the G1-peak is broader for the ROSC-treated cells and after 18 h obviously many cells started to synthesize DNA (second right column, Fig.21).

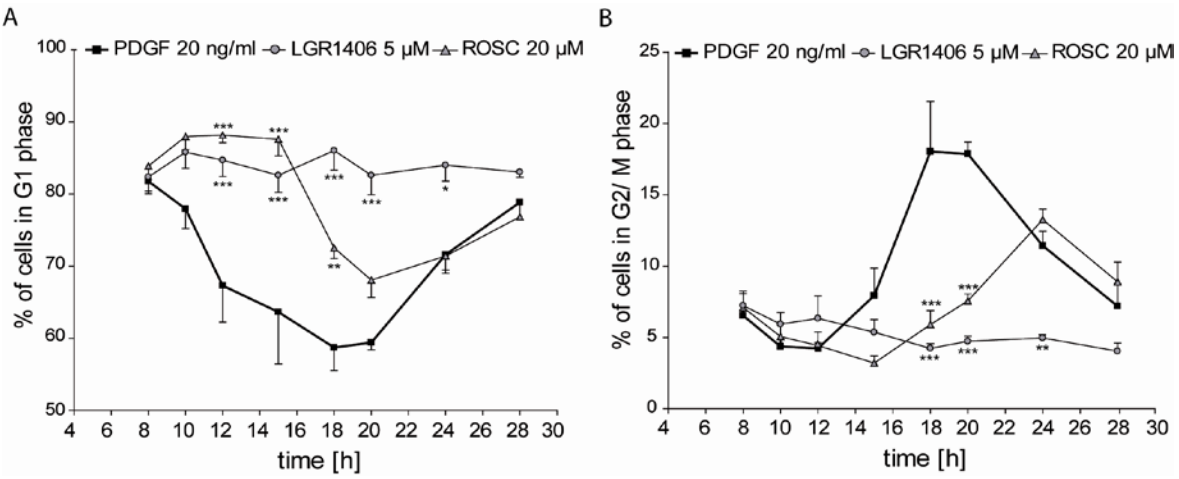

Fig.22. Influence of LGR1406 and ROSC on cell cycle distribution of VSMC upon PDGF-BB stimulation*. Serum-starved VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) and cultured in the presence of PDGF (20 ng/ml). After the indicated periods of time the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI-stained nuclei. (**A**) Percentage of cells in G1-phase (**B**) Percentage of cells in G2/M phase. The means of three independent experiments ± SEM are shown. *P<0.05, **P<0.01, ***P<0.001 (two way ANOVA followed by Bonferroni post-test versus PDGF-treatment).*

To confirm that ROSC does not specifically arrest VSMC in one phase of the cell cycle, we performed further experiments using even up to 25 µM ROSC or prolonged treatment for up to 48 h. We observed an accumulation of cells in G1-phase after 20 h with the highest concentration of 25 µM (82%) but ROSC-treated cells never reached the level of starved control cells (87%, see Fig.23 and Table VII). LGR1406 on the other hand accumulates cells in G1 phase already at 3 µM more potently than ROSC does at 25 µM.

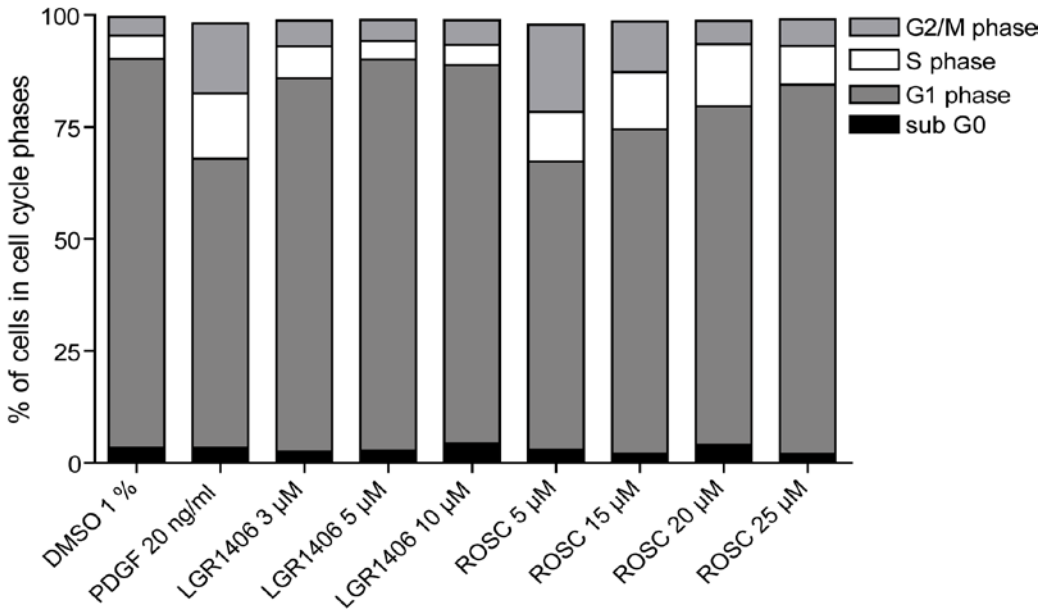

Fig.23. Concentration-dependent alterations in cell cycle distribution of LGR1406 and ROSC-treated VSMC. *Serum-starved VSMC were pretreated for 30 min with LGR1406 (3-10 µM), ROSC (5-25 µM) or vehicle (DMSO 1%) and cultured in the presence of PDGF (20 ng/ml). After 20 h the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI-stained nuclei. Shown are the means of three independent experiments. The exact values and SD are shown in Table VII.*

	sub G1	G1	S	G2/ M
DMSO	3,40 +/- 2,62	86,78 +/- 3,32	5,19 +/- 2,22	4,20 +/- 1,00
PDGF	3,38 +/- 2,45	64,51 +/- 8,30	14,62 +/- 6,81	15,61 +/- 2,36
LGR1406 3 µM	2,55 +/- 0,54	83,32 +/- 3,30	7,12 +/- 2,85	5,75 +/- 0,77
LGR1406 5 µM	2,69 +/- 0,27	87,34 +/- 2,32	4,14 +/- 1,32	4,68 +/- 1,75
LGR1406 10 µM	4,38 +/- 1,01	84,44 +/- 2,28	4,50 +/- 0,22	5,51 +/- 1,78
ROSC 5 µM	2,85 +/- 0,78	64,40 +/- 4,71	11,08 +/- 2,49	19,52 +/- 4,71
ROSC 15 µM	2,01 +/- 0,57	72,44 +/- 5,29	12,84 +/- 1,34	11,22 +/- 7,07
ROSC 20 µM	3,98 +/- 3,91	75,64 +/- 6,66	13,84 +/- 2,78	5,24 +/- 1,01
ROSC 25 µM	1,95 +/- 0,44	82,48 +/- 2,46	8,66 +/- 2,56	5,93 +/- 0,60

Table VII. Concentration-dependent alterations in cell cycle distribution of LGR1406 and ROSC-treated VSMC

ROSC does not lead to an arrest in S- or G2/M-phase either, since we have not observed an accumulation of cells in these phases of the cell cycle after 48 h (Fig.24). LGR1406 obviously stops PDGF-BB-induced cell cycle progression when used in concentrations around its IC_{50} for inhibition of DNA synthesis (3 µM, Fig.14). ROSC, however, when used in the corresponding concentration (IC_{50} ~ 17 µM) seems only to slow down cell cycle progression in general rather than to stop it at a distinct point. Maybe concentrations of

ROSC higher than 25 µM would lead to a cell cycle arrest, but microscopical observations of cells treated with 30 µM indicated that higher concentrations would already lead to cytotoxic effects (data not shown). Therefore we conclude that LGR1406 most likely leads to a G1-arrest of VSMC whereas ROSC delays cell cycle progression in response to PDGF-BB.

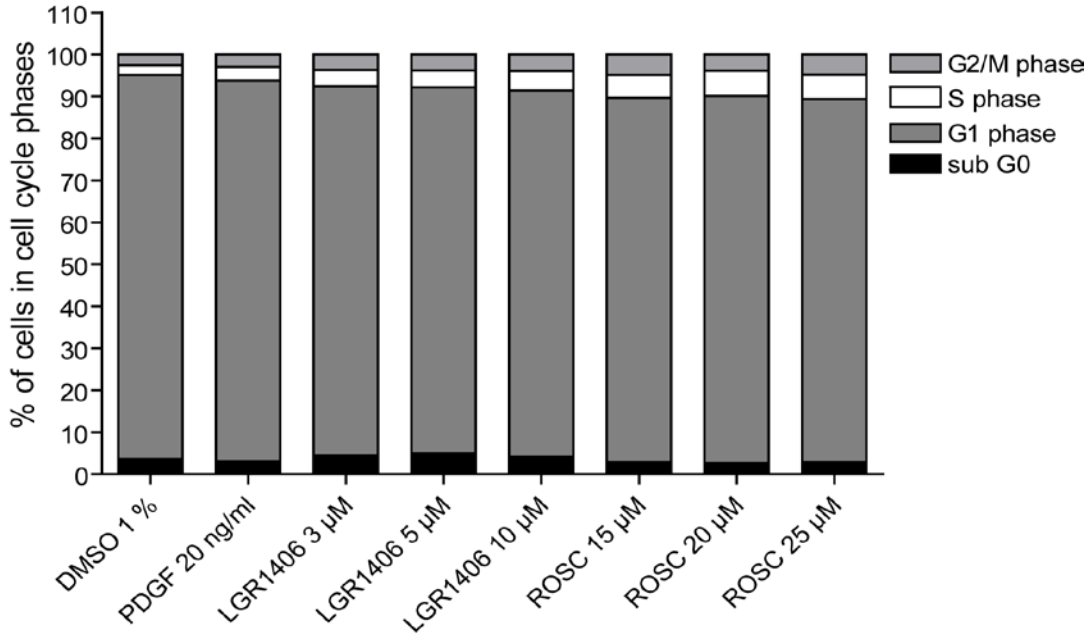

Fig.24. Cell cycle distribution of LGR1406 and ROSC-treated VSMC after 48 h. *Serum-starved VSMC were pretreated for 30 min with LGR1406 (3-10 µM), ROSC (5-25 µM) or vehicle (DMSO 1%) and cultured in the presence of PDGF (20 ng/ml). After 48 h the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI-stained nuclei. Shown are the means of three independent experiments. The exact values and SD are shown in Table VII.*

	sub G1	G1	S	G2/ M
DMSO	3.52 +/- 1.55	89.70 +/- 0.89	2.25 +/- 0.24	2.52 +/- 0.29
PDGF	3.04 +/- 0.78	89.27 +/- 1.32	3.17 +/- 0.83	2.95 +/- 0.23
LGR1406 3 µM	4.42 +/- 3.41	86.18 +/- 3.49	3.84 +/- 1.26	3.58 +/- 0.52
LGR1406 5 µM	4.88 +/- 4.00	86.31 +/- 4.00	4.03 +/- 1.16	3.74 +/- 0.69
LGR1406 10 µM	4.17 +/- 0.99	86.87 +/- 1.60	4.59 +/- 0.29	3.95 +/- 0.48
ROSC 15 µM	2.89 +/- 0.86	85.94 +/- 1.77	5.39 +/- 1.18	4.86 +/- 0.53
ROSC 20 µM	2.57 +/- 0.71	86.07 +/- 2.78	5.94 +/- 2.50	3.83 +/- 0.17
ROSC 25 µM	2.89 +/- 1.22	85.57 +/- 2.95	5.71 +/- 2.05	4.79 +/- 0.34

Table VIII. Concentration-dependent alterations in cell cycle distribution of LGR1406 and ROSC-treated VSMC

Influence of LGR1406 and Roscovitine PDGF-BB-signalling in VSMC

Early PDGF-BB-induced signalling events

As cells treated with LGR1406 seemed to arrest during G1-phase, we subsequently investigated whether either of the substances interferes with early signalling events downstream of the PDGF receptor. Using western blot analyses we could exclude any inhibitory influence of ROSC or LGR1406 on activation of Akt and STAT3 or phosphorylation of the mitogen activated kinases (MAPK) p38, ERK and JNK (Fig.25). In contrast, treatment with LGR1406 slightly but significantly enhanced activation of the MAPK p38 and ERK.

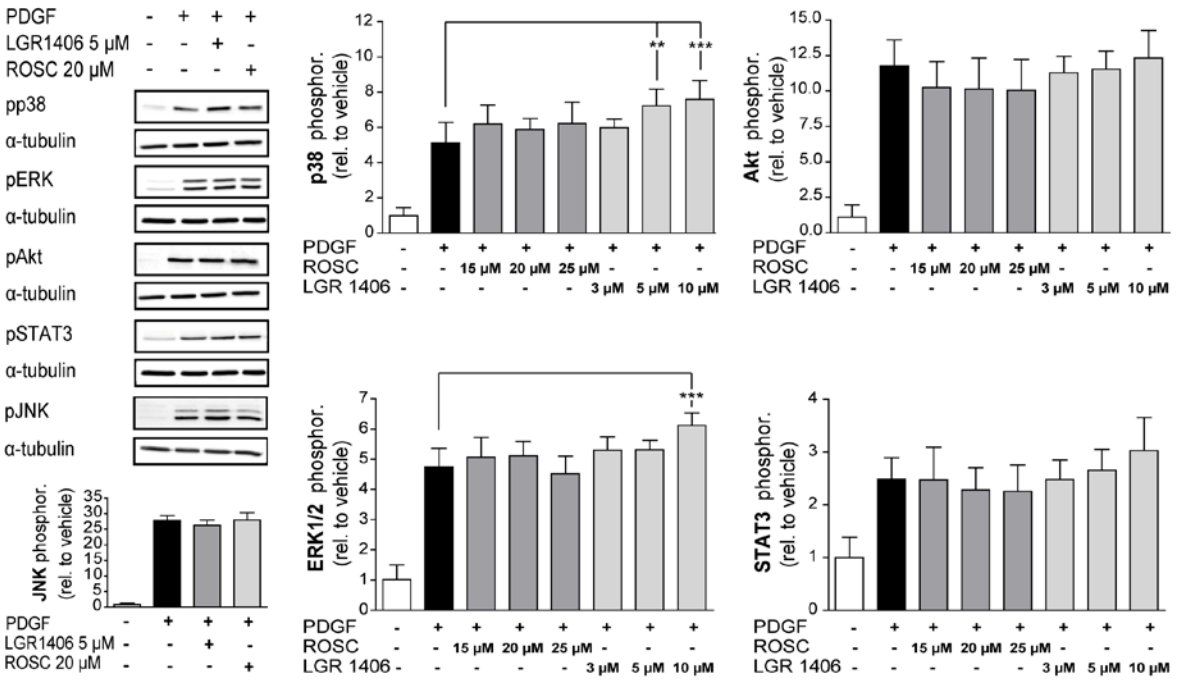

Fig.25. Influence of LGR1406 and ROSC on early signalling events in PDGF-activated VSMC*. Serum-starved VSMC were pretreated for 30 min with LGR1406 (3-10 µM), ROSC (15-25 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for 10 min. Total cell lysates were then subjected to western blot analysis for phosphorylation of p38, ERK, JNK, Akt, STAT3 and total α-tubulin. Representative western blots for LGR1406 5 µM and ROSC 20 µM out of at least four independent experiments with consistent results are shown. Graphs depict compiled data of the densitometrically evaluated phospho-protein/tubulin ratios. Shown are means ± SEM. ** P<0.01 *** P<0.001 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment).*

Since previous reports implicated an interference of ROSC with MAPK activation [127, 144] we wanted to additionally confirm these results by using a prolonged time of preincubation (15 h) for ROSC as well as higher concentrations of both compounds. With concentrations up to 10 µM LGR1406 and 25 µM ROSC, respectively, we could not detect any negative impact of either of the substances on the activation of early signalling cascades (Fig.25). Also we observed no inhibitory effect of ROSC on the MAPK p38, ERK or JNK when preincubating the cells for 15 h (Fig.26).

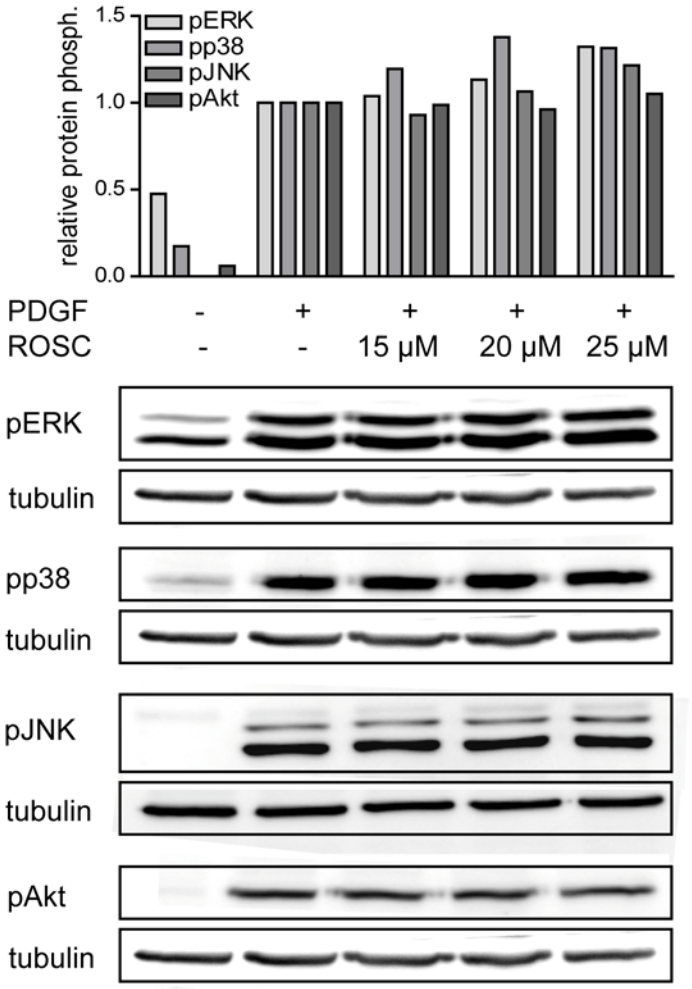

Fig.26. Influence of ROSC on MAPK and Akt activation after 15 h of preincubation*. Serum-starved VSMC were pretreated for 15 h with ROSC (20-25 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for 10 min. Total cell lysates were then subjected to western blot analysis for phosphorylation of p38, ERK, JNK, Akt and total α-tubulin. Western blots and densitometrically evaluated phospho-protein/tubulin ratios of a single experiment are shown.*

In conclusion, the most common immediate early signaling events downstream of the PDGFR were not affected by LGR1406 or ROSC.

Cyclins and pocket proteins

Because we could not detect changes in early signalling events we aimed to study proteins directly driving cell cycle progression. We therefore dissected the time-dependent changes in expression and activity-linked phosphorylation of key regulators of the cell cycle in VSMC upon PDGF-BB stimulation.

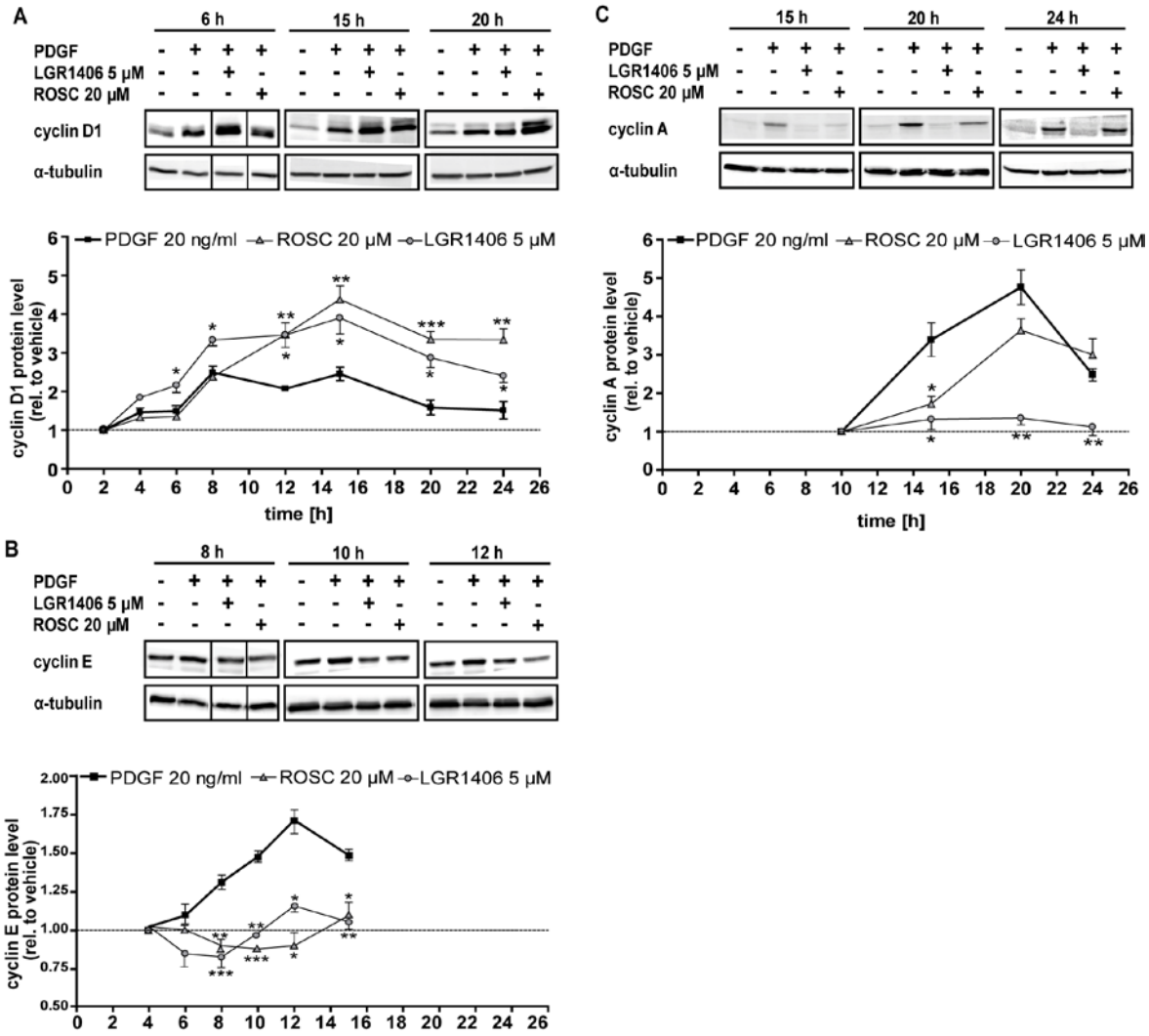

Fig.27. Impact of LGR1406 and ROSC on expression levels of cyclins. *Serum-starved VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for the indicated periods of time. Total cell lysates were then subjected to western blot analysis for cyclin D1, A, E and α-tubulin. Representative western blots out of at least three independent experiments with consistent results are shown for cyclin D1 (**a**), cyclin A (**b**) and cyclin E (**c**). Graphs depict compiled data of densitometrically evaluated cyclin/tubulin ratios for each timepoint normalised to the respective unstimulated vehicle control. Shown are means ± SEM. * P<0.05, ** P<0.01, *** P<0.001 (two-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment).*

First we studied the expression of cyclin D1 responsible for CDK4/ 6 activation and hence for progression through G1-phase. In control cells cyclin D1 levels were increased starting from 4 h on up to 24 h after PDGF-BB stimulation reaching a peak after 8 h (Fig.27). In ROSC- and LGR1406-treated cells cyclin D1 levels stayed elevated until 24 h after PDGF-BB treatment in contrast to control cells, where cyclin D1-protein levels decreased from 10 h on. Neither ROSC- nor LGR1406-treatment did reduce but both even markedly increased cyclin D1 levels compared to control cells from 12 h on, confirming that there was no interference with early signaling events leading to cyclin D1 expression. The prolonged expression of the G1-phase cyclin D1 could implicate an interference with feedback loops during late G1/ S-phase that normally lead to the downregulation of cyclin D1 levels in control cells.

In PDGF-BB-treated control cells the S-phase cyclin A is expressed from 15 h-24 h peaking at 20 h. In agreement with the flow cytometric data on S-phase entry LGR1406 inhibited expression of cyclin A, whereas ROSC only delayed expression of the cyclin (Fig.27). This result confirms our assumption that LGR1406 arrests cells in G1-phase because we could not detect any expression of the S-phase cyclin A.

Cyclin E is usually expressed during G1 to S-phase transition. However, in our experimental setup vehicle treated cells already displayed a high level of cyclin E that was only slightly increased after PDGF-BB treatment (Fig.27). Both compounds inhibited this slight cyclin E increase to control levels or even below. However, it remains unclear whether this effect, given the high basal level, contributes to the impaired cell cycle progression.

In summary, LGR1406 and ROSC do not reduce or prevent cyclin D1 expression and hence do not interfere with very early events in cell cycle progression. However, both compounds reduce cyclin E levels but only LGR1406 prevents cyclin A expression.

Activation of CDK4/6 by binding to cyclin D1 as well as CDK2 by complexing with cyclin A/ E leads to the hyper-phosphorylation of the pocket proteins Retinoblastoma protein (Rb) and p107. Indeed, both proteins are hyper-phosphorylated from late G1-phase (6-8 h) onwards in PDGF-BB-treated control cells (Fig.28) whereas in starved control cells the levels of hyper-phosphorylated pocket proteins are almost undetectable. During S-phase both proteins are even stronger phosphorylated. The p107 phosphorylation peaks after 15 h and Rb phosphorylation is further increasing up to 24 h upon PDGF-BB stimulation.

LGR1406 treatment completely abolished even early hyper-phosphorylation of both proteins again supporting that LGR1406 leads to a G1-phase arrest. ROSC reduced the early phosphorylation of p107 and Rb but in summary again only delayed the onset of PDGF-BB induced pocket protein hyper-phosphorylation (Fig.28).

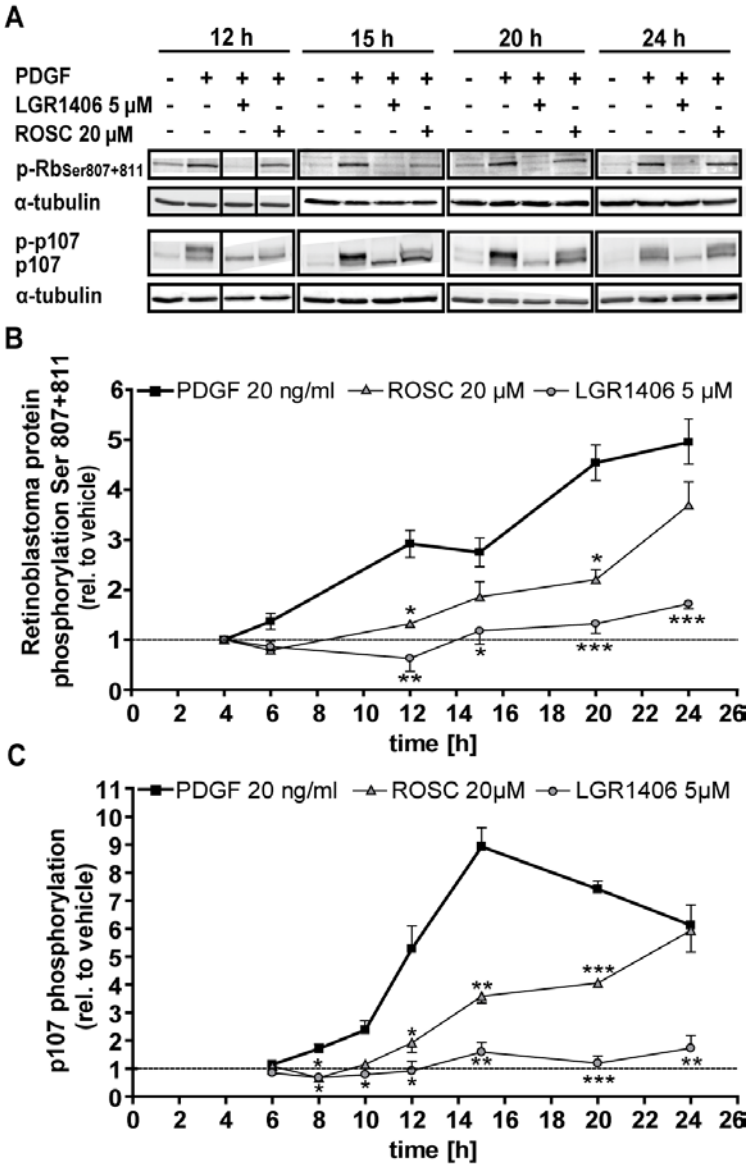

Fig.28. Influence of LGR1406 and ROSC on phosphorylation of the pocket proteins Retinoblastoma protein and p107*. Serum-starved VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for the indicated periods of time. Total cell lysates were then subjected to western blot analysis of retinoblastoma protein phosphorylation (pRb$^{Ser807+811}$), p107/phospho-p107 (p-p107) and α-tubulin. (**a**) Representative western blots out of at least three independent experiments with consistent results are shown for pRb$^{Ser807+811}$ and p-p107. (**b**, **c**) Graphs depict compiled data of the densitometrically evaluated phospho-protein/tubulin ratios for each timepoint normalised to the respective unstimulated vehicle control. Shown are means ± SEM. * P<0.05, ** P<0.01, *** P<0.001 (two-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment).*

With this detailed western blot analyses we corroborate our finding that LGR1406 treatment leads to an arrest of VSMC in late G1-phase as it is affecting pocket protein phosphorylation and expression of S-phase cyclin A. ROSC, however, obviously only delays cell cycle progression and associated events.

In vitro kinase assays for LGR1406 and Roscovitine

Altogether the studies on early signalling events, cyclin expression and pocket protein phosphorylation suggest that ROSC and LGR1406 directly interfere with CDK activity. We therefore performed in cooperation with ProQinase GmbH (Freiburg, Germany) *in vitro* kinase assays. Next to LGR1406 we included LGR1407 and LGR1492, two of the other ROSC-derivatives that gave promising results in the first screening experiments for antiproliferative activity (Fig.14, Fig.15). A panel of kinases was analysed using the standardised ProQinase© screening platform, that had already been used for detailed profiling of ROSC (Bach et al.)[127]. Unfortunately only for a few of the kinases that we tested with the ProQinase© kinase assay, the corresponding data for ROSC were available from Bach et al. who screened mainly different kinases. The kinases that have been tested for all derivatives as well as for ROSC with the ProQinase© kinase assay are shown in Table IX. In Table X we show the compiled data for all kinases that we tested with the new derivatives, including those where no data from Bach et al. were available.

IC50 [µM]	CDK1/ cyclin B	CDK2/ cyclin A	CDK2/ cyclin E	CDK4/ cyclin D1	CDK6/ cyclin D1	NLK	Aurora-A
LGR1406	3.2	1.0	0.6	14.7	>100	>100	0.6
LGR1407	5.7	1.5	1.0	> 50	> 50	>100	2.6
LGR1492	3.1	0,1	0.1	21.7	>100	>100	0.4
ROSC	23.0	1.5	1.2	> 50	> 50	>100	>100

for abbreviations see section 8.1

Table IX. In vitro kinase assays comparing ProQinase data for ROSC an derivatives

	IC50 [µM]		
kinase	LGR1406	LGR1407	LGR1492
ALK	20.7	19.6	>100
AMPK-α1	32.9	17.4	>100
Aurora-A	0.6	2.6	0.4
BRK	>100	51,0	>100
CDK1/ CycB	3.2	5.7	3.1
CDK2/ CycA	1,0	1.5	0.1
CDK2/ CycE	0.6	1.0	0.1
CDK4/ CycD1	14.7	65.9	21.7
CDK5/ p25NCK	0.4	2.0	0.3
CDK6/ CycD1	>100	90,3	>100
CDK7/ CycH	>100	>100	>100
CDK9/ CycT	1.1	1.9	1.9
EGF-R	>100	>100	>100
FAK	12.6	21.6	59.9
FGF-R1	76.2	19.9	63.2
FGF-R2	>100	>100	98.9
MEK1	>100	>100	>100
NLK	>100	>100	>100
PAK4	6.3	17.4	14.9
RAF-1	>100	>100	>100
ROCK1	>100	>100	>100
RSK3	9.1	29.6	31.7
VEGF-R1	>100	>100	>100
VEGF-R2	>100	>100	>100

for abbreviations see section 8.1

Table X. In vitro kinase assays-complete ProQinase data for ROSC-derivatives

Our screening for antiproliferative activity of the different derivatives revealed that LGR1492 was a little more potent than LGR1407 but both were not as potent as LGR1406 (Fig.13, Fig.14, Fig.15). Comparing the *in vitro* kinase data it is interesting that although all of the derivatives show similar IC_{50}s against CDK1/2/5/6/7/9, LGR1406 and LGR1492 additionally inhibited CDK4 (Table X). Moreover, LGR1406, the most potent compound shows an even lower IC_{50} against CDK4 than LGR1492 (15 µM for LGR1406 compared to 22 µM for LGR1492). However, the IC_{50}s were higher than the concentrations used for the cell based testings (max. 10 µM). Looking at Table X also reveals that LGR1406 has lower IC_{50}s against FAK, PAK4 and RSK3 compared to the other derivatives but those kinases are to our knowledge not involved in cell cycle progression. Also it should be noted that we tested the derivatives only against a restricted panel of kinases. Since LGR1406 in general shows a little lower IC_{50}s against several kinases it is possible that this is true for some other kinases as well and that one of those is responsible for the better performance of the compound in cell based assays.

Comparing ROSC and its most potent derivative LGR1406 it becomes clear that LGR1406 had overall lower IC_{50} values against depicted CDKs than ROSC, especially for CDK1 and CDK4.

Influence of LGR1406 and Roscovitine on PDGF-BB-induced migration of VSMC

As both, proliferation and migration contribute to neointima formation and PDGF-BB is not only the most potent proliferative but also the most effective migratory stimulus for VSMC, we additionally examined the influence of LGR1406 and ROSC on chemotaxis.

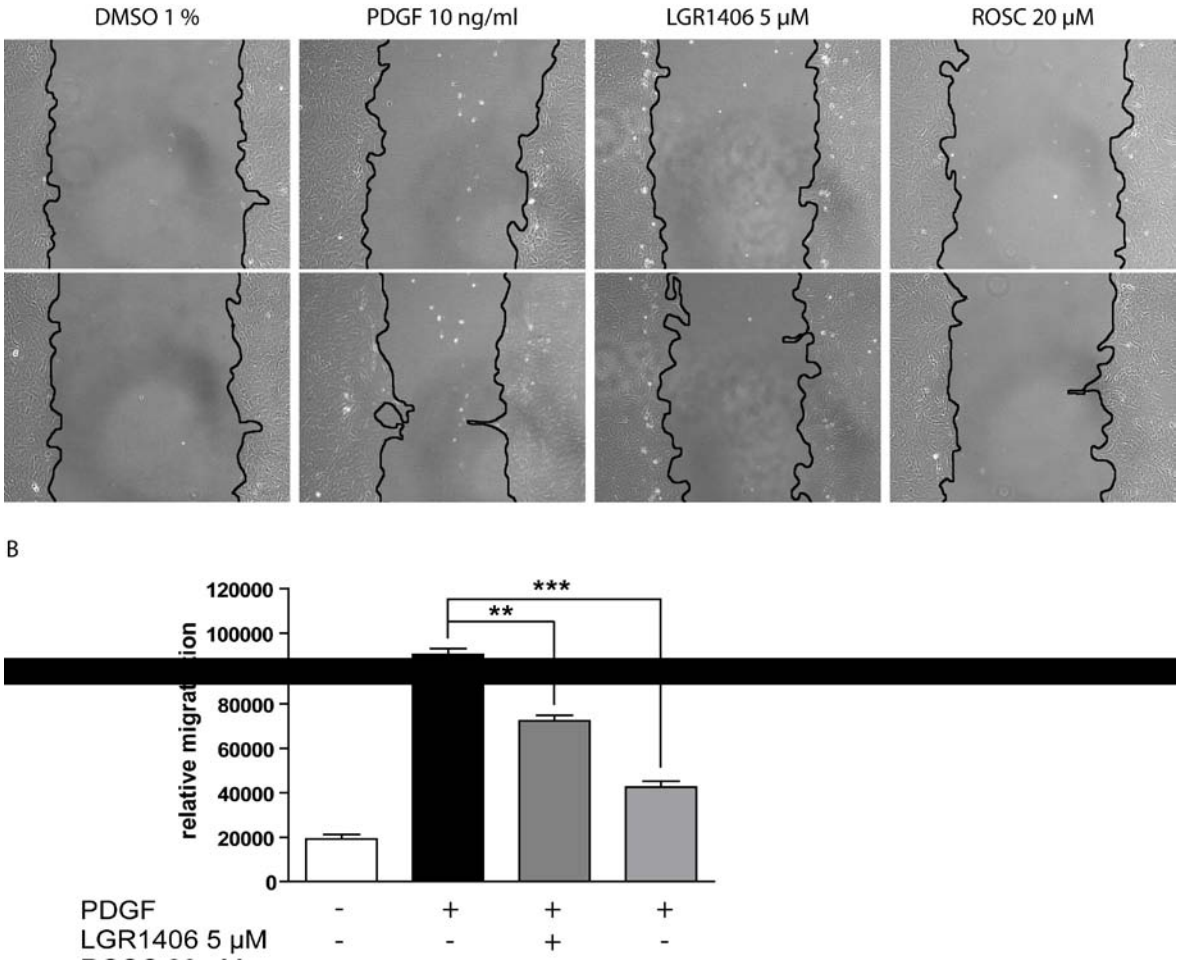

Fig.29. Influence of LGR1406 and ROSC on PDGF-BB-induced migration*. Serum-starved, scratched VSMC were pretreated for 30 min with LGR1406 (5 µM), ROSC (20 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 20 h. Scratches were captured at 0 h and after 20 h and cell free areas were calculated using Cellprofiler software (www.cellprofiler.org). (**A**) Representative photographs of scratches. (**B**) Means of five independent experiments ± SEM. ** P<0.01, *** P<0.001 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment).*

In contrast to the antiproliferative effect, ROSC showed a much stronger antimigratory potential in a scratch assay (discussed in more detail in chapter 4.3.1) than LGR1406. ROSC treatment is reducing the PDGF-BB-stimulated migration by 65% compared to only a slight inhibition of 23% observed for LGR1406 (Fig.29). Whether this inhibition is linked to CDKs or other side targets of these compounds still needs to be investigated.

In conclusion we show that LGR1406 potently inhibits VSMC proliferation in response to PDGF-BB by arresting cells in G1-phase of the cell cycle. Most likely this is due to a direct CDK inhibition. ROSC on the other hand does lead to a delay in cell cycle progression without leading to a cell cycle arrest. The effect of ROSC on VSMC is most likely also due to direct inhibition of CDKs. Additionally ROSC limits VSMC migration in response to PDGF-BB much stronger than LGR1406.

Indirubine-3'-monoxime

Since an antiproliferative effect of I3MO has been already shown by Andrea Schwaiberger [154], we additionally wanted to investigate the potential influence of I3MO on PDGF-BB-induced migration of VSMC.

I3MO inhibits PDGF-BB-induced migration of VSMC

For VSMC migration assays 10 ng/ml of PDGF-BB was used instead of 20 ng/ml because it has been shown, that a lower amount of PDGF elicits rather a migratory response whereas higher concentrations cause proliferation [180]. This was supported in our lab by Andrea Schwaiberger showing that 10 ng/ml of PDGF only slightly enhances cell number after 48 h whereas cells treated with 20 ng/ml clearly proliferate [154]. Additionally we showed that PDGF-BB from 1 ng/ml on leads to migration of VSMC reaching a significant peak at 10 ng/ ml (Fig.30 data partly generated by Mario Kumerz, PhD student).

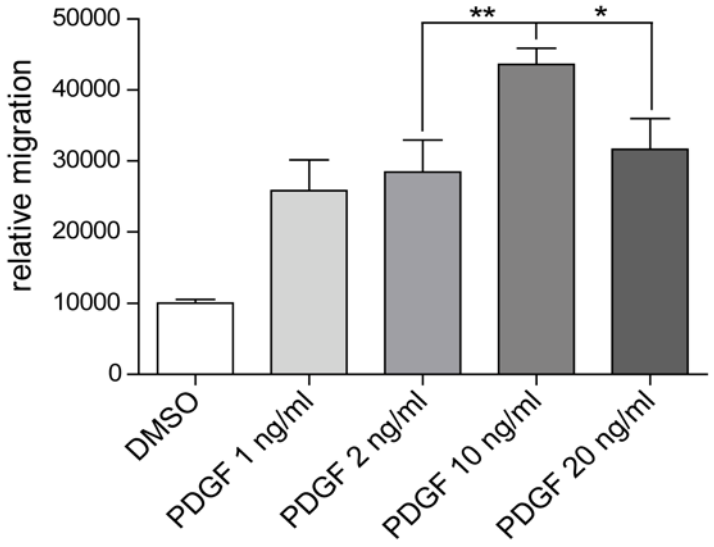

Fig.30. Concentration-dependent increase in PDGF-BB-induced VSMC migration. *Serum-starved, scratched VSMC were pretreated for 30 min vehicle (DMSO 1%) prior to stimulation with PDGF-BB (1-20 ng/ml) for 20 h. Scratches were captured at 0 h and after 20 h and cell free areas were calculated using Cellprofiler software (www.cellprofiler.org). Means of 3 independent experiments ± SEM. * P<0.05, ** P<0.01 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment). Data were in part generated by Mario Kumerz, PhD student.*

Therefore 10 ng/ml of PDGF-BB is a reasonable concentration to trigger VSMC migration. We used two different models to assess the antimigratory potential of I3MO. First we studied a transwell or modified Boyden-chamber model often referred to as model for directed migration towards a chemoattractant [176]. In this assay the cells have to actively migrate through a membrane with a defined pore size along a PDGF-BB gradient. Fig.31

shows, that I3MO potently and dose-dependently inhibited PDGF-BB directed migration. Interestingly we found that I3MO inhibited migration within the same concentration range as it inhibited proliferation shown by Andrea Schwaiberger (around 3 µM).

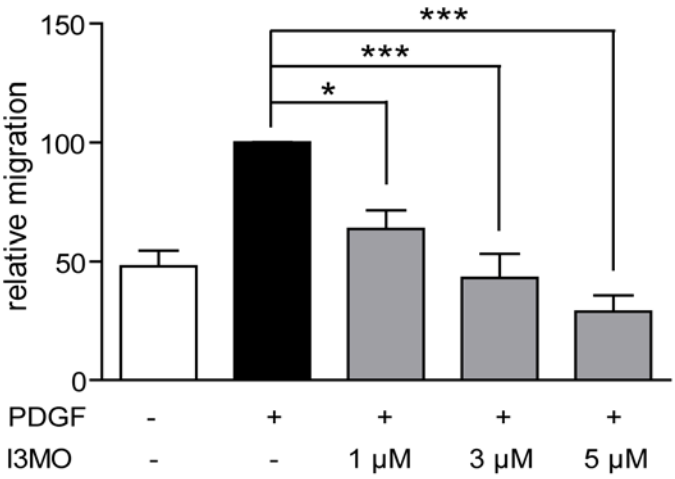

Fig.31. Influence of I3MO on PDGF-BB directed migration*. Serum-starved and calcein AM stained VSMC were seeded into transwell cell culture inserts (pore size 8 µM), pretreated for 30 min with I3MO (1-5 µM) or vehicle (DMSO 1%) and stimulated with PDGF-BB (10 ng/ml) for 5 h. Cells were trypsinized and washed of the bottom side of the inserts. Luminescence of migrated cells was measured in a 96-well plate reader (TECAN GENios Pro™). The means of 3 independent experiments ± SEM are shown. * P<0.05, *** P<0.001 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment).*

Additionally we employed a simpler scratch or wound healing assay, where a scratched cell monolayer is closed by migrating cells in the presence of PDGF-BB. Here no gradient of the chemoattractant is present, and therefore the model is used to assess rather undirected migration. Also in this assay I3MO potently inhibited migration within the same concentration range as in the transwell system (Fig.32).

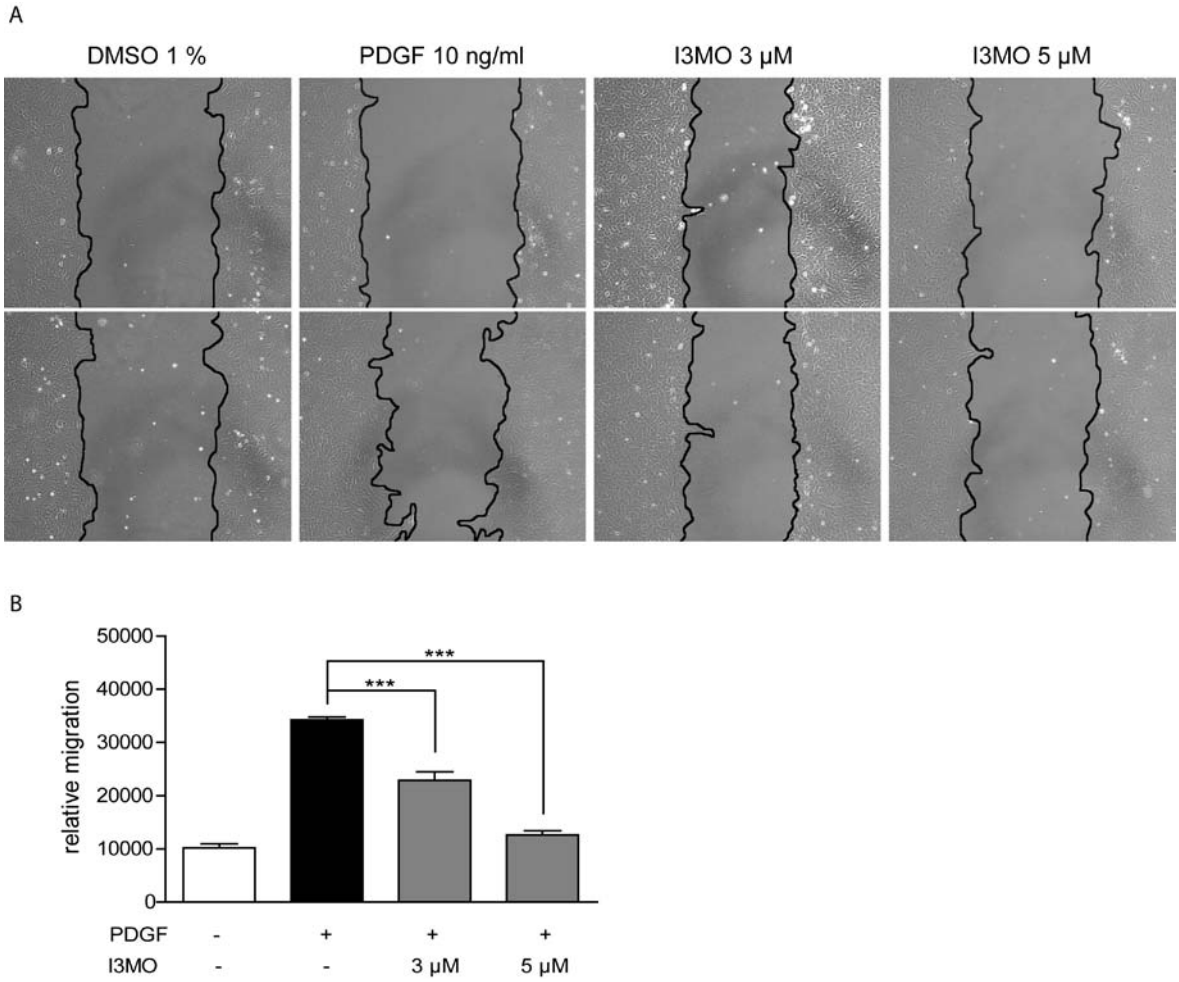

Fig.32. Influence of I3MO on PDGF-BB induced migration in a wound healing assay*. Serum-starved, scratched VSMC were pretreated for 30 min with I3MO (3-5 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 20 h. Scratches were captured at 0 h and after 20 h and cell free areas were calculated using Cellprofiler software. (**A**) Representative photographs of scratches. (**B**) Means of 5 independent experiments ± SEM. *** P<0.001 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment).*

In contrast to the transwell assay where cells are seeded and immediately allowed to migrate, cells have to detach, migrate and reattach to the surface in the scratch assay. Therefore the migration is measured after 20 h in contrast to the rather short migration time of 5 h in the transwell assay. Although VSMC show a generation time exceeding 20 h (26-28 h, see Fig.22), it had to be ruled out that proliferation contributes to the closure of the scratch. Therefore we used Mitomycin C (MitoC), a well known antiproliferative agent, which is widely used to block cell division in migration assays. It is known to induce a S-phase arrest in many different cell types by inhibiting DNA-synthesis. MitoC has been described to inhibit VSMC proliferation when used in doses from 1-300 nM by an arrest in

S-phase [179]. Fig.33 shows that 100 nM of MitoC limit cell proliferation but not migration of PDGF-BB-stimulated VSMC. Hence our experimental setup indeed allows us to study exclusively cell migration not proliferation.

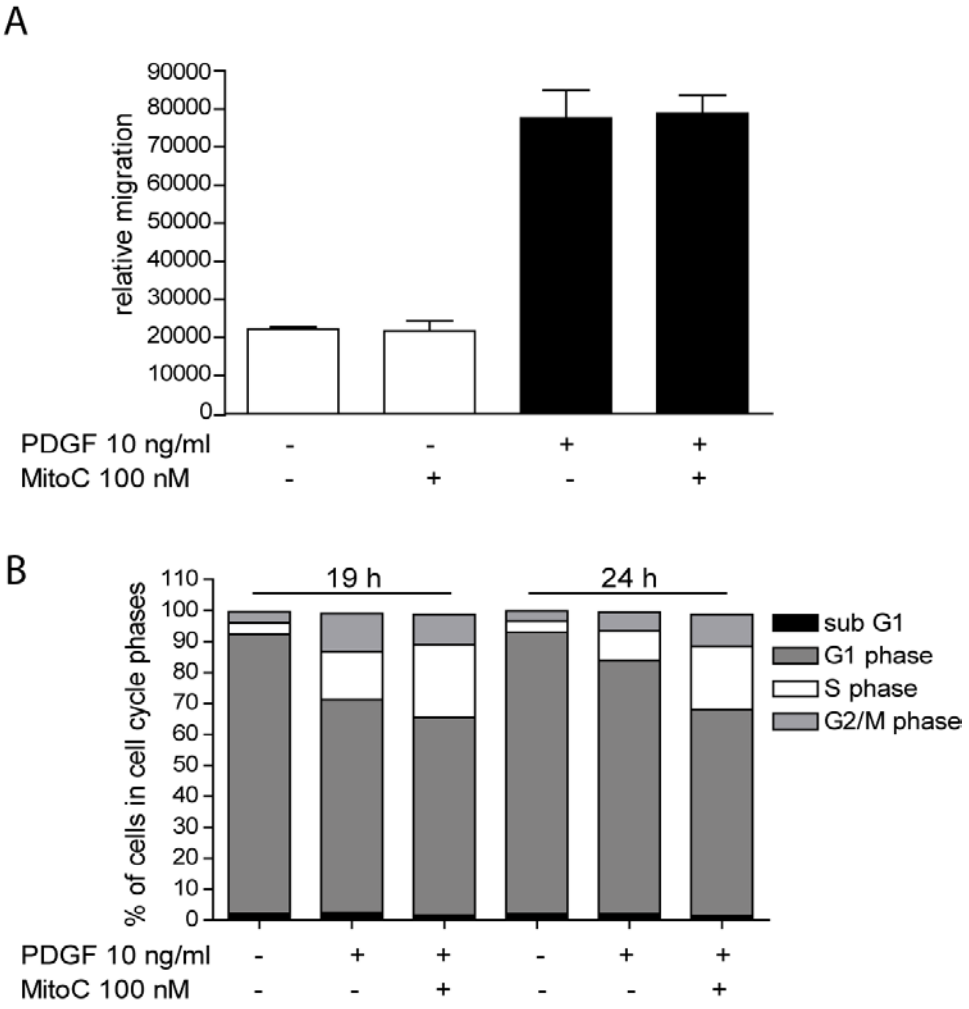

***Fig.33. Influence of Mitomycin C on PDGF-BB-induced migration and proliferation**. (**A**) Serum-starved, scratched VSMC were pretreated for 30 min with 100 nM Mitomycin C or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 20 h. Scratches were captured at 0 h and after 20 h and cell free areas were calculated using Cellprofiler software. Shown are the means of three independent experiments ± SEM. *** P<0.001 (one-way ANOVA followed Bonferroni post-test versus PDGF-treatment). (**B**) Serum-starved VSMC were pretreated for 30 min with 100 nM Mitomycin C or vehicle (DMSO 1%) and cultured in the presence of PDGF (20 ng/ml). After 20 h the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI-stained nuclei. Shown are the means of three independent experiments.*

I3MO clearly inhibited PDGF-BB-induced VSMC migration but as impaired migration could at least partly be explained by impaired adhesion, we wanted to investigate whether I3MO would interfere with cell adhesion. For this purpose serum-starved cells were pretreated

with I3MO or vehicle, seeded into collagen I-coated 96-well plates and allowed to attach for 3 h. Afterwards the non-adherent cells were washed away and remaining cells were stained with crystal violet. Fig.34 shows that I3MO did not interfere with cell adhesion. Hence the observed antimigratory effect of I3MO is not due to its antiproliferative activity, or an interference with VSMC adhesion.

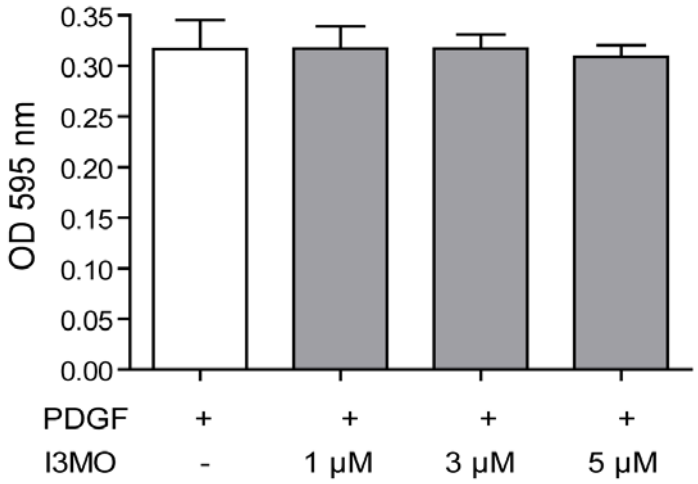

Fig.34. Influence of I3MO on cell adhesion*. Serum-starved VSMC were pretreated for 30 min with I3MO (1-5 µM) or vehicle (DMSO 1%), seeded into collagen I-coated 96-well plates and stimulated with PDGF-BB (10 ng/ml) for 3 h. Cells were stained with crystal violet and solubilised dye was spectroscopically quantified at OD 595 nm. Shown are the means of 3 independent experiments ± SEM.*

Influence of I3MO on Rac-1 activation

Key molecules in early migratory signalling are the small G-proteins Rac-1, Rho and cdc42 (Fig.6). Rac-1 has additionally been reported to play a critical role in VSMC proliferation and neointima formation [181]. As I3MO impairs both migration and proliferation, we investigated the potential influence of I3MO on Rac-1 activation by GTP pulldown assay. Fig.35 shows there was no such inhibition by I3MO upon PDGF-BB stimulation.

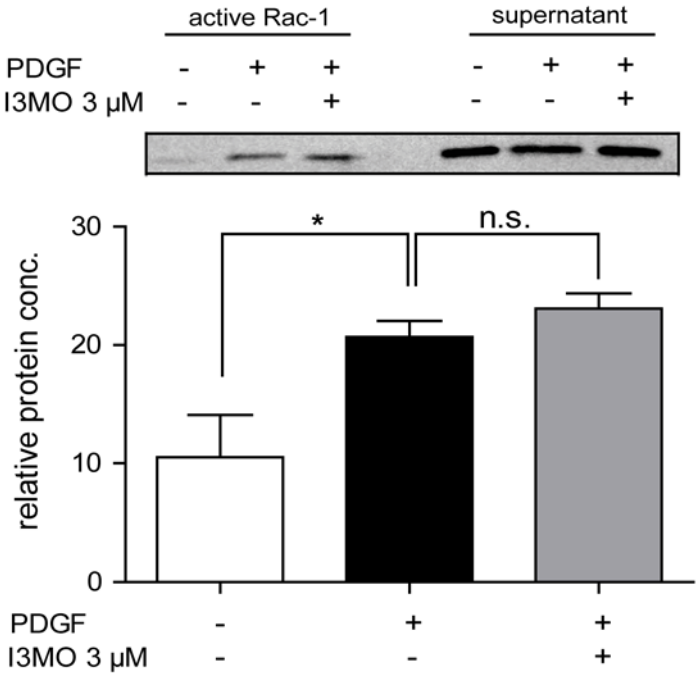

Fig.35. Influence of I3MO on Rac-1 activation in PDGF-activated VSMC*. Serum-starved VSMC were pretreated for 30 min with I3MO (3-5 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (20 ng/ml) for 4 min. Total cell lysates were then used for a Rac-1 activity assay where active Rac-1 is pulled down and subjected to western blot analysis. A representative western blot out of three independent experiments with consistent results is shown. Graph depicts compiled data of the densitometric evaluation. Shown are means ± SEM. n.s. not significant, *p<0.05 (paired t-test versus PDGF-treatment).*

Furthermore we wanted to investigate RhoA and cdc42 as well, but we failed to pull down active RhoA and found that PDGF-BB did not change the activity of cdc42 (data not shown, experiments done by Mario Kumerz, PhD student).

In summary we found no interference of I3MO with PDGF-BB-induced small GTPase signalling. Other immediate early signalling events downstream of the activated PDGFR play a role in migration, too (as discussed in section 2.7.2). However, Andrea Schwaiberger had already excluded inhibitory effects of I3MO on MAPK and Akt signalling but had found an inhibition of STAT phosphorylation. Therefore we next asked the question whether the inhibitory effect of I3MO on VSMC migration is linked to STATs.

I3MO inhibits STAT3 phosphorylation without affecting other common early signalling pathways

As mentioned previously it has been shown in our lab that I3MO specifically inhibits phosphorylation of STAT1/3/5 without affecting activation of other common early signalling pathways downstream of the PDGF-BB receptor (p38, ERK, Akt) [154]. Andrea Schwaiberger in her thesis stated that the antiproliferative activity of I3MO is most likely linked to the inhibition of STAT3. There are indeed some hints from literature suggesting that STAT3 also is involved in cell migration (see section 2.7.2.2).

First of all we aimed to repeat the key experiment of Andrea Schwaiberger on STAT3 phosphorylation using 10 ng/ml of PDGF-BB as used for the migration assays instead of 20 ng/ml used by Andrea Schwaiberger (data generated by Christa Czaloun, diploma student 2009). PDGF-BB in this concentration also led to the activation of ERK and STAT3 and I3MO again completely abolished STAT3 phosphorylation even below the basal level without affecting ERK (Fig.36). Since STAT3 phosphorylation is enhanced even with the lower amount of PDGF-BB it is possible that STAT3 indeed plays a role in mediating the antimigratory effect of I3MO.

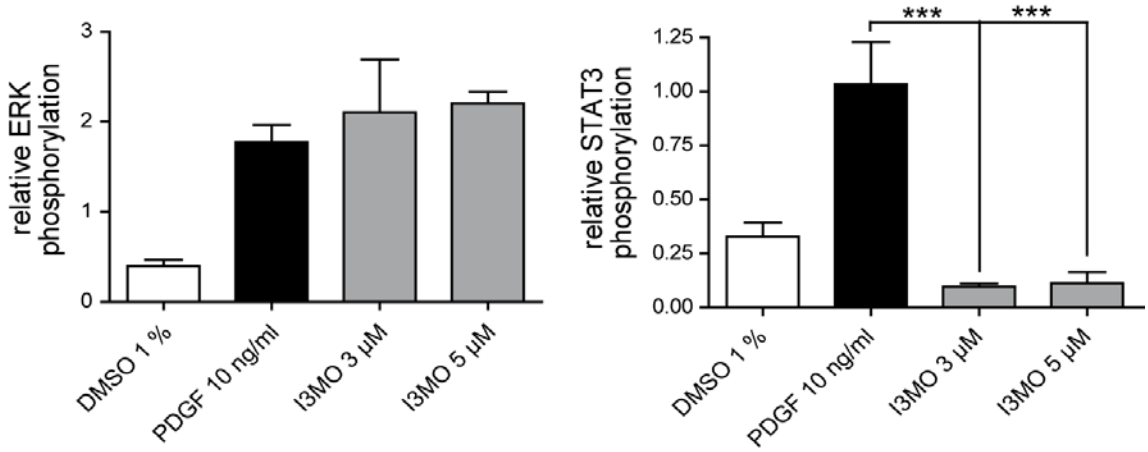

Fig.36. Influence of I3MO on early signalling events in PDGF-activated VSMC*. Serum-starved VSMC were pretreated for 30 min with I3MO (3-5 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 10 min. Total cell lysates were then subjected to western blot analysis for phosphorylation of ERK, STAT3 and total α-tubulin. Graphs depict compiled data of the densitometrically evaluated phospho-protein/tubulin ratios. Shown are means of three independent experiments ± SEM. ***p<0.001 (one-way ANOVA followed by a Bonferroni post-test versus PDGF-treatment). Data were generated by Christa Czaloun, diploma student.*

Comparison of I3MO with chemical inhibitors of common signalling pathways

Influence on PDGF-BB-induced migration

Since we hypothesised that STAT3 plays a role in I3MO effects, we next wanted to compare the antimigratory potential of I3MO to chemical inhibitors of signalling pathways potentially involved in the PDGF-BB-stimulated activation of STAT3.

Basically there are two kinases suspected to be responsible for STAT3 phosphorylation in response to PDGF-BB, namely JAK2 and Src (see chapter 2.7.2.2). Therefore we wanted to investigate whether inhibitors of these kinases would also affect migration. In Fig.37 it can be seen, that the Src inhibitor SU6656 indeed inhibits migration to the same extent as I3MO does. For the JAK-2 inhibitor AG490 we also observed an inhibition. However, this was only seen using rather high concentrations (25-50 µM). However, AG490 is reported in the literature to be used from 10-100 µM [103, 182-184].

As Andrea Schwaiberger showed that STAT3 activation was redox-dependent we additionally employed the general flavoprotein inhibitor DPI in the migration assay. This compound affects several proteins, amongst others also the Nox-family of proteins which is in part responsible for ROS formation in response to PDGF-BB. Again we observed an inhibitory effect on VSMC migration when pretreating the cells with 3 µM of DPI.

In conclusion several inhibitors of STAT3 activation, namely a Src, a JAK2 and a Nox inhibitor showed a comparable antimigratory effect to I3MO on PDGF-BB induced migration of VSMC.

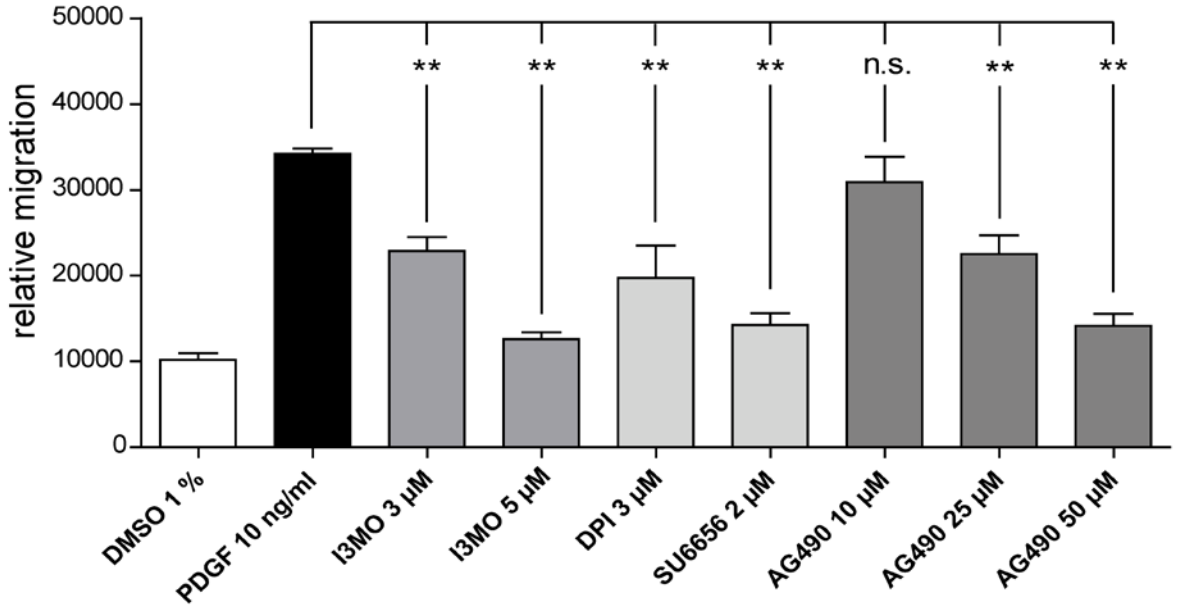

Fig.37. Influence of chemical inhibitors on PDGF-BB-induced migration in a wound healing assay*. Serum-starved, scratched VSMC were pretreated for 30 min with I3MO (3-5 µM), DPI (3 µM), SU6656 (2 µM), AG490 (10-50 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 20 h. Scratches were captured at 0 h and after 20 h and cell free areas were calculated using Cellprofiler software (www.cellprofiler.org). The means of three independent experiments ± SEM are shown. n.s. not significant, ** P<0.01 (one-way ANOVA followed by a Dunnett post-test versus PDGF-treatment).*

As I3MO is also sold as a GSK3 inhibitor, we investigated the influence of the known GSK3 inhibitor LiCl on PDGF-BB induced migration (data generated by Christa Czaloun, diploma student) [185]. Indeed LiCl at a concentration of 20 mM potently inhibits migration (Fig.38). This could not simply be explained by osmotic changes because the same concentrations of MgCl or NaCl did not lead to the same results.

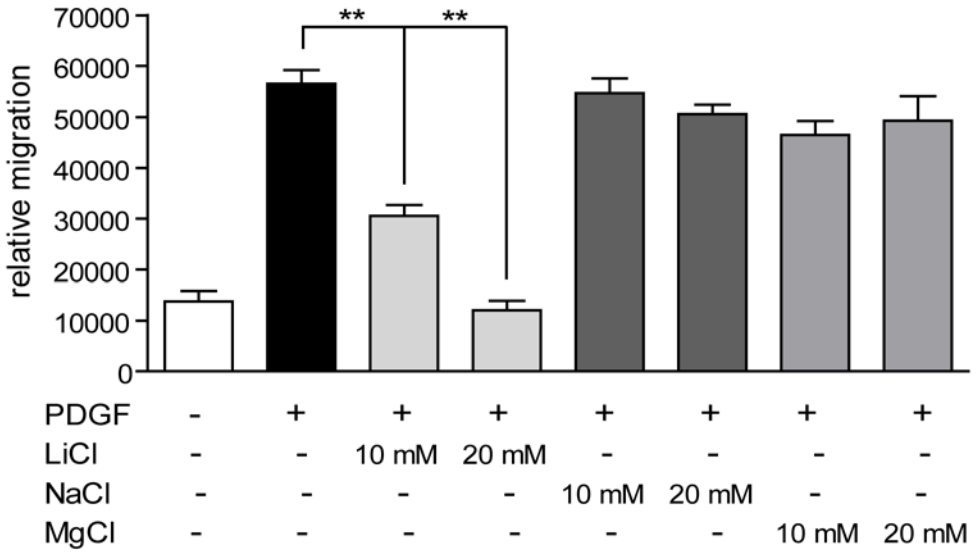

Fig.38. Influence of LiCl, NaCl and MgCl on PDGF-BB induced migration. *Serum-starved, scratched VSMC were pretreated for 30 min with 10 mM/ 20 mM of LiCl, NaCl or MgCl or with vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 22 h. Scratches were captured at 0 h and after 22 h, and cell free areas were calculated using Cellprofiler software (www.cellprofiler.org). Means of 5 independent experiments ± SEM. ** P<0.001 (one-way ANOVA followed by Dunnett post-test versus PDGF-treatment). Data generated by Christa Czaloun, diploma student.*

All together the data generated using chemical inhibitors on VSMC migration point in the direction of STAT3 inhibition. Several inhibitors of STAT3 signalling showed the same effects as I3MO. But an involvement of other I3MO targets as e.g. GSK3 can not be ruled out at this point.

Influence on PDGF-BB-induced phosphorylation of STAT3

Consequently we wanted to explore whether next to the ability to inhibit migration the chemical inhibitors would indeed also impair STAT3 phosphorylation as I3MO does. Andrea Schwaiberger showed that both 2 µM SU6656 and 3 µM DPI inhibited STAT3 phosphorylation whereas AG490 in a rather low dose (10 µM) had no influence [154]. We therefore repeated the experiment only for AG490 in higher concentrations (up to 50 µM). In Fig.39 it can be seen, that AG490 even at the highest concentration of 50 µM only slightly and not significantly reduces STAT3 phosphorylation (data generated by Christa Czaloun). This makes it not very likely that AG490 and I3MO share the very same target, namely JAK2.

LiCl did not affect STAT3 phosphorylation either (Fig.39) (data generated by Christa Czaloun). This was not very surprising because there are only a few articles linking GSK3 to STAT3 and none of them investigated VSMC or PDGF signalling. Where a link has been shown it was attributed to a kind of platform function for GSK3 bringing together STAT3 and its upstream kinase [186]. This function would not require GSK3 kinase activity which is inhibited by LiCl.

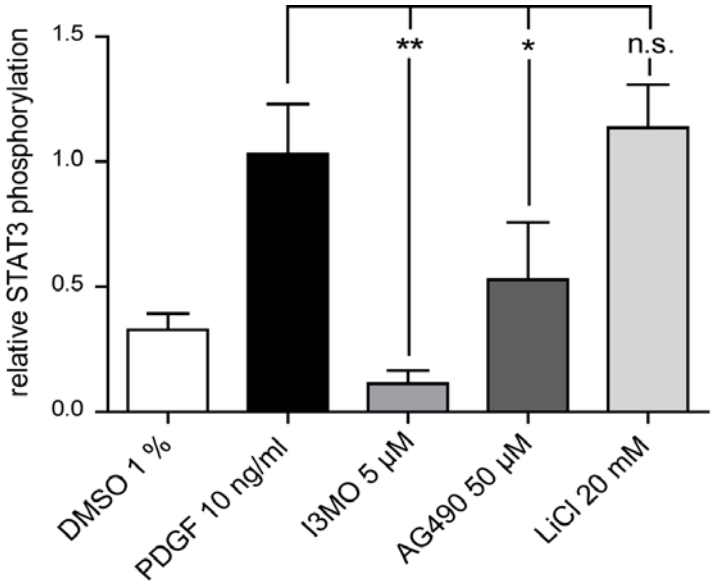

Fig.39. Influence of AG490 and LiCl on STAT3 phosphorylation in PDGF-activated VSMC*. Serum-starved VSMC were pretreated for 30 min with I3MO (5 µM), AG490 (50 µM), LiCl (20 mM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 10 min. Total cell lysates were then subjected to western blot analysis for phosphorylated STAT3 and total α-tubulin. Graphs depict compiled data of the densitometrically evaluated phospho-protein/tubulin ratios of three independent experiments. The means ± SEM are shown. n.s. not significant, **p<0.01 (paired t-test versus PDGF-treatment). Data were generated by Christa Czaloun*

Influence of I3MO on STAT3 target genes

We showed that several chemical inhibitors of STAT3 signalling (DPI, SU6656, AG490) inhibited VSMC migration as well as STAT3 phosphorylation comparable to I3MO. As STAT3 is a transcription factor we next wanted to investigate the influence of I3MO on STAT3 target genes. STAT3 affects amongst others the expression of the cytosolic phospholipase A2 (cPLA2) that has been shown to be involved in PDGF-BB migration [103]. Also it influences the expression of cyclinD1 that is important not only for cell cycle progression but has been reported to be involved in migration, too [187, 188].

We therefore tested the ability of I3MO to interfere with cPLA2 and cyclin D1 expression under the same conditions used for the transwell migration assay. Consequently we used

freshly seeded cells and a stimulation time of 5 h. Fig.40 shows that I3MO indeed inhibited the expression of cPLA2 and cyclin D1 upon PDGF-BB stimulation. However, the induction of cPLA2 by PDGF-BB was low (about 1.5 fold). Therefore we studied longer timeframes (6-20 h) with standard conditions resembling the situation of the scratch assay (Christa Czaloun, diploma thesis). Under these conditions the induction of cPLA2 by PDGF-BB was a little bit stronger (about 1.7 fold). Again I3MO inhibited the PDGF-BB-induced expression of this STAT3 target gene (data in Christa Czaloun, 2009, diploma thesis).

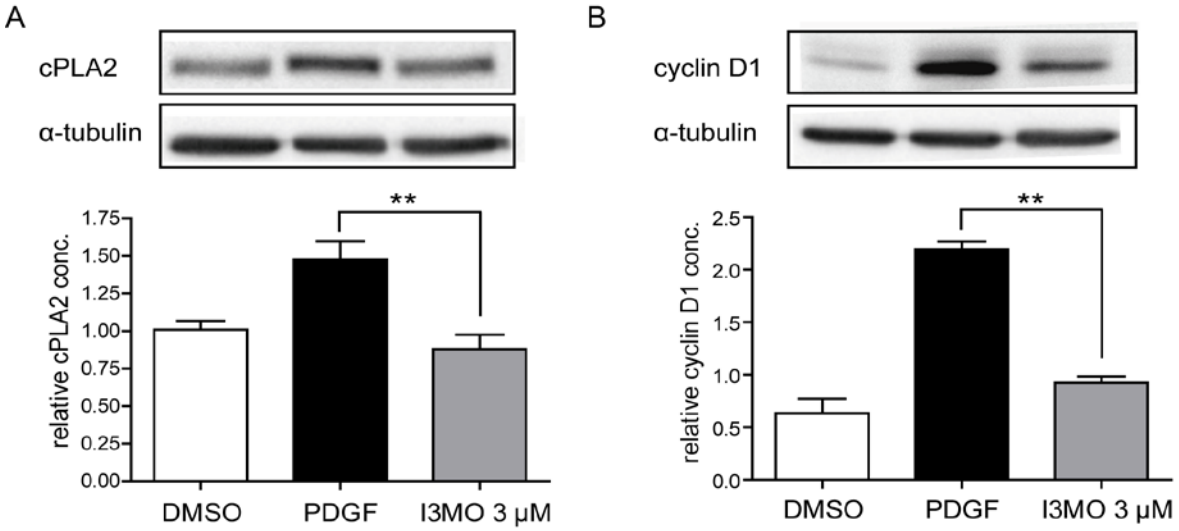

Fig.40. Influence of I3MO on cPLA2 and cyclin D1 expression in PDGF-activated VSMC*. Serum-starved VSMC were seeded freshly and subsequently pretreated for 30 min with I3MO (3 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for 5 h. Total cell lysates were then subjected to western blot analysis for expression of (**A**) cPLA2, (**B**) cyclin D1 and total α-tubulin. Representative western blots out of at least three independent experiments with consistent results are shown. Graphs depict compiled data of the densitometrically evaluated protein/tubulin ratios. Shown are means ± SEM. n.s. not significant, ** p<0.01 (paired t-test versus PDGF-treatment).*

In summary we showed that I3MO not only inhibits PDGF-BB-induced migration and phosphorylation of STAT3 but also expression of STAT3 target genes that are linked to migration.

Influence of I3MO on reorganisation of the actin cytoskeleton

We showed that I3MO inhibits VSMC migration and that this effect could probably be linked to STAT3 or GSK3 but a link between early effects on signalling and migration was

still missing. To further limit the possible targets of I3MO, or to identify possible mediators of its effects we aimed to investigate its influence on early steps of migration. During the first hours of PDGF-BB activation the VSMC start to rearrange the cytoskeleton in order to detach from the surface and migrate. This can be visualized using the actin binding fungal toxin phalloidin. Already as early as 1 h upon PDGF-BB treatment actin ruffles and stress fibres can be observed (Fig.41). But the effects are much better visible after 2 h. I3MO blocks the formation of actin ruffles and seems to reduce the number of actin stress fibres observed in the PDGF-BB-activated VSMC. This is indicating an interference with early steps in cell migration.

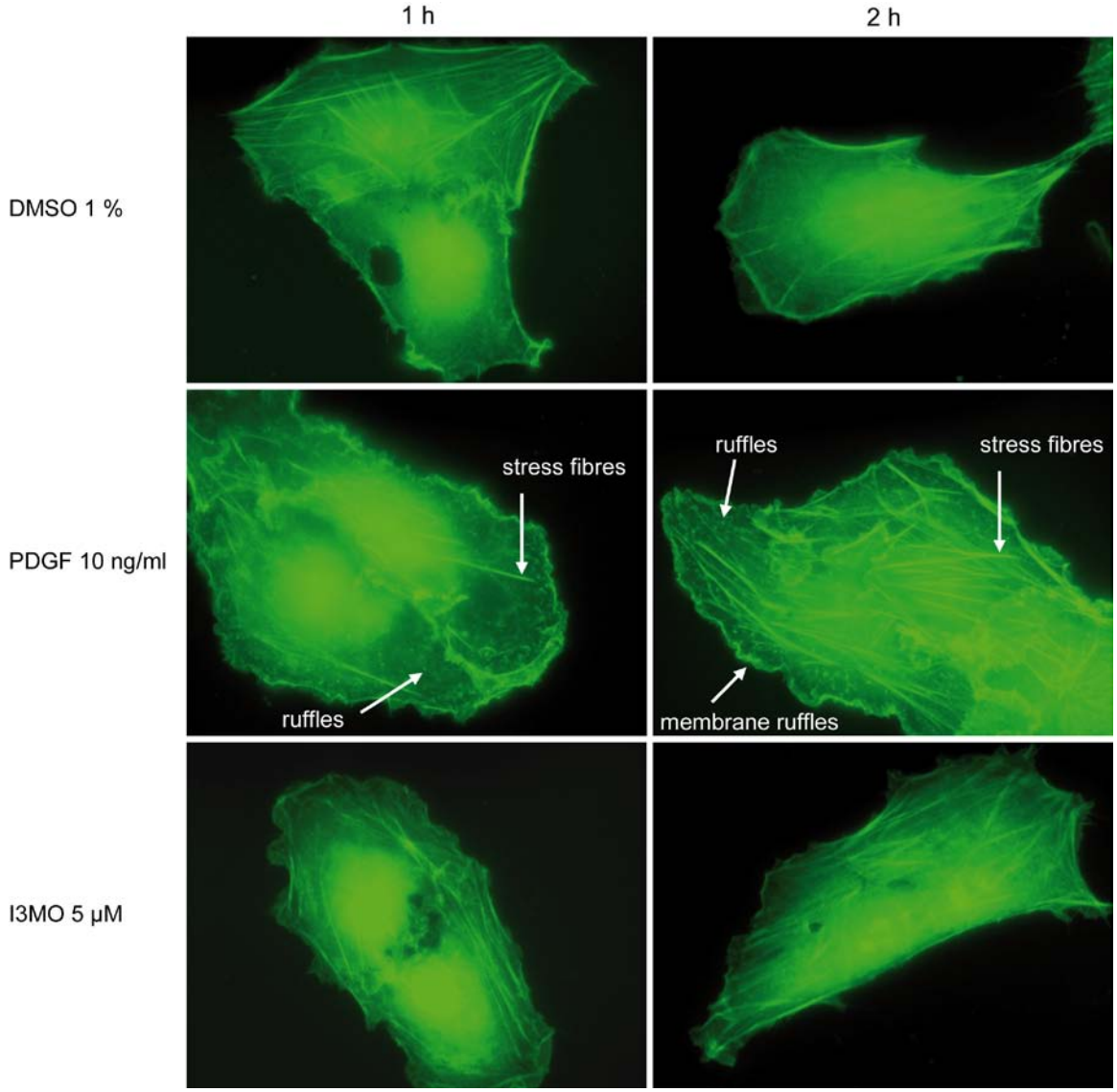

Fig.41. Influence of I3MO on rearrangement of actin cytoskeleton in PDGF-activated VSMC*. Serum-starved VSMC were pretreated for 30 min with I3MO (3-5 µM) or vehicle (DMSO 1%) prior to stimulation with PDGF-BB (10 ng/ml) for the indicated periods of time. Cells were fixed and stained with fluorescence-labled phalloidin. Membrane ruffles and big actin fibres (stress fibres) are marked with arrows.*

Later on cells also form thin protrusions (filopodia) and broader ones (lamellipodia) (data not shown). However, so far we did not analyse the influence of I3MO on these cellular rearrangements. The results shown here on cytoskeletal rearrangements are rather preliminary and further research with more sophisticated staining methods is needed to determine the exact effects of I3MO on VSMC cytoskeleton.

In conclusion we report for the first time that I3MO not only inhibits PDGF-BB-induced proliferation but also migration of VSMC. This effect is probably linked to the specific inhibition of STAT3 phosphorylation exerted by I3MO. We furthermore showed that I3MO also inhibits the expression of STAT3 target genes that are linked to migration.

Influence of LiCl on PDGF-BB induced cell cycle progression of VSMC

The effect of LiCl on migration was impressive and we therefore wanted to examine whether the salt can also interfere with PDGF-BB-induced cell cycle progression. Indeed LiCl leads to an accumulation of cells in G1-phase of the cell cycle (Fig.42). Whether both effects of LiCl (inhibition of migration and proliferation) are due to inhibition of GSK3 or another target would be an interesting question to answer in future studies. Also it would be worthwhile to investigate whether I3MO and LiCl share the very same target as their effects on PDGF-BB-induced proliferation and migration of VSMC seems to be very similar.

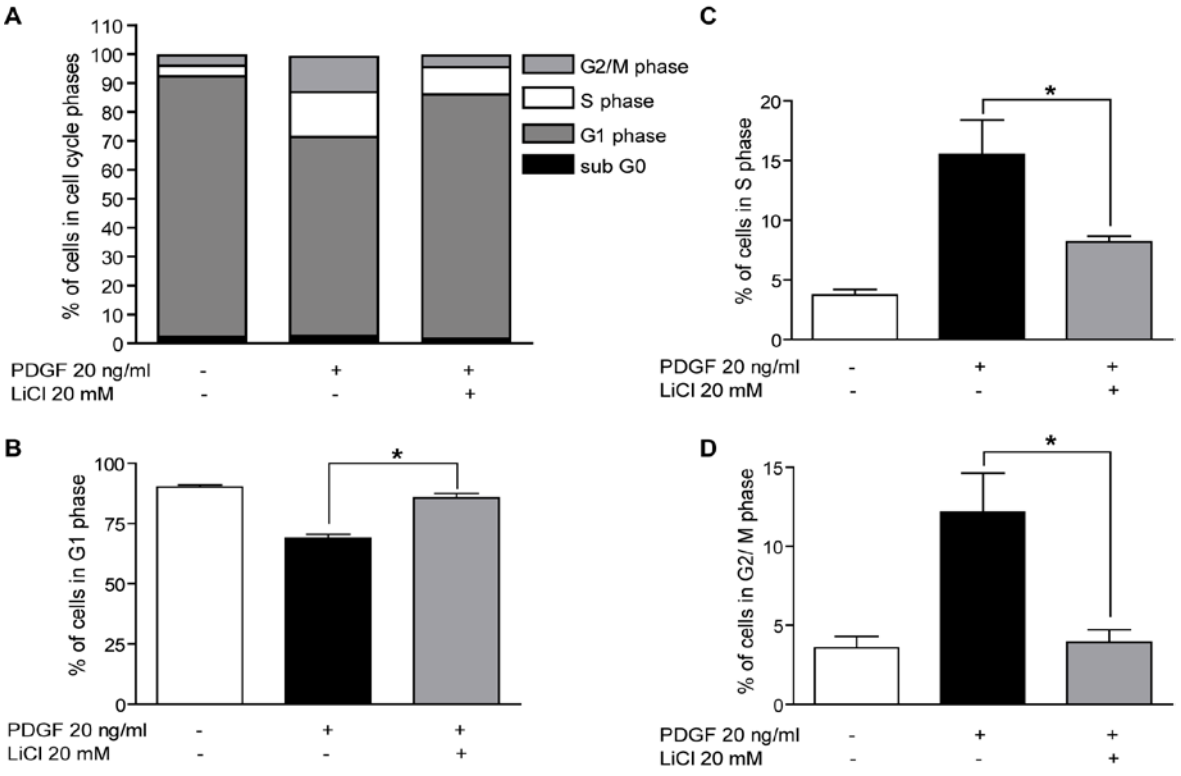

***Fig.42. Influence of LiCl on cell cycle distribution upon PDGF-BB-stimulation**. Serum-starved VSMC were pretreated for 30 min with 20 mM LiCl or vehicle (DMSO 1%) and cultured in the presence of PDGF (20 ng/ml). After 18 h the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI-stained nuclei. The graphs show (**A**) an overview of all cell cycle phases, (**B**) cells in G1-phase, (**C**) cells in S-phase and (**D**) cells in G2/ M phase. Shown are the means of three independent experiments ± SEM, *p<0.05 (paired t-test versus PDGF-treatment).*

5. DISCUSSION

Roscovitine and Roscovitine derivatives

Searching for novel antiproliferative compounds acting on VSMC we investigated for the first time the antiproliferative effect of ROSC and its derivative LGR1406 on PDGF-BB-mediated vascular smooth muscle proliferation. We show that both compounds inhibit proliferation in VSMC without affecting early signalling pathways downstream of the activated PDGFR (Fig.25). LGR1406 is hereby the far more potent antimitotic agent with an apparent IC_{50} of 3.0 µM in the BrdU assay compared with an IC_{50} of 17 µM of ROSC (Fig.16). A detailed molecular analysis showed that both compounds affect entry of cells into S-phase of the cell cycle, expression of cyclin A and E as well as phosphorylation of pocket proteins presumably by inhibition of CDK activity (Fig.20, Fig.27, Fig.28). But whereas ROSC only delayed these events at 20 µM when compared to control cells, LGR1406 elicited complete inhibition at 5 µM.

Although ROSC has been studied extensively in different cell types for its antiproliferative influence there are only two publications investigating its effect on VSMC [37]. Both studies do not provide data about ROSC and PDGF signalling, although PDGF-BB is considered the major proliferative stimulus for VSMC, also in pathological hyperplasia [144].

However, one study uses the hypertrophic factor Angiotensin II (AngII) and identifies ROSC as inhibitor of ERK activity [189]. Besides the distinct stimulus our study also differs in the treatment protocol from the work of Li et al. Whereas Li et al. pretreated their cells for 15 h with ROSC prior to AngII stimulation, we only preincubated VSMC with ROSC and LGR1406 for 30 min prior to PDGF-stimulation. 15 h of preincubation with ROSC allows changes in protein expression pattern between treated and untreated cells that make a distinction between direct and indirect effects on AngII-triggered signalling rather difficult. Moreover, Li et al. used 30 µM of ROSC which already elicited first signs of toxicity in our cells. In order to rule out a common mode of action, namely inhibition of ERK activity, underlying our and their observation, we also pretreated our cells for 15 h or with up to 25 µM of ROSC and 10 µM of LGR1406 and examined PDGF-mediated phosphorylation of ERK and other MAPK (Fig.26). Even with these altered settings we did not find a reduced phosphorylation of either of the kinases. Furthermore the expression of cyclin D1, a known target gene of ERK is not inhibited by ROSC or LGR1406 (Fig.27)[145]. Therefore we conclude that the effects on PDGF-BB-induced cell cycle progression we observed for ROSC and LGR1406 were not due to interference with ERK signalling. Notably cyclin D1 levels are even enhanced by ROSC and LGR1406 which may be due to a disturbed negative feedback loop between cell cycle progression and cyclin D1 expression.

The second study investigating ROSC and VSMC used serum as proliferative stimulus [121, 190, 191]. Here the investigators focused on differences in growth of human VSMC from healthy vessels and in-stent-restenosis (ISS) lesions. Since ISS-VSMC expressed high levels of cyclin E and A, they employed ROSC as known CDK2 inhibitor. Consistent with our results, ROSC led to a delayed cell cycle progression and most surprisingly ISS VSMC were more susceptible to ROSC treatment than those isolated from healthy vessels. These findings additionally highlight ROSC as an interesting lead for an anti-restenotic therapy. They also showed that higher concentrations of ROSC (30-40 µM) led to reduced viability of VSMC which supports the use of lower concentrations as we did in our study (15-25 µM).

Comparing ROSC and LGR1406 we clearly demonstrate that ROSC does not completely block PDGF-mediated cell cycle progression of VSMC whereas its novel derivative LGR1406 does. More precisely, the S-phase transition is blocked by LGR1406 but only delayed by ROSC. Given the similar IC_{50} values *in vitro* and thus a comparable affinity of both compounds for CDK2-the kinase responsible for S-phase entry-the observed distinct effects of ROSC and LGR1406 might be either due to differences in their ability to permeate cells, their chemical or metabolic stability or due to targets that might be affected by LG1406 in addition to CDK2. Since ROSC is currently undergoing phase-II clinical trials against breast cancer, there are multiple publications addressing its metabolism, pharmacokinetic and stability issues [190]. ROSC is stable in human plasma up to 48 h at 0, 25 and 37 °C and insensitive to multiple freeze-thawing cycles but easily binds to serum albumin [192]. Therefore it is likely that chemical stability in cell culture medium is not responsible for the weaker activity of ROSC.

Recently a detailed study on ROSC metabolism *in vitro* and *in vivo* has been published [193]. McClue et al. showed that ROSC is rapidly metabolised by rat liver microsomes, leading to a 50% reduction of parent compound after 90 min incubation. The major metabolite found was a carboxylate (PMF30-128), which has been shown to have exceedingly higher IC_{50} values against CDK1/4 than ROSC itself but only slightly lower activity against CDK2 [194, 195]. McClue et al. also identified the responsible P450 proteins, namely CYP3A4 and CYP2B6. A panel of different P450 proteins including CYP3A4 has also been found to be expressed in VSMC [196]. It is therefore conceivable, that in VSMC ROSC is differently or faster metabolised than LGR1406 and thus exerts reduced or only transient activity towards its CDK targets. In contrast to LGR1406 ROSC is carrying a hydroxyl group which is preferentially used for metabolising steps.

Another explanation for the different impact of ROSC and LGR1406 on PDGF-BB induced growth of VSMC could be the lower IC_{50} of LGR1406 against CDK4 (15 µM vs. > 75 µM). Although the IC_{50} value (15 µM) is higher than the concentration used for our experiments (5 µM) it is possible that LGR1406, at least to some extent inhibits CDK4 activity and hereby blocks progression through the cell cycle earlier than ROSC. This notion is supported to some extent by the results of kinase assays for another ROSC-derivative which was less potent concerning antiproliferative activity than LGR1406 but more potent than ROSC, namely LGR1492. LGR1492 showed with 22 µM an intermediate IC_{50} against CDK4 compared to 15 µM for LGR1406 and 75 µM for ROSC (Table II, Table X) reflecting the results for antiproliferative activity. Nevertheless this could not account for the fact, that ROSC does not completely block cell cycle progression in later CDK4-independent stages.

Finally, an alternative reason for the differences between ROSC and LGR1406 could be found in the redundancy of CDKs themselves. It has been shown that in CDK2 gene targeted mice CDK1 can back-up for CDK2 [197]. As both ROSC and its major metabolite display higher IC_{50} values against CDK1 than LGR1406 it is plausible that although ROSC blocks CDK2, CDK1 compensates to some extent for its activity and drives cell cycle progression, whereas in LGR1406-treated cells CDK1 is also inhibited.

It can, however, at this point not be ruled out that LGR1406 has an additional, exclusive and so far unknown target. For example LGR1406 strongly affects Aurora A (Aur-A) kinase that is not inhibited at all by ROSC. Aur-A, however, is involved only in very late stages of cell cycle progression and its activity peaks during the G2/ M transition where it is critical for proper formation of the mitotic spindle [198]. It should be noted that there is a recent publication showing that Aurora B in a complex with survivin and mTOR regulates G1 to S-phase transition in T-cells by promoting Rb phosphorylation, cyclin A expression as well as Cdk1 and Cdk2 activity. Since Aur-A and Aur-B are structurally very similar (they share 71% identity in their C-terminal catalytic domain), it is likely that LGR1406 inhibits both kinases what would have to be confirmed in future experiments [199]. But there is no evidence yet that either of them play a role in VSMC G1 to S-phase transition [200]. There has been another dual inhibitor of CDKs and Aurora kinases described (JNJ-7706621) and it might be worth investigating whether this compound has similar effects on VSMC as LGR1406 [201, 202].

Taken together, our data on key regulators of the cell cycle and *in vitro* kinase assays support the conclusion that LGR1406 is a more potent CDK inhibitor than ROSC. Possibly LGR1406 is more resistant against metabolism and/ or more prone to cross the cell membrane. The latter points have to be confirmed in further studies.

An interesting final outcome was the observation that ROSC exerts a stronger antimigratory effect than LGR1406 in concentrations that were also used for antiproliferative assays. ROSC (20 µM) inhibited PDGF-BB-induced migration by 65% compared to only 23% of inhibition by LGR1406 (5µM) (Fig.29). It is not a complete novel finding that ROSC is able to inhibit migration because there are some publications addressing this issue in cancer cells (e.g.[201]). However, we are the first to demonstrate such an effect in VSMC.

In prostate cancer cells ROSC treatment was shown to result in changes in the cytoskeleton and hence loss of cellular polarity and motility [202]. Moreover Miyamoto and colleagues showed that ROSC impaired the PDGF-dependent migration of primary rat oligodendrocyte precursor cells and could mimic this effect using retroviral CDK5 shRNA knockdown [203]. The authors of these and other studies therefore linked the antimigratory effect of ROSC to inhibition of CDK5. In keratinocytes ROSC inhibited adhesion of cells to fibronectin and hence migration, associated with reduced active beta1 integrin [127]. Also here the authors state that the effects observed were due to CDK5 inhibition. As shown in Table II and Table X, ROSC and LGR1406 inhibit CDK5 with comparable IC_{50} values of 0.16 µM and 0.4 µM, respectively. Therefore we would expect to see similar effects of both substances on VSMC migration if they were CDK5 mediated. Moreover we were not able to detect CDK5 protein in western blot analysis in VSMC (data not shown), and there are no data available from literature showing CDK5 protein levels in VSMC. However, there were two publications in the early nineties showing mRNA levels for CDK5 in VSMC. Hence it can not be ruled out that ROSC indeed impairs VSMC migration via the inhibition of CDK5. If this is the case it would be interesting to investigate why LGR1406 does not exert the very same effect. It is also still possible that ROSC inhibits another kinase that is not affected by LGR1406. Among the published 150 kinases tested for inhibition by ROSC in enzyme-based assays there is none that seems really promising concerning migration [117]. But as there are much more protein kinases known in mammals (> 850 present in human genome [34]) this possibility can not be excluded at this point. Alternatively LGR1406 may additionally inhibit an antimigratory kinase that counteracts its action on CDK5, but

this is highly speculative. Therefore more research concerning open questions related to ROSC and LGR1406 and migration is needed.

The ideal stent-coating agent is still to be found and most probably is not a single compound but rather a mixture of anti-proliferative/ anti-migratory compounds on the one hand and endothelium-protective factors on the other hand in order to achieve re-endothelialisation without neointima formation [154]. However, with this study we provide first evidence that optimised ROSC-derivatives such as LGR1406 could serve as anti-proliferative agents against VSMC in drug eluting stents.

Indirubine-3'-monoxime

In her PhD Thesis Andrea Schwaiberger showed that I3MO attenuated PDGF-BB-induced proliferation of VSMC and neointimal hyperplasia in an experimental animal model [180]. We demonstrated in the present study that I3MO not only acts as antiproliferative compound but also strongly inhibits PDGF-BB-induced VSMC migration (Fig.31, Fig.32). This was shown using two different approaches, namely the scratch and transwell assay, and interestingly I3MO exerts both the antimigratory and antiproliferative effect within the same concentration range (3-5 µM), possibly suggesting a common target for both. We could exclude any inhibitory activity on cell adhesion (Fig.34). As discussed in section 4.3.1, we used 10 ng/ml of PDGF-BB for migration assays instead of 20 ng/ml that was used for the studies on proliferation because we achieved maximal migratory response with this concentration (Fig.30). Also it has been shown, that a lower amount of PDGF elicits rather a migratory response whereas higher concentrations rather cause proliferation [204-206]. We repeated some crucial experiments with this lower amount of PDGF-BB and confirmed that I3MO completely abolishes STAT3 phosphorylation without affecting ERK phosphorylation (Fig.36).

Although indirubin is intensively investigated there is very poor knowledge about its antimigratory potential. In fact only three publications addressed this question up to now [205]. Just recently it has been reported that I3MO is able to block microglial migration in a scratch assay using a mouse cell line [64, 166]. This finding could be easily explained since I3MO is known to inhibit amongst others CDK5 which in turn has been reported to be essential for neuronal migration [204]. However, Yuskaitis and colleagues claim that their observation is solely due to inhibition of GSK3 and do not mention CDK5 or other possible targets at all.

There is also one article describing the influence of I3MO on migration in the context of the cardiovascular system [206]. Tran et al. reported that I3MO did not significantly affect human endothelial cell migration in a transwell assay but inhibited angiogenesis in an *in vitro* angiogenesis assay for tube formation.
It has also been published that I3MO can even augment migration in response to several interleukins in a transwell assay but this was only reported for human leukaemia cancer cells [207]. The authors of this study showed that I3MO had differentiation-promoting activity and promoted expression of typical neutrophil receptors via CDK inhibition. They concluded that the suppressive effect against leukaemia of I3MO is mediated through its differentiation-promoting activity.

Taken together I3MO elicits antimigratory or promigratory responses or does not affect migration at all depending either on cell type, organism or stimulus used. Also the authors of the different studies do not agree on the underlying mechanism of action of I3MO and either suggest CDK or GSK3 inhibition to be responsible. Obviously much more research is needed to understand the mechanistic and influence of I3MO on cell migration.

Therefore our study adds on important information showing for the first time an antimigratory potential of I3MO in VSMC.

Key regulators of VSMC migration are the small GTPases of the Rho protein family (Rac-1, cdc42, RhoA) [208, 209]. They all play crucial roles in cytoskeletal rearrangements upon stimulation with chemotactic agents such as PDGF-BB. We found that I3MO inhibits such rearrangements as e.g. membrane ruffling or stress fibre formation (Fig.41). Therefore we addressed the question whether I3MO influences small GTPases. We could exclude any inhibitory activity on the activation of Rac-1 (Fig.35). Cdc42 has been reported to be constitutively activated in VSMC and it has been shown that PDGF-BB does not enhance the levels of activated (GTP-bound) Cdc42 [208, 210]. Also in our lab we found that cdc42 was constitutively activated and failed to induce an increase in active cdc42 with PDGF-BB (data not shown). Therefore it is unlikely that I3MO suppresses VSMC migration via the inhibition of cdc42. Unfortunately we failed to detect active RhoA (personal communication Mario Kumerz, PhD student, data not shown). However, RhoA activity in response to PDGF-BB has been reported for VSMC, and future investigations are warranted [154].

In her thesis Andrea Schwaiberger demonstrated that I3MO impaired the activation of STAT1/3/5/6 without affecting other common early signalling pathways (p38, ERK, Akt) and suggested that the effect on STAT3 may be at least partially responsible for the antiproliferative activity of I3MO [211]. Substantial evidence is found in the literature supporting that STAT3 is involved in cell migration, motility, and invasiveness under normal and pathological situations [212, 213]. Especially for cancer cells where it is often overexpressed or constitutively active, STAT3 has been shown to mediate not only cell proliferation but also migration and to be involved in neoangiogenesis [103, 112, 214, 215]. Studies in other cell types including keratinocytes, fibroblasts, epithelial cells and VSMC have been shown that STAT3 is essential for migration there, too [216]. Its role in migration has been attributed mostly to its function as transcription factor that is dependent on its tyrosine phosphorylation. Among the STAT3 target genes are indeed several that are implicated in cell migration and cell adhesion such as e.g. metalloproteinases, platelet-endothelial cell adhesion molecule (PECAM-1), vav-2 (another member of the Rho GTPase family) and others [102, 103]. Moreover, Rao and colleagues reported that STAT3 is indispensable for VSMC migration in response to thrombin and PDGF-BB because it is responsible for the transcription of the cytosolic phospholipase A2 (cPLA2) that in turn cleaves membrane lipids and leads to the accumulation of arachidonic acid and its metabolites [103]. These metabolites have been implicated in VSMC migration. Neeli et al. showed that expression of a dominant negative STAT3 abolished PDGF-BB-induced migration of VSMC and that this effect could be rescued by adding arachidonic acid [211]. The relevance of other STAT3 target genes and direct evidences for their role in migration has, however, not been established so far [217]. Additionally, it should be noted that there are also publications showing that STAT3 exerts effects on cell migration that are independent of tyrosine-phosphorylation and transcription. Ng et al. demonstrated that non–tyrosine-phosphorylated STAT3 can stabilize the polymerization of microtubules through a direct interaction with the tubulin-binding protein stathmin [154]. However, for our setting the latter aspect does not seem to be important because we only observed an impaired STAT3 phosphorylation with I3MO but no changes in overall STAT3 levels [39].

We further supported the role of STAT3 for PDGF-BB induced migration using the JAK2 inhibitor AG490 as well as the Src inhibitor SU6656 that both impaired VSMC migration (Fig.37). JAK2 and Src have also been implicated in PDGF-BB induced phosphorylation of STAT3 [154]. We confirmed this in our group by showing that both compounds indeed impair STAT3 phosphorylation (see Fig.39 for AG490 and Andrea Schwaiberger, 2008 for SU6656 [188]). As the Src inhibitor completely abrogated STAT3 phosphorylation but the

JAK2 inhibitor only reduced it by about 70% we conclude that in our settings the Src kinase takes over the major part in the phosphorylation of STAT3 but that both kinases contribute to STAT3 activation as well as to VSMC migration.

Since we found several hints suggesting that STAT3 is important for VSMC migration, we further focused on the transcription of STAT3 target genes that might be involved. We found that I3MO reduced the PDGF-BB induced expression of cPLA2 as well as cyclin D1 (Fig.40). The role of cPLA2 in migration has already been pointed out. Cyclin D1 has been shown to play a role in migration next to its proliferative function in embryonic fibroblasts but data on VSMC migration are missing [205, 218]. However, it is not clear whether the reduced levels of cPLA2 and cyclinD1 could mediate the antimigratory effect of I3MO we observed. First, the basal levels of cPLA2 are already rather high and PDGF-BB only elicits a small enhancement, and therefore it is questionable whether this reduction of cPLA2 levels could lead to the complete abolishment of migration seen in our experiments. Second, we monitored differences in cytoskeletal rearrangements of I3MO treated cells as early as 1-2 h upon PDGF-BB stimulation. This timeframe is clearly too short to include protein expression in the considerations (Fig.41). Nevertheless, it seems plausible that I3MO exerts its antimigratory effect through multiple ways and that one of those is mediated via the downregulation of cPLA2 and/ or cyclin D1 or other so far unknown STAT3 target genes.

Whether there is a causal link between inhibition of STAT3 phosphorylation and inhibited migration still needs to be shown. For this purpose we want to study the impact of a constitutively active mutant of STAT3 on PDGF-BB-induced VSMC migration. Additionally we will use gene silencing strategies for STAT3 and study the influence on VSMC migration. It is also possible that STAT3 does not play a role at all in mediating I3MO effects. It should be noted that STAT5 shares many properties with STAT3, for example it also influences cyclin D1 expression and is phosphorylated by Src. Moreover suppression of STAT5b has been shown to reduce neointima formation as well as VSMC migration [219, 220].

As I3MO is marketed as GSK3 inhibitor and a few publications suggest a role for this kinase in migration, we addressed the question whether other GSK3 inhibitors could mimic the antimigratory effect of I3MO [221-223]. LiCl, a known GSK3 inhibitor strongly impairs PDGF-BB induced migration of VSMC (Fig.38). Other salts did not lead to such an effect ruling out osmotic changes as reason for the inhibition seen with LiCl. However, LiCl

did not mimic the effect of I3MO on STAT3 phosphorylation (Fig.39). Therefore we exclude STAT3 as common antimigratory target of LiCl and I3MO. However, it can not be ruled out at this stage that the inhibition of migration by I3MO is independent of inhibition of STAT3 phosphorylation and that I3MO indeed inhibits migration just as LiCl most likely does via GSK3 inhibition.

The strong effect of LiCl on VSMC migration is completely novel, and we furthermore were interested whether LiCl would also influence VSMC proliferation. Indeed LiCl treatment led to an accumulation of cells in G1 phase (Fig.42). Further investigations should address whether GSK3 inhibition is really the underlying mechanism for our observations by using other GSK3 inhibitors, siRNA approaches or expression of a constitutively active GSK3.

Another interesting finding of Andrea Schwaiberger was the antioxidative effect of I3MO in activated VSMC, especially because the generation of ROS has been shown to be very important for VSMC migration [223]. Weber et al. showed that PDGF-induced VSMC migration is ROS dependent and identified a Src-dependent signalling pathway as an important ROS-sensitive mediator of VSMC migration [154]. Although in this article STAT3 is not mentioned it could be involved because we found that Src inhibition leads to STAT3 inhibition and that STAT3 activation is ROS sensitive (Andrea Schwaiberger, 2008 [224]). ROS are generated through multiple pathways and enzymes in a cell. In VSMC the NAD(P)H oxidases (Noxes), xanthine oxidase, lipoxygenases, the mitochondrial respiratory chain and nitric oxide synthases have been identified so far as sources of ROS [225]. Particularly the Nox protein family which belongs to the flavoproteins plays a pivotal role in the controlled production of ROS in response to the activation of numerous receptors [206]. We therefore used the rather unspecific flavoprotein inhibitor DPI to block ROS generation (data on DPI and ROS production in Schwaiberger, PhD Thesis, 2008) and could again mimic the effect of I3MO on PDGF-BB-induced migration. An antioxidative effect of I3MO could definitely play a role in its antimigratory activity. This should be followed in future experiments using other antioxidants or siRNA approaches targeting ROS-producing proteins. Additionally it should be investigated whether STAT3, as its activation is redox-sensitive, is involved in connecting the antioxidative and antimigratory effects or not.

Next to STAT3, GSK3 inhibition and the antioxidative effect of I3MO, there is at least one other possible alternative explanation for its antimigratory effect. As already mentioned it

has been suggested that I3MO exerts its antimigratory activity in cancer cells through inhibition of CDK5 [51]. In our studies we found that two different CDK5 inhibitors, I3MO and ROSC inhibit VSMC migration. But LGR1406 did not exert a potent antimigratory effect, although it is able to inhibit CDK5 as well. Moreover as discussed in section 5.1 we were neither able to detect CDK5 in western blot analysis in VSMC (data not shown) nor did we find data published showing CDK5 protein levels in VSMC. However, it can at this point not completely be ruled out that I3MO impairs VSMC migration at least partly via the inhibition of CDK5.

Taken together, we clearly demonstrated that I3MO inhibits PDGF-BB-induced migration of VSMC, and our data suggests that this effect is probably mediated via the reduction of ROS production in the cell and consequent inhibition of Src and subsequent STAT3 phosphorylation. We already had shown in previous studies, that I3MO inhibits VSMC proliferation and neointima formation *in vivo*. Also I3MO has been shown to exert antiinflammatory activities, which would also be beneficial for the treatment of restenosis. The present study shows that I3MO additionally has antimigratory activity against VSMC and hereby further substantiates that I3MO is a promising compound for e.g. stent coating. However, there are still some open questions regarding its direct molecular targets. Especially the role of STAT3 or other STATs in I3MO effects seen in *in vitro* and *in vivo* experiments needs to be clarified.

6. SUMMARY

Abnormal vascular smooth muscle cell (VSMC) proliferation and migration contribute to vascular narrowing during the pathogenesis of atherosclerosis. There are various treatment modalities either preventing the progression of atherosclerosis or re-widening diseased arteries. However, even if an artery has been re-widened its lumen is often reduced again, a process called restenosis. The latter is also characterized by the proliferation and migration of VSMC and the subsequent formation of a new inner layer of the vessel wall (neointima). To maintain the vessel lumen often mesh-like structures, so called stents are implanted. Eventually these stents are coated with antiatherogenic drugs. An ideal compound for treating atherosclerosis and restenosis should impair both proliferation and migration of VSMC. Therefore, finding drugs that specifically inhibit these processes is an important subject of research.

A major proliferative stimulus and the strongest chemoattractant for VSMC is the Platelet Derived Growth Factor-BB (PDGF-BB). PDGF-BB-stimulation leads to the activation of several signalling cascades and ultimately to cell migration as well as to cell cycle progression. The latter requires the activation of cyclin-dependent kinases (CDKs) through their association with regulatory subunits called cyclins. During each phase of the cell cycle different CDK/cyclin holoenzymes are specifically activated and in turn hyperphosphorylate pocket proteins. Reinduction of positive cell-cycle control genes in VSMC, like CDK2 and cyclin A and E is a hallmark of atherosclerosis and restenosis and therefore CDKs are expedient drug targets to combat vascular proliferative diseases.

Roscovitine (ROSC) is a derivative of the plant hormone Isopentylalanin that is able to unspecifically inhibit kinases. However ROSC itself shows a high specificity for CDKs and has been cocrystalized with CDK2. Research has been primarily focused on ROSC (Seliciclib) as an anti cancer drug and is currently in clinical trials phase II against breast cancer. ROSC is well known to act as a CDK inhibitor but most of the publications only show *in vitro* data and other potential targets have been published as well. There are only two publications mentioning effects on VSMC and none of them solved its mechanism of action satisfyingly. We therefore wanted to investigate the mode of action of ROSC and promising derivatives in PDGF-BB stimulated VSMC and define the changes in cell signaling. During screening a panel of synthetic ROSC-derivatives for a general antiproliferative activity in VSMC we identified several derivatives with significant higher potencies than ROSC itself. The most potent of which was LGR1406. We, therefore, compared the influence of ROSC and its most promising derivative LGR1406 on PDGF-BB induced proliferation and migration of VSMC. We showed that LGR1406 and ROSC

both inhibit proliferation of VSMC without affecting early signalling pathways downstream the activated PDGF receptor. LGR1406 is hereby the far more potent compound with an apparent IC_{50} of 3.0 µM in the BrdU assay compared with an IC_{50} of 17 µM of ROSC. A detailed molecular analysis using western blot analysis as well as *in vitro* kinase assays showed that both compounds affect entry of cells in to S-phase of the cell cycle, expression of cyclin A and E as well as phosphorylation of pocket proteins presumably by inhibition of CDK activity. However, ROSC only delayed these events whereas LGR1406 permanently blocked cell cycle progression in G1-phase of the cell cycle. We conclude that LGR1406 could be used for further development as e.g. stent coating agent but *in vivo* studies as well as further investigations on its molecular mechanism are needed. With this study we provide first evidence that optimised ROSC-derivatives such as LGR1406 could serve as anti-proliferative agents against VSMC growth in drug eluting stents.

Indirubin-3'-monoxime (I3MO) is a derivative of indirubin which is an active component from a recipe of traditional chinese medicine exerting anti-cancer function as well as inhibiting inflammatory reactions. All together there have been a number of biological activities contributed to I3MO many of which seem to be cell type specific. However, studies on VSMC were missing until Andrea Schwaiberger in our group addressed this issue (PhD thesis, Vienna, 2008). She found that I3MO used at 3-5 µM potently blocks VSMC cell cycle progression in response to PDGF-BB. In cooperation with Prof. Binder from the Medical University of Vienna she furthermore showed that I3MO inhibits neointima formation in a mouse model. However, the molecular mechanism of action of I3MO is not completely understood so far. *In vitro* kinase assays showed that indirubin can target different kinases and therefore may affect several targets in a cell. Furthermore Andrea Schwaiberger showed that is suppresses the activation of members of the STAT protein family in response to PDGF-BB without interfering with other common early signalling events. The work was further focused on STAT3 and it was suggested that the inhibitory effect on STAT3 may be at least partially responsible for the antiproliferative activity of I3MO. As both proliferation and migration contribute to neointima-formation we therefore not only wanted to further investigate the underlying molecular mechanism of I3MO-effects on proliferation but also its potential influence on migration of VSMC. In this study we now showed that I3MO dose-dependently inhibits PDGF-BB-induced migration of VSMC in the same concentration range as it limits PDGF-BB-induced proliferation. Also we found that I3MO interferes with early cytoskeletal rearrangements involved in cell migration upon PDGF-BB-stimulation. I3MO did not affect the activation of the small

GTPase Rac-1 that is crucial for VSMC migration. We also found that I3MO reduces the expression of STAT3 target genes involved in migration. Additionally we were comparing the effects exerted by I3MO with chemical inhibitors of common PDGF-BB-induced signalling pathways involved in migration. We found that I3MO exerts similar effects as a Src kinase inhibitor and a flavoprotein inhibitor which reduces the levels of reactive oxygen species (ROS) in a cell. We clearly demonstrated that I3MO inhibits PDGF-BB-induced migration of VSMC and our data suggests that this effect is probably mediated via the reduction of ROS production in the cell and consequent inhibition of STAT3 phosphorylation. Our studies further substantiate that I3MO is a promising compound for e.g. stent coating but still there are some open questions regarding its molecular targets. Especially the role of STAT3 or other STATs in conducting the effects of I3MO seen in *in vitro* and *in vivo* experiments needs to be clarified.

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8. APPENDIX

Abbreviations

	Abbreviations
4EBP1	eIF4E binding protein 1
A	
ABP	Actin-binding proteins
ACE	Angiotensin-converting enzyme
AhR	Co-transcriptional factor aryl hydrocarbon receptor
Akt	AKT8 virus oncogene cellular homolog
ALK	Anaplastic lymphoma kinase
AMPK	AMP-activated protein kinase
Ang II	Angiotensin II
ANOVA	Analysis of variance between groups
APS	Ammonium persulphate
Arp2/ 3	Actin-related protein 2/3
ATP	Adenosine triphosphate
Aur-A	Aurora A
Aur-B	Aurora B
B	
bFGF	basic fibroblast growth factor
BrdU	BrdU Bromodeoxyuridine
BRK	Breast tumor kinase
BSA	Bovine serum albumin
C	
cdc42	A small GTPase of Rho family
CDK	Cyclin dependent kinase
CAK	CDK activating kinase
CKI	CDK inhibitor proteins
c-myc	Similar to myelocytomatosis viral oncogene
COX	Cyclooxygenase
CRM	Chromosome region maintenance 1
CRP	C-reacitve protein
CS	Calf serum
cyc	Cyclin

Abbreviations

D

ddH_2O	Double distilled water
DNA	Desoxyribonucleic acid
DES	Drug-eluting stent
DMSO	Dimethylsulphoxide
DPI	Diphenylen iodonium
DTT	Dithiothreitol

E

EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamintetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethylether)-tetraacetic acid
ERK	Extracellular signal-regulated kinase

F

FACS	Fluorescence-activated cell sorter
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
Fyn	A src family tyrosine-protein kinase

G

GAP	GTPase activating protein
GATA-6	Gastric transcription factor-6
GAX	Growth Arrest Homeobox
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 3
GSK-3	Glycogen synthase kinase-3
GST	Glutathion-S-Transferasen

Abbreviations

H

HBSS	Hanks` balanced salt solution
HEPES	N-(2-hydroxyethyl)piperazine-'L-(2-ethanesulphonic acid)
HFS	Hypotonic fluorochrome solution
HRP	Horse radish peroxidase

I

I3MO	Indirubine-3'-monoxime
IFN	Interferon
IFN-γ	Interferon γ
IGF	Insulin like growth factor
IL	Interleukin 1
INK4	Inhibitor of CDK4
IRSE	IFN-stimulated response element

J

JNK	c-jun N-terminal kinase

K

kDa	Kilo Dalton
KIP	Kinase Inhibitor Protein

L

LCK	Lymphocyte-specific protein tyrosine kinase
LDL	Low density lipoprotein
Lyn	A src family tyrosine-protein kinase

M

MAP	Microtubule-associated protein
MAPK	MAPK Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Monocyte-colony stimulating factor
mDia1/ 2	Mammalian Diaphanous-related formins 1/2

Abbreviations

M

MEK	MAPK/ERK kinase
MMP	Metalloproteinase
MTOC	Microtubule-organisation centres
mTOR	Mammalian target of rapamycin

N

NCK	Nck adaptor protein
NFAT	Nuclear factor of activated T cell
NF-κB	Nuclear factor-κB
NLK	Nemo-like kinase
NO	Nitric oxide
Nox	NAD(P)H-oxidase

P

PAA	Polyacrylamide
PAK	p21-activated protein kinase
PBD	p21-binding domain
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDXK	Pyridoxalkinase
PI	Propidium iodide
PI3K	Phosphoinositol 3 kinase
PIP3	Phosphatidylinositol-3-phosphat
PLC	Phospholipase C
PMSF	Phenylmethylsulphonylfluoride
PTA	Percutaneous transluminal angioplasty
p-TEFb	Positive transcription elongation factor b
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PTP	Protein tyrosine phosphatase

Abbreviations

R

Rac	A small GTPase of Rho family
Raf	Ras-activated factor
Ras	Rat sarcoma GTP protein
RAS	Renin-angiotensin system
Rb	Retinoblastoma protein
RhoA	A small GTPase of Rho family
RLU	Relative light units
ROCK	Rho-associated, coiled-coil-containing protein kinase
ROS	Reactive oxygen species
ROSC	Roscovitine
RSK3	Ribosome S6 Kinase 3
RTK	Receptor tyrosine kinase domain

S

S6K1	p70 ribosomal protein S6 kinase 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SGK	Serum/glucocorticoid-regulated kinase
SH2	Domain Src-homology 2 domain
SHP	SH2-containing protein-tyrosine phosphatase
α-SMA	Smooth muscle alpha actin (α-SMA)
SMPC	Smooth muscle progenitor cells
Sos	Son of sevenless guanine nucleotide exchange factor
Src	Rous sarcoma oncogene cellular homolog
SSC	Side scatter
STAT	Signal transducer and activator of transcription

T

TBS-T	Tris-buffered saline containing Tween 20
TEMED	N,N,N’,N’-tetramethylethylene diamine
TGF	Transforming growth factor
TGFα /β	Ttransforming growth factor α and β

Abbreviations

T

TIMP	Tissue inhibitors of metalloproteinase
TNF	Tumor necrosis factor
TNF-α	Tumour necrosis factor-α
Tyr	Tyrosine

V

VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cells

W

WASP	Wiskott-Aldrich syndrome protein
WAVE	Verprolin-homologous protein

Y

Yes	A src family tyrosine-protein kinase

Alphabetical list of companies

Bachem (Weil am Rhein, Germany)

BD Bioscience Pharmingen (San Diego, CA, USA)

Beckman Coulter (Fullerton, CA, USA)

Biorad Laboratories (Hercules, CA, USA)

Calbiochem (La Jolla, CA, USA)

Carl Roth (Karlsruhe, Germany)

Cell Biolabs (San Diego, CA, USA)

Fluka (Buchs, Switzerland)

Fujifilm (Tokyo, Japan)

GraphPad Software Inc (San Diego, CA, USA)

Invitrogen (Carlsbad, CA, USA)

Lonza Group Ltd. (Basel, Switzerland)

New England Biolabs (Beverly, MA, USA)

Olympus Europa GmbH (Hamburg, Germany)

Promega (Mannheim, Germany)

ProQinase GmbH (Freiburg, Germany)

Qiagen AG (Hilden, Germany)

Quattro Research GmbH (Munich, Germany)

Raytest GmbH (Straubenhardt, Germany)

Roche Diagnostics (Basel, Switzerland)

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Thomson ResearchSoft (Carlsbad, CA, USA)

Whatman plc (Kent, UK)

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Der Mensch empfängt unendlich mehr, als er gibt. Dankbarkeit macht das Leben erst reich.

In ordinary life we hardly realize that we receive a great deal more than we give, and that it is only with gratitude that life becomes rich

Dietrich Bonhoeffer