Novel Approaches for Cardiovascular Drug Eluting Devices Based on Cellular Pharmacokinetic Determinants of Coronary Artery Cells

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Dekan

Für meine Eltern und Großeltern

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Abbreviations

CTP cytidine triphosphate

CVD cardiovascular disease

EC(s) endothelial cell(s)

FDA United States Food and Drug Administration

FKBP12 FK506 binding protein 12

HCAEC human coronary artery endothelial cells

HCASMC human coronary artery smooth muscle cells

HMG-CoA hydroxy methylglutaryl coenzyme A

IFI4 2′-5′-oligoadenylate synthetase

IFIT1 interferon-induced protein with tetratricopeptide repeats 1

IFIT3 interferon-induced protein with tetratricopeptide repeats 3

IL interleukin

IRF9 interferon regulator factor 9

ISR in-stent restenosis

LDL low-density lipoprotein

LST late stent thrombosis

mTOR mammalian target of rapamycin

NFAT nuclear transcription factor of activated T cells

NF-κB nuclear factor kappa B

NO nitric oxide

OATP2B1 organic anion transporting polypeptide 2B1

OCT1 organic cation transporter 1

P27Kip1 cyclin-dependent kinase inhibitor 1B

PCR polymerase chain reaction

PLLA poly-L-lactic acid

PTCA percutaneous transluminal coronary angiography

SMC(s) smooth muscle cell(s)

SLC solute carrier transporter

TGF-β transforming growth factor beta

TNF-α tumor necrosis factor alpha

Summary

Cardiovascular diseases represent the main cause of mortality in industrialized countries; clinical manifestations include angina pectoris, myocardial infarction, and chronic coronary heart disease. Changes in fluid dynamics disturb the physiological functions of the vascular endothelium [1] and subsequent vascular remodeling accompanied by proliferation of cells and infiltration of inflammatory cells results in atherosclerosis within the vessel. The formation of atherosclerotic plaques causes a flow-limiting stenosis, thus restraining the coronary blood flow [2].

The most frequently performed invasive procedure to reopen a stenotic vessel in clinics is percutaneous transluminal coronary angioplasty. To prevent a spontaneous occlusion and reduce restenosis rates, a coronary stent is deployed. However, the use of bare-metal stents (BMS) and drug-eluting stents (DES) is associated with two severe complications. A mechanical-induced injury provokes a remodeling of the arterial wall resulting in a neointima formation within the stented segment, namely in-stent restenosis (ISR). This has been defined as one major drawback of BMS. Restenosis evolves by increased vascular smooth muscle cells (SMC) migration and proliferation from the intimal layer of the vessel wall that ultimately obstruct the vessel lumen. Even if the use of DES reduces the incidence of ISR, the unspecific cytotoxicity of the loaded substances is believed to promote the development of the rare but more severe complication, known as late stent-thrombosis (LST) [3]. Based on the current understanding, a permanent inhibition of endothelial cell (EC) proliferation and migration hampers the re-endothelialization of the stent struts. Additionally, a hypersensitive reaction to the stent material and polymer supports the development of thrombosis.

Considering the pathophysiological basis for development of ISR and LST it seems evident that stent material and especially the drug coating are key features that should be modulated to inhibit the progressive proliferation of SMCs and to promote the re-endothelialization.

Within the context of dual-drug technology that combines the different cellular effects of two compounds [4], we developed a DES with a luminal located atorvastatin and an abluminal applied sirolimus. This approach inhibits ISR without provoking a long-term impact on re-endothelialization. Novel concepts predict the use of an abluminal located antiproliferative drug to ensure a targeted tissue release while reducing the systemic exposure [5]. Based on this

concept, we analyzed the effects of atorvastatin and sirolimus on cellular proliferation (**section 3.1**). Atorvastatin was found not to impede the antiproliferative effect of sirolimus on SMCs. Furthermore, atorvastatin revealed a less pronounced effect on ECs proliferation. Given these results, re-endothelialization may be less impaired by using dual DES with an abluminal/luminal coating strategy.

From a pharmacological point of view there are two different strategies to improve cell-specific effects of chemotherapeutics which includes identifying of cellular targets and modulating the pharmacokinetics of candidate drugs. One mechanism that contributes to the drug`s pharmacokinetic profile is the expression of drug transporters that mediate the uptake or efflux of compounds [6]. Specifically, the expression of efflux transporters, including P-glycoprotein, modulates the biological cellular activity of chemotherapeutics thus hampering the therapeutic effect [7]. The uptake transporter OCT1 has been shown to transport a variety of substances including the established DES-compound paclitaxel [8]. Therefore, we tested whether an adenoviral-induced overexpression of OCT1 using the SMC-specific promotor of transgelin (SM22α) would enhance the antiproliferative effects of paclitaxel in vascular SMCs (**section 3.2**). First, the activity of SM22α was assessed in various cell types; a muscle cell-specific expression was demonstrated. The activity of OCT1 was then compared in adenoviral infected ECs and SMCs, with a higher accumulation of OCT1 substrates found in SMCs. To test the findings from the concept study relating to cell-specific drug effects, we studied the impact of paclitaxel treatment of ECs and SMCs, finding a significantly increased effect in SMCs. These results suggest that cell-specific expression of transport proteins serves as a mechanism for producing a selective effect on target cells.

Another approach to improve the outcome of DES is the use of drugs that show benefits in the treatment of atherosclerosis. Since pleiotropic activities of statins were associated with a high potential in restenosis reduction after systemic therapy [9, 10], statins were suggested as suitable drug candidates for local application. Following data that shows a high impact of cerivastatin in inhibiting SMCs proliferation and neointima formation without impairing ECs cellular behavior [11, 12], we studied which cellular mechanism may account for the cell-specific activity (**section 3.3**). Endothelial cells and SMCs were treated with different statins, with atorvastatin especially presenting an SMC-specific inhibition of proliferation. Quantifying the expression of the primary drug target revealed comparable levels of HMG-CoA reductase mRNA and protein expression, leading to the assumption that pharmacokinetics may account for different cellular activity. We detected a higher accumulation of atorvastatin in SMCs; this has been

associated with a higher endogenous expression of OATP2B1, a high affinity transporter for atorvastatin [13]. Adenoviral-induced overexpression of OATP2B1 supported our previous finding. Assuming that the expression of OATP2B1 is a determinant of drug effects in SMCs, we used a cell line overexpressing OATP2B1 to identify cytotoxic drugs suitable for SMC specific inhibition. The screening provided evidence that teniposide may be an OATP2B1 substrate. This was supported by subsequent proliferation assays demonstrating a higher efficacy of teniposide on SMC proliferation in the presence of heterogeneously expressed OATP2B1.

A variety of limus agents have entered clinics for local application on DES. While several drugs including sirolimus, zotarolimus, and everolimus demonstrated high efficiency and safety, some failed to do so [14]. The anti-inflammatory and immunomodulatory compound pimecrolimus especially showed excessive neointimal growth in humans despite promising data from a preclinical assessment [15, 16]. Nevertheless, the underlying mechanisms contributing to the failure of pimecrolimus eluting stents are unknown. We therefore studied the impact of pimecrolimus on SMCs and ECs proliferation and viability (**section 3.4**). According to our study, pimecrolimus had a cytostatic effect in both cells. Since preliminary data from an mRNA microarray suggested that pimecrolimus induced the expression of genes involved in the interferon signaling pathway, we analyzed their expression by real-time quantitative PCR. Importantly, pimecrolimus but not sirolimus led to an upregulation of these genes. This could in part be associated with inhibition of the phosphatase calcineurin, a downstream target of the pimecrolimus/FK506-binding protein 12-complex and known to modulate the interferon pathway [17]. Specifically, the interaction of calcineurin with toll-like receptor 4 may modulate the expression of interferon-inducible genes upon pimecrolimus treatment. In accordance were our findings showing that silencing of the toll-like receptor 4 reduced the activation of gene expression. This crosstalk between the interferon and toll-like receptor 4 signaling may be a molecular mechanisms explaining the failure of pimecrolimus-eluting stents in clinical trials.

Chapter 1

Introduction

This chapter introduces coronary artery disease and its pathophysiological basis – namely atherosclerosis. Furthermore, it provides information about therapeutic strategies and highlights the pros and cons, and need for coronary artery stents. Finally, we offer a glimpse into novel approaches to improve efficacy and safety of these devices.

1.1. Cardiovascular disease (CVD) - Coronary artery disease

Cardiovascular diseases (CVD) represent the leading cause of mortality in developed countries. CVD are disorders of the heart and blood vessels including for instance cerebrovascular disease or coronary heart disease, also known as coronary artery disease, which is the most common cause of death in Europe [18]. The clinical manifestations of CVD are diverse and involve stable and unstable angina pectoris, myocardial infarction, stroke, and chronic coronary heart disease. As reviewed by the American Heart Association in "Heart Disease and Stroke Statistics – 2014 Update" the most attributable risk factors contributing to the manifestation and progression of CVD are high blood pressure, smoking, poor diet, insufficient physical activity, or abnormal blood glucose levels [19].

The role of the vascular endothelium in CVD

On a cellular level, various pathophysiological mechanisms are assumed to be associated with the development of CVD. Although blood vessels histologically consist of three layers, (1) the tunica intima, a monolayer of endothelial cells (EC), (2) the tunica media, mainly represented by smooth muscle cells (SMC), and (3) the tunica adventitia, it has become evident that ECs especially play a decisive role in pathogenesis of CVD (compare Figure 1a).

Endothelial cells are not only directly exposed to blood and circulating noxious elements [20], but also maintain vascular homeostasis by modulating the vascular tone, controlling the transport of molecules and blood gases through the vascular wall, and regulating cell growth and inflammatory responses [21]. Not surprisingly, changes in fluid dynamics or mechanical forces result in functional and structural alteration of the endothelial layer. The predominant stimulus influencing vascular homeostasis is endothelial shear stress that derives from the friction of the

flowing blood on the luminal surface of the arterial wall [20, 22]. Disturbed or low shear stress induces the expression of transcription factors resulting in suppressed or induced expression of genes modulating cellular functions [23]. In particular, in arterial regions with disturbed flow, low shear stress diminishes the expression and function of the endothelial nitric oxide synthase (eNOS) [24], thereby reducing bioavailability of nitric oxide (NO). The decrease in NO bioavailability substantially contributes to development and progression of CVD since NO is assumed to regulate activity of growth factors released from vascular cells, promote SMC-induced vasodilatation, and protect vessels against platelet aggregation [21]. In addition, NO exhibits anti-inflammatory properties, as it inhibits adhesion of inflammatory cells to the endothelial cell layer.

Taken together, these results suggest that a functional vascular endothelium is essential for cardiovascular homeostasis, and endothelial dysfunction is considered an early marker for atherosclerosis [25], which is the pathophysiological foundation of most forms of CVD.

1.2. Coronary atherosclerosis

There are two terms frequently used in the context of coronary artery or vascular disease - namely atherosclerosis and arteriosclerosis. In general, a thickening or hardening of the artery wall defines arteriosclerosis, which causes stiffness and a loss of elasticity. Arteriosclerosis can be present in every artery of the body, but is fatal when it affects the coronary arteries. The most common type of arteriosclerosis is atherosclerosis mainly characterized by lipid deposition in a focal plaque within the arterial wall.

Atherosclerosis as an inflammatory disease

Based on our current understanding, atherogenesis is assumed to be a multifactorial process as summarized by Libby *et al.* (illustrated in Figure 1b-d) [2]. Interestingly, there was a change in understanding of the pathophysiology of atherosclerosis at the beginning of the 21st century. Previously, atherosclerosis was considered a degenerative disease, but as inflammation has been identified as an essential driving force, atherosclerosis is nowadays classified as an inflammatory disease [26]. Although it is generally accepted that risk factors including hypertension and hyperlipidemia play a role in the initiation and progression of atherosclerosis, atherosclerotic plaques predominantly arise in sites of disturbed blood flow [27]. Based on the "response-to-injury" hypothesis established by Ross et al., changes in blood flow induce a sequence of cellular alterations where inflammation is a substantial factor contributing to all stages of the progressing disease [24, 28]. The complex interplay of resting cells (EC and SMC)

and cells circulating in the blood (thrombocytes, lymphocytes, monocytes) and their secretory products does ultimately change the homeostasis of the balanced system.

The recruitment of inflammatory cells in the area concerned is a major component in atherogenesis. Shear stress induced activation of the transcription factor nuclear factor-κB (NF-κB) regulates the expression of cytokines (TNFα, interleukin-1β, interferon-γ) and adhesion molecules including the vascular adhesion molecule 1, the intercellular adhesion molecule 1 and the monocyte chemoattractant protein 1 [23, 29]. Increased expression of these molecules facilitates the recruitment, attachment, and transmigration of leukocytes. Consequently, after transmigration in the artery wall, monocytes differentiate into macrophages that sustain the pro-inflammatory environment. Simultaneously, changes in the permeability of the endothelium allow the entry of cholesterol-containing low-density lipoproteins (LDL) that accumulate in the arterial wall [30].

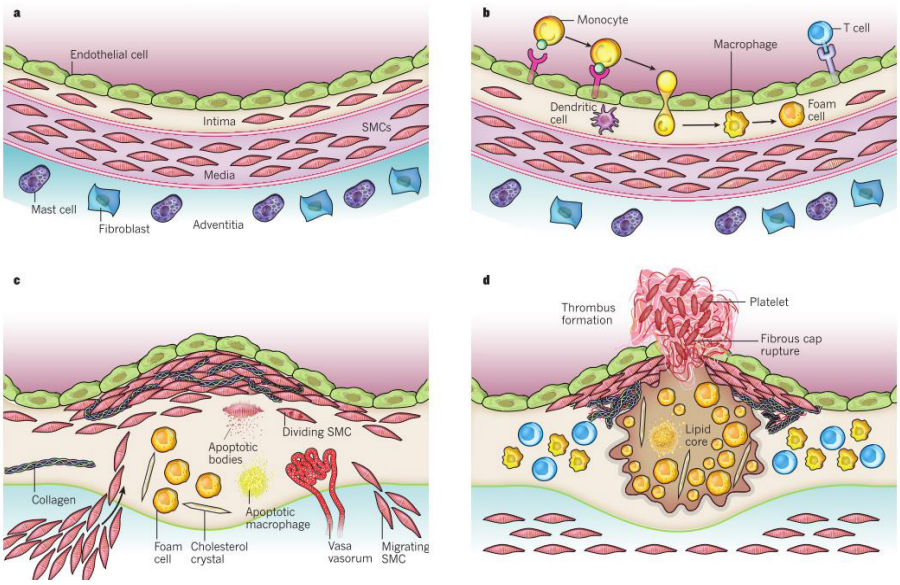

Figure 1. Development of atherosclerotic lesions. (A) Normal structure of an artery consisting of three different layers; (1) the intima containing resident smooth muscle cells and lined by an endothelial cell monolayer, (2) the tunica media represented by smooth muscle cells (SMCs) embedded in extracellular matrix, and (3) the tunica adventitia. **(B)** Changes in vascular homeostasis leads to an expression of adhesion molecules promoting the adhesion and migration of monocytes, and subsequently formation into macrophages, which accumulate LDL to yield foam cells. **(C)** SMCs from the tunica media migrate into the tunica intima and proliferate. They produce extracellular matrix proteins such as collagen. Extracellular lipid from dead SMC accumulate in the plaque known as the necrotic/lipid core. **(D)** Disruption of the plaque results in thrombus formation, a severe complication of atherosclerosis. Figure reproduced from Libby *et al.* 2011 [2]

Activated macrophages take up the LDL particles and become so-called foam cells, whose accumulation reflects the histological appearance of the yellow-white fatty-streak lesions. Additionally, vascular SMC predominantly located in the tunica media migrate into the intima and proliferate. Finally, the secretion of cytokines, growth factors, and extracellular matrix proteins from inflammatory cells and SMC promote the formation of a plaque covered by a

fibrous cap. Moreover, an inefficient clearance of dead cells results in accumulation of cellular debris and extracellular lipid that forms a necrotic lipid-rich core within the plaque [31].
Consequently, plaque formation causes a flow-limiting stenosis in the area concerned, which in turn can promote an exercise-induced myocardial ischemia, or can reduce resting coronary flow to produce unstable angina or myocardial infarction. Moreover, different circumstances lead to digestion of extracellular matrix that involves a thinning of the fibrous cap that is prone to rupture. Subsequently, exposed pro-coagulant material activates and aggregates thrombocytes, forming a thrombus on the lesion's surface that interrupts the blood flow locally or that lodges in distal vessels, finally provoking embolism.

1.3. Percutaneous coronary intervention

Looking at the potential life-threatening consequences of atherosclerosis, therapeutic treatment is a prerequisite to reduce and/or prevent atherogenesis and to improve the prognosis of patients suffering from CVD. However, therapeutic options of atherosclerosis and its clinical manifestations are diverse. Avoiding risk factors such as smoking or physical inactivity and medical treatment of hyperlipidemia, hypercholesterolemia, diabetes, and/or high blood pressure are fundamental strategies to prevent atherosclerosis.
One principle approach to treat functionally relevant vascular stenosis is the mechanical dilation of affected vessels by angioplasty. In 1964 Dotter and Judkins first described the successful transluminal treatment of stenotic femoral arteries, while thirteen years later Andreas Gruntzig performed the first percutaneous transluminal angioplasty (PTCA) as an invasive intervention in cardiology, widening an obstructed coronary blood vessel [32, 33].
In this procedure, a catheter system consisting of a guiding and a dilatation catheter was inserted into the stenotic area of the coronary artery via the arteria femoralis. The balloon at the tip of the dilatation catheter was inflated at the side of the stenosis and pressed the atherosclerotic material into the vessel wall, thereby widening the lumen and reducing the clinical symptoms of stenosis.
Since then the technique of transluminal angioplasty has been constantly improved and has become one of the most often performed invasive procedures in clinical practice.
However, restenosis of the dilated vessels that occurs in 30 – 50% of patients limited the clinical success of PTCA [34-36]. While acute vessel closure immediately after dilatation resulted either from thrombus formation or elastic recoil of the stretched area [37], restenosis occurring within the first months after dilatation is assumed to be associated with proliferation of intimal SMC and extracellular matrix remodeling at the side of the injured endothelial layer [34, 38]. Based on

the idea that a metal scaffold inserted after the procedure of balloon angioplasty would mechanically prevent acute occlusion of PTCA treated vessels, the first bare metal stent (BMS), also known as the WALLSTENT® (Schneider, AG) was developed and implanted in a human coronary artery in the mid-1980s [39].

Bare metal stents – Appearance of in-stent restenosis

The first clinical data reported on BMS - a self-expandable stainless-steel mesh stent (WALLSTENT®) and a balloon-expandable metal stent (Palmaz-Schatz stent) - were promising [39-41]. Unfortunately, despite the unwanted side effect of elastic recoil mechanically prevented by BMS implantation, clinical trials revealed an increase in subacute thrombotic events within the first weeks after implantation; this could in part be reduced by antiplatelet therapy with small molecules such as clopidogrel [42].

In the following years, various clinical studies further validated the improved outcome of PTCA followed by stent implantation [43, 44]; in 1994, this finally led to the FDA approval of the balloon-expandable Palmaz-Schatz stent developed by Johnson & Johnson.

However, as well as thrombus formation, the occurrence of a second side effect hampered the clinical success of BMS. The so-called neointimal hyperplasia led to restenosis within the stented segment. This in-stent restenosis (ISR) occurred at rates of around 30%, varying with the respective stent platform that was implanted [45, 46].

The pathophysiological mechanisms leading to ISR remain only partly understood. However, the most accepted explanation is an adaptation of the "response-to-injury" hypothesis [47-49]. As recently summarized from Simard and colleagues, a mechanical disruption of the endothelial lining by PTCA with stent placement and subsequent inflammatory signals stimulates a remodeling process involving the migration and proliferation of vascular SMCs from the tunica media to the injured area. The secretion of extracellular matrix proteins by SMC with a synthetic phenotype further exacerbates neointimal growth, ultimately obstructing the vessel lumen (Figure 2) [49].

In accordance with the hypothesis that endothelial injury is a stimuli for ISR, findings show that the intensity of neointimal proliferation of SMC is directly associated with stenting and in particular with the stent design [50, 51]. A greater stent strut thickness, which is assumed to be associated with a higher degree of vascular injury results in higher restenosis rates [52]. This has been supported by findings from a porcine model of coronary restenosis demonstrating a strong correlation of neointima formation with severity of vessel injury as defined by "injury scores" [53]. The denudation of the endothelium and subjacent tunica media induced greater neointima

compared to arteries where stenting only impaired the endothelium. The role of inflammation in the process of ISR is supported by studies showing the increase of inflammatory cells in neointimal regions, thereby suggesting that inflammation is a predictor of neointimal growth [54-56].

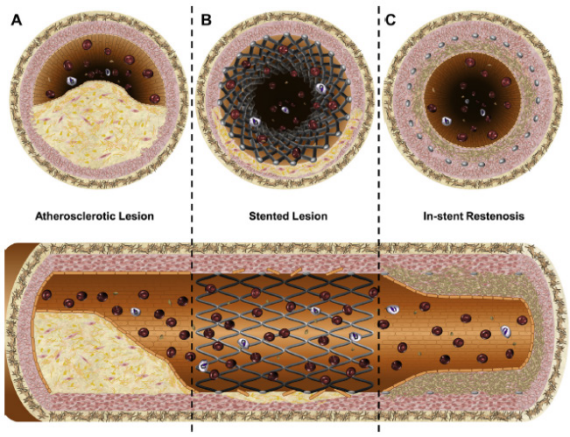

Figure 2. Development of in-stent restenosis after BMS implantation. (A) Atherosclerotic lesion with plaque development. **(B)** BMS implantation compresses the atherosclerotic material into the vessel wall provoking an injury of the endothelial cell layer. **(C)** Proliferation of vascular SMCs and secretion of extracellular matrix proteins leads to in-stent restenosis. Figure reproduced from Simard *et al.* [49]

Drug-eluting stents – Appearance of late stent thrombosis

Considering that PTCA induced injury is not fully avoidable during the procedure, and assuming that inflammatory processes and proliferation of vascular SMCs contribute to in-stent restenosis, the stent material was coated with antiproliferative or immunosuppressant drugs. These drug-eluting coronary implants revolutionized interventional cardiology. The first generation of drug-eluting stents (DES) approved by the FDA were loaded with sirolimus (Cypher®, Cordis, Warren, New Jersey, USA) or paclitaxel (Taxus®, Boston Scientific, Natick, USA) on a stainless steel platform, both inhibiting the mitogen-mediated proliferation of vascular SMCs during neointima formation. While sirolimus inhibits the initial phase of cell cycle progression (G1) [57], paclitaxel directly restrains the spindle microtubule dynamics and therefore inhibits the mitosis phase of the cell cycle [58]. The approval of these devices was based on data from short-term clinical trials, which evaluated the appearance of ISR and adverse cardiovascular events like myocardial infarction. Researchers observed restenosis rates of approximately 10% without adverse systemic effects normally associated with the compounds [59-63]. Even five-year follow-up studies investigating the long-term efficacy of paclitaxel- and sirolimus-eluting stents demonstrated a real benefit with an overall reduction of target vessel revascularization when compared to BMS treated patients [64, 65].

However, despite the successful reduction of neointima formation, the increasing risk of a rare but more severe complication, namely late stent thrombosis (LST, > 30 days after stenting) or very LST (> 1 year) overshadowed the clinical success of first generation DES [66-68]. Stent thrombosis results in an abrupt closure of the stented artery and is associated with a high risk for myocardial infarction and death [69, 70]. One must consider that thrombotic events often occurred with the first DES due to premature antiplatelet therapy discontinuation. In this regard, the FDA prolonged antiplatelet therapy after DES placement from 3-6 months to one year despite this being associated with the risk of bleeding [71-73].

Unfortunately, there is no precise understanding of the mechanisms contributing to late stent thrombosis but findings suggest that a combination of several factors promote its development [49]. Apart from patient- and lesion-specific factors, DES-specific characteristics lead to the increased risk of LST including the hypersensitivity to drug coating or polymer and inflammatory response to stent struts [3]. Nevertheless, the most attributable factor for thrombosis is the disturbance of endothelial cell regeneration (re-endothelialization) by the not cell-specific activity of the loaded compounds. Both, sirolimus and paclitaxel not only inhibit the migration and proliferation of SMCs but also the proliferation and viability of endothelial cells. Since functional vascular endothelium plays a crucial role in vascular homeostasis by preventing inflammation and remodeling processes, these compounds thus create a pro-thrombotic environment with possible fatal consequences one year after stent implantation.

In-stent restenosis vs. late stent thrombosis

In conclusion, the implantation of coronary devices is associated with two severe complications the in-stent restenosis and the late stent thrombosis. In-stent restenosis was first observed in patients treated with BMS (Figure 3A) [74], while late stent thrombosis is the unwanted side effect of DES (compare Figure 3B).

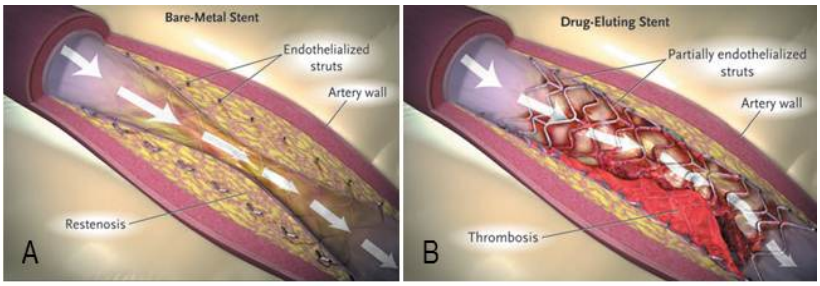

Figure 3. Complications of Coronary Stenting. (A) Restenosis or **(B)** late stent thrombosis in a coronary artery treated with a BMS or DES, respectively. Figure reproduced with permission from Curfman *et al.* [75], Copyright Massachusetts Medical Society.

Even if the current understanding of the factors contributing to ISR and LST suggests that these unwanted effects are device-specific, there is an overlap. Restenosis and thrombosis affect the clinical outcome of both devices, but often at different time-points post stenting [76]. For instance, it has been demonstrated that very LST occurs with a rate of 0.1% per year in patients receiving a BMS even though this rate is much lower than that observed for DES [77, 78].

1.4. Current approaches for improvement of DES

Considering the pathological basis for restenosis and stent thrombosis it seems evident that the stent material and/or drug coating are key features that may be improved to promote a functional vascular endothelium. From a pharmacological point of view, the identification of a compound ideally demonstrating SMC-specific antiproliferative activity appears to be a promising strategy. Hence, special emphasis has been placed on the improvement of coronary stents using novel polymers or stent materials and/or potent anti-inflammatory and antiproliferative drugs. Thus researchers have developed DES with (1) different stent platforms, (2) biocompatible or bioresorbable polymers, (3) or other compounds of the limus group, investigations that resulted in the development of second- and next-generation DES (see Table 1). Different randomized controlled clinical trials have compared the clinical outcome of these DES with the first-generation DES or BMS. The following paragraphs elucidate these recent investigations focusing on stent material and compounds of the limus group and how previous research has led to the approaches adopted in this study.

Investigations on stent platform and polymers

There is emerging evidence that stent design plays a significant role in development of restenosis and/or thrombosis, especially since hypersensitivity reactions to the materials has impaired the success of coronary implants [79, 80]. Current scaffolds of DES are composed of cobalt chromium or platinum chromium both allowing thinner stent struts with a reduction of local inflammatory processes and the development of overall and very LST [81-83].

In addition, biocompatible stent platforms, especially devices that consist of the poly-L-lactic acid (PLLA) demonstrate good safety in coronary applications [84, 85]. Moreover, inflammatory reactions can be reduced using bioresorbable stent platforms and/or drug-eluting polymers. Previous polymers eluting sirolimus or paclitaxel consisted of synthetic materials, which have been associated with LST [86]. Immediately after drug release, the remaining polymer induces an inflammatory reaction as demonstrated from pathological samples of stented vessels where inflammatory cells appear around fragments of polymers [87]. The biolimus-eluting stent Nobori®

consists of a stainless steel platform, biolimus and a bioresorbable polymer, which dissolves into carbon dioxide and water after 6 to 9 months. Studies comparing Nobori® with paclitaxel- or sirolimus-eluting stents have demonstrated promising results [88, 89]. Consequently, stents with complete bioresorbable scaffold were designed. The Absorb®, a fully bioresorbable stent completely dissolves in 3 to 3.5 years. Although investigations yielded no significant concerns with the Absorb® compared to stents with durable material, a multi-center study demonstrated a high risk of scaffold thrombosis within the first 6 months [90, 91]. Accordingly, further studies are warranted to elucidate whether these strategies will improve the safety and efficacy profiles of currently used DES.

Table 1. Overview of former and new Drug-eluting stents and related clinical trials (small selection)

Stent name	Stent platform	Polymer	Drug eluted	Clinical trials
First-Generation DES				
Cypher®	steel	persistent	sirolimus	SIRIUS
Taxus®	steel	persistent	paclitaxel	TAXUS EXPRESS® I-VI
Second-Generation DES				
Xience V®	cobalt	persistent/biocompatible	everolimus	SPIRIT I-V, SORT-OUT
Endeavor®	cobalt	persistent	zotarolimus	ENDEAVOR I-IV
Next-Generation DES				
Nobori®	steel	bioresorbable PLA	biolimus	NOBORI I
Absorb®	bioresorbable PLA	bioresorbable	everolimus	ABSORB II

Everolimus and zotarolimus as new compounds on DES

The efficacy and safety of a coronary device is not only influenced by the stent material, but also by the loaded drug. As inflammation plays a crucial role in the development of atherosclerosis, restenosis and thrombosis, the first DES was loaded with sirolimus, which is an effective immunosuppressive and antiproliferative agent that demonstrates high potential in restenosis reduction. Data from meta-analyses confirmed a more efficient inhibition of ISR compared to paclitaxel-eluting stents [92, 93]. Thus, researchers evaluated the efficacy of sirolimus-derivatives such as everolimus and zotarolimus (Figure 4). Despite everolimus being commonly used as an immunosuppressant in transplantation medicine and exhibiting similar pharmacodynamics as sirolimus with inhibition of cell metabolism and cell proliferation by

blocking the cell cycle at late G1 phase, this drug has a different pharmacokinetic profile with higher polarity and a slightly higher lipophilic character [94, 95]. Consequently, everolimus is assumed to exhibit enhanced cellular absorption and a longer intracellular resident time. After observing promising results with oral everolimus in animal studies [96, 97], a clinical evaluation of an everolimus-eluting stent was carried out. On the basis of results from the SPIRIT trial program, the FDA approved the Xience V® Stent (Abbott Laboratories, Abbott Park, IL, USA) in 2008. When compared to BMS or paclitaxel-eluting stents this device demonstrated enhanced safety with a significant risk reduction in thrombotic events even in the long-term follow up [98-100]. A meta-analysis demonstrated increased benefits with everolimus-eluting stents in long-term clinical outcome with regard to stent thrombosis [101]. However, when compared to sirolimus-eluting stents the risks for myocardial infarction or all-cause death were similar for both stent types.

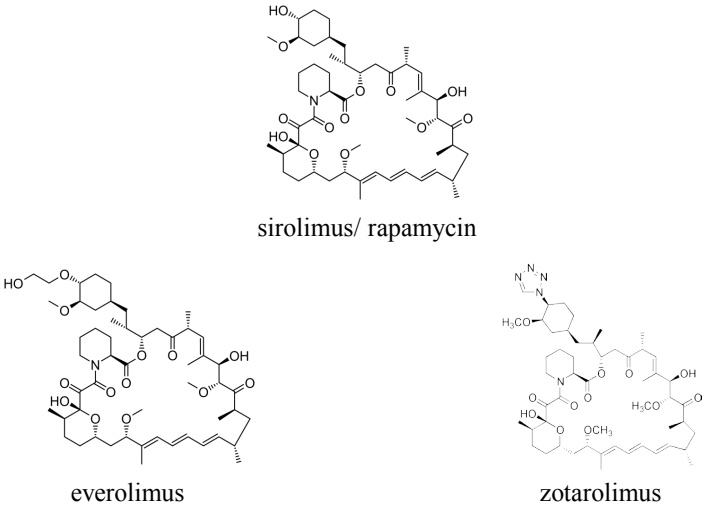

Figure 4. Structures of limus agents.

Another compound of the limus group is zotarolimus, which is a semisynthetic derivative of sirolimus. Zotarolimus is the most lipophilic of the limus analogues synthesized to support higher vessel wall localization, whereby reducing systemic circulation and demonstrating a high impact on vascular SMC proliferation [102]. Data from clinical trials comparing the efficacy and safety of the zotarolimus-eluting stent Endeavor® (Medtronic, Santa Rose, CA, USA) in humans demonstrated this stent system as a safe treatment for obstructive coronary disease [103] with a lower incidence of restenosis compared to BMS [104]. Although Endevaor® failed to promote an improvement in ISR reduction when compared to first-generation DES [105, 106], the low safety-risks enabled an FDA approval. Interestingly, myocardial infarctions and death were

less common with zotarolimus-eluting stents suggesting a potential benefit concerning vascular healing [107, 108].

Brief summary about current approaches

In addition to new stent materials and polymers, the loaded drug remains the main predictor for efficacy and safety of coronary devices. The first DES were developed using agents that mainly exhibited antiproliferative activity in cells. Due to safety issues associated with the lack of cellular specificity of the compounds, there is a need to identify drugs that specifically inhibit the proliferation of vascular SMCs or that restrain the inflammation that arises after stent deployment. Although the use of sirolimus derivatives everolimus and zotarolimus reduced the rates of stent thrombosis compared to first-generation DES, there is still the risk of late events suddenly affecting patients' health; the development of neoatherosclerosis and associated incidence of late restenosis and thrombosis is a common feature in patients with late stent failure [109]. Experience has shown that it is essential to know which cellular mechanisms are responsible for the success or failure of a drug in stent design. For example preclinical studies with the limus agent tacrolimus promised a favorable outcome in patients but clinical data demonstrated a high restenosis rate [110-112]. Following this, *in vitro* data investigating the efficacy of tacrolimus to inhibit SMCs proliferation revealed an induction of cell proliferation by tacrolimus, explaining the failure in clinical assessment (see chapter 1.5 anti-inflammatory compounds) [113]. Thus, there is a need for *in vitro* research to identify the pharmacodynamics in cells specifically from the microcompartment coronary artery, which are involved in restenosis and thrombosis development in order to extrapolate the efficiency *in vivo*.

1.5. Novel approaches for DES based on cellular determinants

Vascular endothelial and smooth muscle cells are those cells that are mainly influenced by dynamic changes and injuries. Both cell types react with structural and functional alterations consequently leading to a remodeling of the vessel. Therefore, in drug development it is necessary to understand the cellular mechanisms in SMCs as well as ECs that contribute to the required effects, especially when a cell-specific activity is warranted.

Dual-loading stents – A luminal/abluminal loading technology

Since clinical data of sirolimus-eluting stents indicated strong potential, long-term safety concerns encouraged researchers to evaluate the effect of other compounds targeting cellular processes involved in ISR development. First, the efficacy of systemically applied substances

including antiplatelet and anticoagulant drugs, vitamins, or statins was assessed [114]. This led to considering whether a combination of these drugs with sirolimus or paclitaxel would be useful for targeting different cellular functions to strengthen the efficacy in ISR reduction and to enhance safety of DES. Accordingly, it is important to note that the antiproliferative drug should be directed to the side where SMCs migrate and proliferate (abluminal). This loading technology ensures a more targeted tissue release as used in the biolimus-eluting Nobori® DES stent [115].

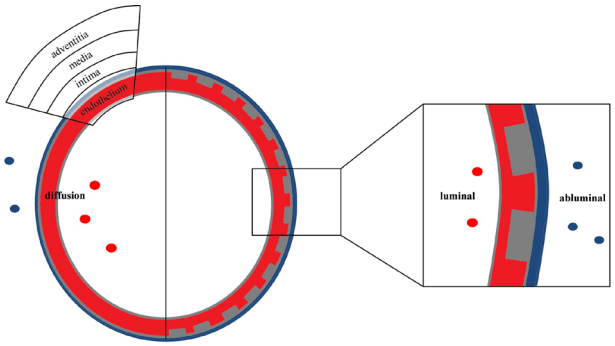

Figure 5. Schematic of a dual-loading DES. The metallic scaffold is coated with a polymer integrating a luminal (red) bloodstream-directed compound and an abluminal (blue) vessel-wall directed drug-release. The DES is integrated into the endothelium. Drug release into the abluminal direction should inhibit the proliferation of intimal and medial smooth muscle cells.

Antioxidants and anti-thrombotic compounds

The stimulation of vascular SMC proliferation is amongst others achieved by oxygen free radicals [116]. Therefore, probucol a lipid-lowering drug with antioxidant properties was tested in local drug delivery based on studies demonstrating a benefit of systemic probucol [117-119]. In detail, probucol reduced inflammatory cell adhesion and inhibited stent thrombosis as well as neointimal hyperplasia, thereby promoting re-endothelialization in rabbit artery models. However, although a polymer free dual-loading DES consisting of probucol and sirolimus demonstrated a good efficiency and safety profile this DES failed to show enhanced clinical benefits [120]. This was assumed to be a result of the polymer free surface [121]. Currently a probucol-releasing anti-thrombogenic DES is under development [122].

Another compound, which has been embedded with sirolimus in a DES, is the anti-thrombotic drug triflusal. *In vitro* release kinetics revealed an inhibition of platelet adhesion and suggested a release of both compounds within a time frame that favors the temporal sequence of thrombosis formation, inflammation, and proliferation *in vivo* [123]. These findings are supported by additional *in vivo* studies in a porcine coronary model showing a reduction in restenosis when compared to controls. However, clinical data are still missing.

HMG-CoA reductase inhibitors – Statins

The activity of the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) is the rate-limiting step in human cholesterol synthesis by reducing HMG-CoA to mevalonate. Since hypercholesterolemia is associated with the development of atherosclerosis and cardiovascular disease, a number of clinical trials have explored whether HMG-CoA reductase inhibitors, namely statins, diminish the progression of atherosclerosis and CVD [9, 10]. Indeed, systemically administered statins reduced overall cardiovascular mortality. Accordingly, research that followed studied the impact of statins in patients receiving a coronary stent, showing lower rates of restenosis and/or thrombotic events. These findings are thought to be based on the "pleiotropic effects" of statins, including anti-inflammatory and anti-thrombotic activity [124-126]. Local application of statins in DES has been tested in order to avoid adverse events and to achieve a higher local drug concentration. Although at first *in vivo* data investigating the effect of statin-eluting stents demonstrated a positive impact on endothelialization compared to BMS or DES [127-129], there was no superiority compared to former DES with regard to neointima reduction. Nevertheless, the activity of statins specifically on endothelial cell regeneration is useful for dual loading-applications, for example based on data showing statin-induced upregulation of endothelial NO synthase activity [130]. Thus, statins were suspected to suppress the delayed arterial healing originating from the cytotoxicity of limus agents. Preliminary efforts have been made testing the concomitant loading of tacrolimus and atorvastatin [131]. Although tacrolimus alone demonstrated a pro-proliferative effect on vascular SMC proliferation by activation of growth factor signaling (TGF-β) [113], Giordano *et al.* detected that dual treatment of SMCs with atorvastatin suppressed the induced proliferation by apparently reinforcing the proper activity of tacrolimus (see also section 1.5 anti-inflammatory compounds) [131]. Furthermore, tacrolimus supplemented with atorvastatin enhanced endothelial cell proliferation due to increased expression of the pro-angiogenic factor endoglin. Nonetheless, the more potent antiproliferative compounds are sirolimus and everolimus. Therefore, future DES design could use these compounds as suitable drug candidates for concomitant use with statins.

Smooth muscle cell-specific drug targeting

As reviewed by Tang and Chen, the identification of SMC-specific drugs may follow different approaches including the identification of proteins regulating cell proliferation, or detection of growth factors which have differential effects on EC and SMC, and/or use of more than one

compound to achieve multiple function for different cells [132]. In the vascular system, target (SMC) and non-target (EC) cells are in close vicinity thus previously used compounds failed to demonstrate cell-type specific efficiency. Therefore, one promising approach is the identification of a compound that specifically targets SMCs proliferation and/or migration during vascular remodeling. One example of a SMC specific drug target is the enzyme CTP synthase -1 that catalyzes CTP biosynthesis, which is essential for DNA and/or RNA replication during cell proliferation [133]. Tang *et al.* showed that proliferating SMCs especially exhibited a high expression of this enzyme; and low dose inhibition of CTP synthase 1 diminishes growth factor induced SMC proliferation while not affecting ECs [132, 134]. Similar specific effects could be obtained using the fatty acid oxidation inhibitor trimetazidine, which was shown to decrease the proliferation and migration of SMCs while exhibiting pro-proliferative effects in endothelial cells [135].

Cell-specific inhibition by statins

Despite lipid-lowering activity, statins are assumed to modulate different cellular functions independently from cholesterol synthesis (see Figure 6). The inhibition of small GTP-binding proteins (including Ras and Rho) by statins plays an especially crucial role in modulation of cell proliferation [136]. Statins inhibit the synthesis of the isoprenoid derivatives geranylgeranyl pyrophosphate, subsequently suppressing the activation the GTPase Rho. If activated, Rho translocate to the cellular membrane. The subsequent modulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ promotes cell-cycle progression and proliferation of vascular SMCs [137]. In quiescent cells, $p27^{Kip1}$ is stably expressed and contributes to growth arrest by inhibition of cyclin/cyclin-dependent kinase complexes. Upon mitogen stimulation, $p27^{Kip1}$ is down-regulated and leads to an induction of cell cycle and proliferation. Consequently, overexpression of this protein attenuated neointima formation following vascular injury [138].

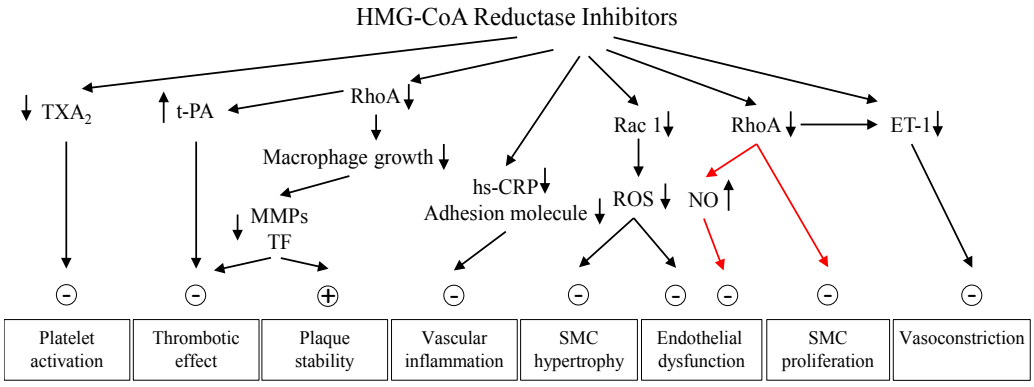

Figure 6. Pleiotropic Effects of Statins Figure reproduced and modified from Takemoto & Liao [136]

Importantly, a previous study using cerivastatin showed differential cell proliferation *in vitro,* with more pronounced proliferation in SMCs compared to ECs [12]. Further *in vivo* investigation using a porcine coronary model supported these findings. From a pharmacological point of view, cerivastatin treatment increased $p27^{Kip1}$ levels in mitogen stimulated SMCs, an effect that was less pronounced in ECs. The authors assume that this cell-specificity is considered a statin effect since they found similar results using fluvastatin. However, the underlying mechanism for the SMC-specific effect remains unanswered.

Inflammation as potent target in stent technology

Based on current understanding, inflammatory reaction plays a significant role in the development of ISR. Cytokine-stimulated release from infiltrating monocytes in injured vessels initiates the migration and proliferation of vascular SMCs. In addition, stent thrombosis associated with DES deployment is a local hypersensitivity reaction accompanied by infiltration of T cells, leukocytes and eosinophils and is histopathological described by a persistent fibrin thrombus between stent and arterial wall [55, 87]. As recently reviewed, although late stent thrombosis occurs with an incidence of 0.5% per year it is a severe complication resulting in acute myocardial infarction or sudden cardiac death with a mortality rate of 45% [139]. It has been assumed that the increased endothelial tissue factor expression caused by either sirolimus or paclitaxel promotes the activation of the coagulation cascade [140, 141]. Therefore, the use of these drugs is no longer reasonable. Accordingly, efforts have been made to use compounds that consistently exhibit anti-inflammatory effects thus inhibiting the activation and proliferation of inflammatory cells including T cells.

The calcium- and calmodulin-dependent serin/threonine phosphatase calcineurin is essential for T cell activation (compare Figure 7). A complex interaction of antigen-presenting cells and toll-like receptors induces the activation of calcineurin which dephosphorylates the nuclear transcription factor of activate T cells (NFAT). Active NFAT translocate to the nucleus and bind promotor elements of genes thus increasing the expression of pro-inflammatory cytokines including interleukin (IL)-2, IL-3, interferon-γ, and TNF-α [142]. In contrast to the immunosuppressive activity of sirolimus achieved by the binding of mTOR, the compounds cyclosporine A, tacrolimus, or pimecrolimus act via inhibition of calcineurin. A comparison of both substance classes *in vitro* demonstrated a markedly smaller effect of tacrolimus on cell proliferation compared to sirolimus [143]. Conversely, tacrolimus seemed to induce the proliferation of vascular SMCs by activation of growth factor signaling cascades [113]. The discrepancy of these *in vitro* data is reflected in *in vivo* studies [110, 112]. Despite the failure of

tacrolimus-eluting stents in a clinical study, a preclinical setting demonstrated a positive impact on neointima formation. Similar observations have been made studying the safety and efficiency of DES loaded with pimecrolimus [15, 16, 144].

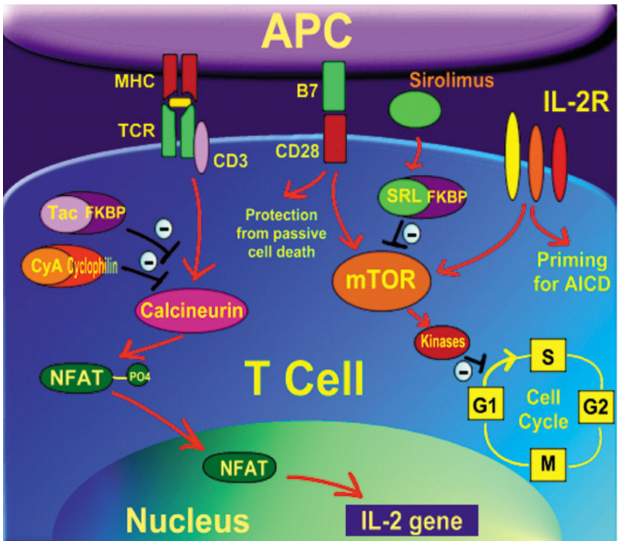

Figure 7. mTOR and calcineurin signaling in T cells The activation of calcineurin results in de-phosphorylation of NFAT (nuclear factor of activated T cells) and a subsequent expression of IL-2 (interleukin 2) and other cytokines. Calcineurin inhibitors cyclosporine and tacrolimus act by binding to their respective immunophilins and induce a blockade of IL-2 production that inhibits T cell proliferation. The sirolimus/FKBP12 complex binds mammalian target of rapamycin (mTOR) subsequently inhibiting cell cycle. Figure reproduced from Dupont and Warrens [145].

1.6. Pharmacokinetics determine pharmacodynamics

The balancing act in drug development is to identify a compound that achieves high drug concentrations on the desired tissue or cellular target while avoiding adverse events often resulting from high systemic concentrations. This means an ideal compound applied on drug-eluting devices should have (1) a wide therapeutic window, (2) lipophilic properties and (3) an appropriate tissue retention time to guarantee complete endothelialization [146]. Accordingly, the therapeutic efficacy of DES compounds depends not only on the drug target but also on the pharmacokinetics in the vascular wall. Pharmacokinetics in general describes the fate of a compound starting from liberation, absorption, distribution, biotransformation, and excretion. All parameters influence the pharmacological activity of the compound as a drug, as they regulate its exposure to the desired tissue. In contrast, pharmacodynamics describes the relation between the drug concentration at its target and the drug effect; specifically, the initial absorption or uptake of compounds into target cells is an important step in biological drug activity.

Mechanisms of drug transport

Endogenous substances or exogenously-administered compounds get into cells by different transport mechanisms depending on their structural nature. The interplay of molecular weight, molecular charge, and surface polarity defines cellular entry including simple diffusion, facilitated diffusion, and active transport.

Impact of pharmacokinetics in DES technology

Physiochemical properties of substances including aqueous solubility, lipophilicity or ionization play an important role in drug potency. Hydrophilic compounds, which are rapidly cleared, are differ from hydrophobic compounds that are retained within the tissue, often with dramatic consequences [147]. A hydrophilic compound is advantageous if this drug possesses a small therapeutic window, thus avoiding a toxic accumulation in tissues. Contrastingly, a lipophilic compound is necessary to be absorbed and to maintain high doses in the right place. The parameters of "Lipinski´s rule of five" predict the permeability of a compound through biomembranes especially after oral drug delivery. It includes the number of H-bond donors and exceptors, molecular weight and the log P value [148]. "P" describes the partition of a compound between two liquid phases, polar water and non-polar octanol and measures the lipophilicity of a compound. A log P > 5 means poor absorption or penetration through the membrane. Furthermore, the molecule passes over into the lipophilic membrane but will be less likely to pass from the membrane on the receptor side into aqueous internal layers [149]. Direct comparison of sirolimus and paclitaxel revealed an equal lipophilic property as characterized by log P values of ~4.3 and ~3.2 for sirolimus or paclitaxel, respectively, which is associated with an efficient accumulation in the vascular wall [150]. However, the effect of compounds is not exclusively due to the tissue distribution achieved by pharmacokinetics. Sirolimus accumulates particularly in the ISR-originating intima-media zone whereas paclitaxel distributes heterogeneously through the arterial wall, explaining the better efficacy of sirolimus [151]. This has been associated with the different distribution of their cellular targets. The target for limus agents FKBP12 is abundantly expressed in SMCs and upregulated after vascular injury [152, 153]. Based on the current understanding of ISR development, drug concentrations should be highest in the medial and intimal layer of the vascular wall. Nevertheless, drug transporters extend the possibilities for compounds to be distributed. Hence, they reduce or enhance drug absorption through the membranes. However, the impact of these proteins on drug absorption is not predicted by Lipinski´s rule of five.

The first identified drug transporters are members of the ATP-binding cassette superfamily facilitating the efflux of their substrates. More precisely, an overexpression of P-glycoprotein has been associated with the incidence of multidrug resistance in cancer cells to overcome chemoresistance [154]. However, drug transporters are not exclusively expressed in malignant transformed tissue. Hence, they mediate absorption, elimination, and distribution of a variety of its substrates, thereby modulating their biological activity.

Chapter 2

Aim of the thesis

Considering the data from various clinical studies there is still a need to improve coronary stent technology. Although the incidence of in-stent restenosis is reduced, the cytotoxic compounds applied in DES induce a delayed healing characterized by incomplete strut endothelialization [69]. Delayed arterial healing is assumed to contribute to the development of late thrombotic events [155]. Thus, one approach is the identification of compounds, which specifically inhibit SMCs proliferation while not affecting ECs.

The limus compound sirolimus has been widely used for many years as an immunosuppressive and antiproliferative agent in DES. It inhibits the function of mTOR, resulting in the inhibition of cell cycle progression [57]. Statins exert pleiotropic effects including antiproliferation, anti-inflammation, and anti-thrombosis [136]. Cerivastatin and atorvastatin especially reduced the proliferation of vascular cells [129, 156]. Additionally, atorvastatin activates NO synthesis by enhanced expression of the endothelial NO synthase [130], and accelerated the neointimal coverage and re-endothelialization if systemically-administered after sirolimus-eluting stent implantation in a porcine coronary model [157].

The **first aim** of the present thesis is to study the effects of a concomitant treatment of sirolimus and atorvastatin both applied on a dual-loading DES. Stents were produced at the University of Rostock, Germany and analyzed with respect to the release kinetics of both drugs. The effects on cells are studied *in vitro*. Here, the impact on SMCs and ECs proliferation and viability are taken into consideration.

Despite passive membrane transport induced by the lipophilic character of a xenobiotic, one mechanism contributing to pharmacokinetics is the expression of drug transporters that facilitate the transport of substrates [6]. The organic cation transporters (OCT) are one subfamily within the superfamily group of SLC transporters. OCT1 plays a key role in drug distribution of exogenous and endogenous cations including paclitaxel [8].

The aim of our **second study** is to analyze whether overexpression of OCT1 will enhance the cellular effects of paclitaxel particularly in SMCs. Therefore we used the promotor of transgelin

(SM22α) a commonly used marker for SMCs, which has been previously identified as a mechanistic tool for performing a cell-type specific overexpression of protein in a mouse model [158, 159].

Cell proliferation is affected by a variety of compounds. Cerivastatin and fluvastatin demonstrate a more pronounced effect on SMCs compared to ECs *in vitro* [12]. However, pravastatin does not share this antiproliferative activity [160]. Although statins primarily target HMG-CoA reductase, they exert different physiochemical and pharmacokinetic properties including active transport or passive permeability [161]. Hence, comparing all statins, especially different lipophilicity can account for a modulation of cellular distribution. Assuming that the distribution of cells of the coronary artery surrounding the drug-delivery devices also depends on passive diffusion or active transport, those mechanisms may significantly influence cell-type specificity of drugs used in DES.

In the **third part** of this thesis, the impact of different statins on cell proliferation is compared, focusing on whether differences in pharmacodynamics or pharmacokinetics are responsible for the observed cellular effects.

Inflammation characterized by an increased level of inflammatory cells and cytokines is believed to be the major driving force in the development of restenosis and thrombosis after vascular injury [162, 163]. Accordingly, treating inflammation is one of the approaches in stent technology. First generation DES used sirolimus showing a more pronounced effect on cell proliferation. Hence, other limus agents including everolimus, tacrolimus or pimecrolimus were tested in preclinical studies. However, tacrolimus and pimecrolimus did not enter clinics due to failure in neointima reduction [16, 110]. Since the underlying mechanism with tacrolimus was based on growth factor induced proliferation of SMCs, little is known about the pharmacodynamics of pimecrolimus in human coronary cells.

In our **final project** we studied the impact of pimecrolimus on cell proliferation and viability of SMCs and ECs in order to identify the molecular mechanisms that may explain the observed clinical outcome in patients.

In vitro cell model – Microcompartment of the coronary artery

In our recent work we used primary endothelial and smooth muscle cells isolated from the human coronary artery, namely HCAEC and HCASMC (Promocell GmbH, Heidelberg, Germany), characterized for their cell-specific proteins (Figure 8). Endothelial and smooth muscle cells, which are in close vicinity, represent the cellular part of the microcompartment coronary artery that is mainly involved in the development of restenosis and thrombosis after stenting. The effects of different compounds were studied in various assays in order to identify pharmacokinetics and pharmacodynamics that account for the activity of the tested substances. To simulate the situation *in vivo* before stent placement, HCAEC and HCASMC were cultured in a medium containing low concentrations of fetal calf serum for 24 h or 48 h, respectively. Thereafter cells were treated with the compounds in presence of growth factors to mimic the situation post stenting. Results from our *in vitro* studies were used to extrapolate the situation *in vivo* or to explain previous data that arised from *in vivo* studies.

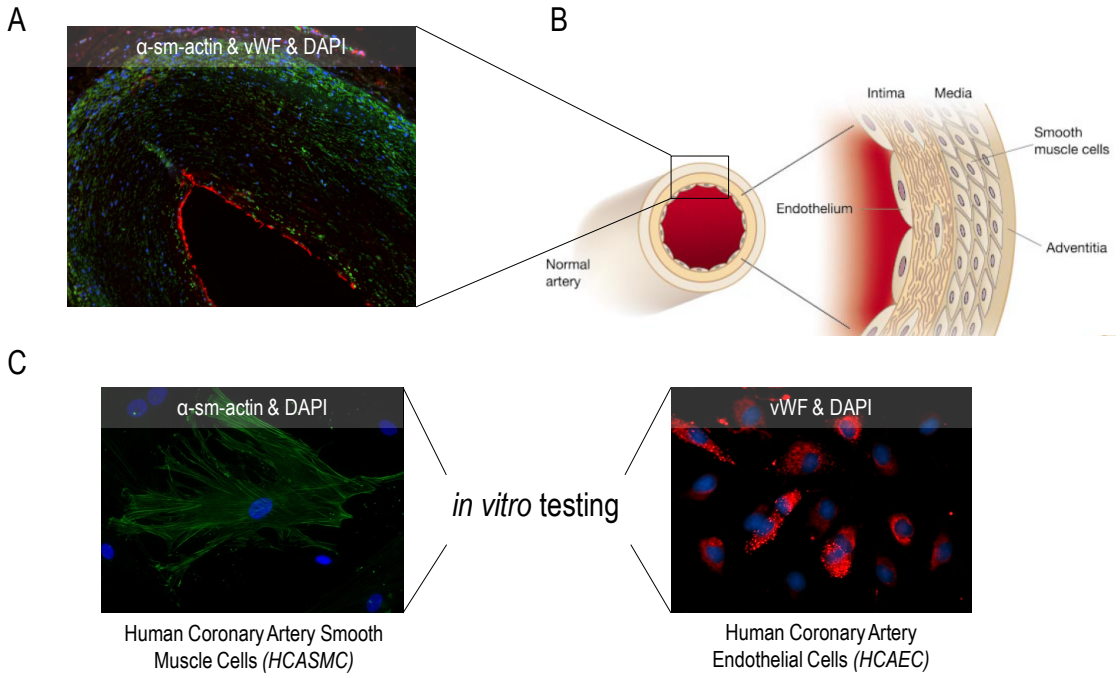

Figure 8. Coronary artery. (A) Immunofluorescence of a histological section from human coronary artery. Tissue slide of paraffin-embedded coronary artery was stained for expression of SMC specific alpha-smooth muscle actin (α-sm-actin, green color) and for van Willebrand factor (vWF, red color) characteristic for ECs. Nuclei were stained with 4′,6-Diamidin-2-phenylindol, DAPI. (400x) **(B)** Schematic of a human artery. Figure reproduced and modified from Libby [164]. **(C)** Immunofluorescence of human coronary artery smooth muscle cells (HCASMC) and endothelial cells (HCAEC). Cells were stained for expression of α-sm-actin (HCASMC, green color) and vWF (HCAEC, red color). Nuclei were stained with DAPI. (HCASMC, 200x; HCAEC, 400x).

List of Publications

This doctoral thesis is based on four publications:

1. Svea Petersen, **Janine Hussner**, Thomas Reske, Niels Grabow, Volkmar Senz, Robert Begunk, Daniela Arbeiter, Heyo K. Kroemer, Klaus-Peter Schmitz, Henriette E. Meyer zu Schwabedissen[2], Katrin Sternberg
   In vitro study of dual drug-eluting stents with locally focused sirolimus and atorvastatin release
   Journal of Material Science: Materials in Medicine (2013) 24: 2589-2600

2. Henriette E. Meyer zu Schwabedissen, Robert Begunk, **Janine Hussner**, B. Ole Juhnke, Daniel Gliesche, Kerstin Böttcher, Katrin Sternberg, Klaus-Peter Schmitz, Heyo K. Kroemer
   Cell-specific expression of uptake transporters – A potential approach for cardiovascular drug delivery devices
   Molecular Pharmaceutics (2014) 11, 665-672

3. **Janine Hussner**, Robert Begunk, Kerstin Boettcher, Daniel Gliesche, Katharina Prestin, Henriette Meyer zu Schwabedissen
   Expression of OATP2B1 as determinant of drug effects in the microcompartment of the coronary artery
   Vascular Pharmacology (2015) 72, 25-34

4. **Janine Hussner**, Juliane Sünwoldt, Isabell Seibert, Daniel G. Gliesche, Henriette E. Meyer zu Schwabedissen
   Pimecrolimus increases the expression of interferon-inducible genes that modulate human coronary artery cells proliferation
   European Journal of Pharmacology (2016) 137-146

Additionally, another publication and two abstracts of congress contributions are cited here but do not contribute to the draft of this doctoral thesis and are not entirely presented but whose data reflect research that contribute in form and content to the improvement of DES.

Daniel Gliesche, **Janine Hussner**, Dominik Witzigmann, Fabiola Porta, Timo Glatter, Alexander Schmidt, Jörg Huwyler, Henriette Meyer zu Schwabedissen
Secreted Matrix Metalloproteinase-9 of proliferating smooth muscle cells a trigger for drug release from stent surface polymers in coronary arteries
Submitted in Molecular Pharmaceutics

Hussner J, Begunk R, Iaroshenko V, Mkrtchyan S, Hein M, Supe L, Sternberg K, Langer P, Bien S, Schmitz K.P, Kroember H.K, Meyer zu Schwabedissen H. *Pyrazolinone-Derivatives selectively influence the proliferation of HCASMC – A new approach of prevention in-stent restenosis.* 77th Annual Meeting of DGPT, Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie e.V. Frankfurt, Germany, March 31 – April 1, 2011 (poster presentation)

Hussner J, Begunk R, Bien S, Koeck K, Kroember H.K, Meyer zu Schwabedissen H. *HDAC9 – A potential drug target in preventing smooth muscle cell proliferation.* Annual Scientific Session of the ATVB (Arteriosclerosis, Thrombosis, and Vascular Biology). Chicago, IL, USA, April 28 - 30, 2011 (poster presentation)

Chapter 3

Results and discussion

3.1. In vitro study of dual drug-eluting stents with locally focused sirolimus and atorvastatin release

Svea Petersen[1], Janine Hussner[2], Thomas Reske[1], Niels Grabow[1], Volkmar Senz[1], Robert Begunk[2], Daniela Arbeiter[1], Heyo K. Kroemer[1], Klaus-Peter Schmitz[1], Henriette E. Meyer zu Schwabedissen[2], Katrin Sternberg[1]

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Contribution J. Hussner: co-author and experimental part (*in vitro* studies)

Journal of Material Science: Materials in Medicine 2013; 24(11): 2589-2600.

J Mater Sci: Mater Med (2013) 24:2589–2600
DOI 10.1007/s10856-013-5001-7

In vitro study of dual drug-eluting stents with locally focused sirolimus and atorvastatin release

Svea Petersen · Janine Hussner · Thomas Reske · Niels Grabow · Volkmar Senz · Robert Begunk · Daniela Arbeiter · Heyo K. Kroemer · Klaus-Peter Schmitz · Henriette E. Meyer zu Schwabedissen · Katrin Sternberg

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Abstract Within the context of novel stent designs we developed a dual drug-eluting stent (DDES) with an abluminally focussed release of the potent anti-proliferative drug sirolimus and a luminally focussed release of atorvastatin with stabilizing effect on atherosclerotic deposits and stimulating impact on endothelial function, both from biodegradable poly(L-lactide)-based stent coatings. With this concept we aim at simultaneous inhibition of in-stent restenosis as a result of disproportionally increased smooth muscle cell proliferation and migration as well as thrombosis due to failed or incomplete endothelialisation. The especially adapted spray-coating processes allowed the formation of smooth form-fit polymer coatings at the abluminal and luminal side with 70 % respectively 90 % of the drug/polymer solution being deposited at the intended stent surface. The impacts of tempering, sterilization, and layer composition on drug release are thoroughly discussed making use of a semi-empirical model. While tempering at 80 °C seems to be necessary for the achievement of adequate and sustained drug release, the coating sequence for DDES should be rather abluminal–luminal than luminal–abluminal, as reduction of the amount of sirolimus eluted luminally could then potentially minimize the provocation of endothelial dysfunction. In vitro proliferation and viability assays with smooth muscle and endothelial cells underline the high potential of the developed DDES.

1 Introduction

After entering the European market in 2002, drug-eluting stents (DES) revolutionized the treatment of coronary artery disease. DES are specialized vascular stents which allow a long-term local drug delivery with the purpose to reduce or prevent in-stent restenosis (ISR), which has been identified as one major drawback of bare metal stents (BMS). In this context, DES of the so called first generation provided a controlled release of either the immunosuppressant sirolimus (SIR, e.g. Cypher™, Cordis) or the cytostatic paclitaxel (e.g. Taxus™, Boston Scientific) from a permanent polymeric coating. However, despite their high efficacy regarding the inhibition of ISR, the first generation came under scrutiny, in particular when late thrombosis and delayed healing were identified as potential risks associated with DES [1–3]. As this was on one side dedicated to the anti-proliferative effect of both drugs on endothelial cells (EC) in addition to the targeted smooth muscle cells (SMC) [4] and on the other side to local hypersensitivity with regard to the permanent polymeric coating [5], new drugs and biodegradable polymers have been recently introduced. Commonly used polymers are nowadays polylactides (PLA) [6, 7] and copolymers, such as poly(lactide-co-glycolide) (PLGA) [8], while innovative drugs are often SIR analogues including zotarolimus [9–11], everolimus [12, 13] and biolimus A9 [14]. Moreover, in order to simultaneously inhibit ISR as a result of disproportionally increased smooth muscle cell migration

Electronic supplementary material The online version of this article (doi:10.1007/s10856-013-5001-7) contains supplementary material, which is available to authorized users.

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as well as thrombosis due to failed or incomplete endothelialization, drug combinations such as SIR-heparin, zotarolimus-dexamethasone, SIR-17β-estradiol and SIR-genistein are currently under investigation [15]. An experimental approach in this context was published by Huang et al. in 2010 who reported the development of a dual drug-eluting stent (DDES) with SIR and the antithrombotic drug triflusal embedded in a biodegradable PLGA coating [16]. The in vitro results demonstrated that the developed 2-layered dual-drug coated stents cause a sustained release of both drugs in rates which effectively reduce thrombosis and proliferation within the same time frame. The migration of SMC and re-endothelialization are however locally focussed processes. While SMC originate from the vessel, the endothelial layer might be built up by endothelial progenitor cells circulating within the blood stream [17, 18]. Novel DES concepts hence foresee an abluminal location of anti-proliferative drugs in order to ensure more targeted tissue release and reduced systemic exposure. Among these, the BioMatrix DES (Biosensors) with its asymmetric and abluminal PLA coating elutes biolimus A9 into the vessel wall [14]. A similar drug-polymer combination only on the abluminal stent surface is used in coronary Nobori DES (Terumo Europe MV [19]).

In the present study, we combined both approaches in a DDES concept, allowing an abluminally focused release of SIR and a luminally focused release of atorvastatin (ATOR) from biodegradable poly(L-lactide) (PLLA) stent coatings. ATOR has been chosen as additive drug as clinical data suggest a positive impact of statins on the clinical outcome of patients treated with DES. For instance, Wang et al. [20] demonstrated in minipigs that treatment with orally administered ATOR prior to stenting with SIR-eluting stents led to enhanced re-endothelialization and neointimal coverage compared to BMS and SIR-eluting stents without ATOR pretreatment. Next to preclinical studies, a prospective cohort study from Eindhoven et al. [21] showed that patients undergoing percutane coronary intervention treated with ATOR had a lower mortality rate than using simvastatin. While little is known about the mechanisms involved in these observations, the stabilizing effect on atherosclerotic deposits [22] and stimulating impact on endothelial function [23] are assumed promising properties for the application of ATOR in the developed DDES allowing higher local concentrations at the injury site than by systemic administration.

In this context, we report on the in vitro characterization of DDES with locally focused SIR and ATOR release including effects on SMC and EC proliferation and viability, morphology, coating thickness and distribution as well as in vitro release of SIR and ATOR. The impacts of processing conditions (tempering, sterilization) and layer composition are thoroughly discussed making use of a semi-empirical model introduced by Gallagher et al. [24], giving insights into the mechanisms behind drug release and the stent design requirements.

2 Materials and methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Mallinckrodt Baker (Griesheim, Germany), SERVA Feinbiochemica (Heidelberg, Germany), Thermo Scientific (Karlsruhe, Germany) or Merck (Darmstadt, Germany) in p. a. quality or higher if not indicated differently. ATOR was obtained from Pfizer Pharma (Karlsruhe, Germany), and SIR from Sigma-Aldrich (Taufkirchen, Germany) for in vitro cell culture experiments and stent coating. PLLA (Resomer® L210, M_w = 310,000 g/mol, Boehringer Ingelheim, Ingelheim, Germany) was used as polymeric coating for coronary cobalt-chromium (CoCr) stents of 3 mm in diameter and 13 mm in length with a complete surface area of 57 mm^2. Stents and 3 mm × 15 mm balloon catheter for stent dilatation were kindly provided by Biotronik SE & Co. KG (Erlangen, Germany).

2.2 Cell assays

2.2.1 Cell culture

HCASMC and HCAEC of different cell donors were obtained from PromoCell GmbH (Heidelberg, Germany) and maintained in culture using optimized culture media namely smooth muscle cell growth medium 2 and endothelial cell growth medium MV, respectively. The media with supplements were purchased from PromoCell GmbH (Heidelberg, Germany). For analysis of proliferation, HCASMC and HCAEC were seeded at a density of 5×10^3 cells/well in 96-well plates and incubated in the respective growth medium for 24 h. Subsequently, the cells were treated for 24 h with basal medium supplemented with reduced concentrations of FCS (0.5 % for HCAEC and 0.05 % for HCASMC). Afterwards the cells were treated for 48 h at 37 °C with ATOR (0.1–100 μM) and/or SIR (1 pM–100 nM) in presence of the respective growth media. Cell culture was performed at 37 °C, with 5 % CO_2 in a humidified atmosphere.

2.2.2 H&E staining

For haematoxylin and eosin staining HCASMC were seeded on cover slips at a density of 3×10^5 cells/well in 12-well plates. After treatment with ATOR (1 μM) and/or SIR (1 μM) cells were fixed with methanol:acetone

(1:1, v:v) prior to H&E staining. Briefly, cells were incubated with haematoxylin (Merck KGaA, Karlsruhe, Germany), rinsed with tap water and incubated in eosin solution (Merck KGaA). After dehydration in ascending ethanol concentrations, the cover slips were mounted on slides using Roti® Histokitt (Carl ROTH GmbH & Co. KG Karlsruhe, Germany). Staining was visualized using the Axio Observer.D1 microscope (Carl Zeiss Jena GmbH, Jena, Germany).

2.2.3 Cell viability assay

Cell viability was assessed using the commercially available Fluorometric Cell Viability Kit I (PromoKine GmbH, Heidelberg, Germany) as described by the manufacturer. In brief, after treatment the cells were exposed to fresh medium containing 10 % resazurin. After incubation for 3 h at 37 °C and 5 % CO_2, fluorescence of resorufin was determined using a microplate reader (Infinite® M200, Tecan Deutschland GmbH, Crailsheim, Germany) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Data are expressed as percentage of control cells treated with the solvent (DMSO) only.

2.2.4 Cell proliferation assay

Cellular proliferation was determined using the commercially available BrdU Cell Proliferation Kit (Roche, Basel, Switzerland). After 24 h treatment the BrdU labelling solution was added to the medium followed by 24 h incubation at 37 °C. Subsequently, BrdU incorporation was measured by ELISA according to the manufacturer's instructions. Cells treated with the solvent (DMSO) served as reference; data are expressed as percent of control. IC_{50} values were determined calculating sigmoidal dose response curves using the GraphPad Prism© software (LaJolla, CA, USA).

2.3 Sample preparation

2.3.1 Stent processing

PLLA was dissolved in chloroform to yield a concentration of 2.5 mg/mL. SIR for abluminal and ATOR for luminal coating were mixed into the polymer solution to obtain a final drug concentration of 17.5 % in PLLA (w/w). The polymer drug solution was then applied to the metallic stent surface via three different self-developed spray coating processes based on 2-component jet atomization technology: complete, selective abluminal and selective luminal coating. For complete and selective abluminal coating, stents were inserted into a holder of an electropneumatic spray coating system, which applied a homogeneous coating from the outside by continuous stent rotation along their longitudinal axis. During selective abluminal coating an additional longitudinal air stream inside the tubular stent structure was applied in order to selectively deposit the coating solution on the outer stent surface. In contrast, selective luminal coating was achieved by generation of an outward radial spray inside the tubular stent structure according to our own patent application [25]. In order to allow for homogenous coating the stent is translated along its longitudinal axis. For DDES, the selective abluminal and luminal coating with SIR and ATOR respectively were performed successively in both orders. Intermediate weighing using a microbalance (UMX5, Mettler Toledo, Giessen, Germany) during all processes allowed for the adjustment of coating mass. While 240 ± 20 µg polymeric coating were deposited on DDES during each process, DES with complete coating received 460 ± 20 µg coating mass in total. Hence, DDES and DES received a total SIR load of 1.4 µg/mm^2 in accordance to the SIR-eluting stent Orsiro (Biotronik, Berlin, Germany), which evidenced excellent late luminal loss results in a first-in-man experience in treatment of patients with single de novo coronary artery lesions [26]. A defined coating surplus was applied during both DDES coating processes to achieve a form fit of the coating, implicating additional deposition of coating mass on the respective opposite stent surface. If not indicated differently, coated stents were tempered in vacuum for 13.5 h at 80 °C after each spraying process, and terminally sterilized by a common ethylene oxide sterilization process.

2.3.2 PLLA film preparation

The polymer films for thermal and molecular weight analysis were prepared using the following procedure: 1 g PLLA was dissolved in 25 mL chloroform and poured into a glass petri dish (Ø = 9 cm). The chloroform was allowed to evaporate until a 100 µm thick film, as measured by means of a thickness gauge (2109 Mitutoyo, Mitutoyo Europe GmbH, Neuss, Germany), had formed. For further experiments PLLA samples of 5 mm in diameter were prepared and if indicated tempered at 40 or 80 °C and/or sterilized following the same protocol as during stent processing.

2.4 In vitro characterization

2.4.1 Electron microscopy

The surface morphology of tempered and sterilized polymeric stent coating was assessed by means of a Philips XL 30 ESEM (Philips Electron Optics, Eindhoven, Netherlands) operating in the ESEM mode. For analysis of the structural integrity after expansion, stents were mounted on

3 mm × 15 mm balloon catheter and then dilated to their nominal diameter with 8 bar.

2.4.2 Coating thickness

Determination of coating thicknesses and distribution with regard to side-selectivity of the process was performed with stents which have only received either luminal or abluminal coating. $N = 3$ stents per subgroup were embedded in an epoxy resin (EpoThin, Buehler GmbH, Düsseldorf, Germany) and cured for 8 h. Then the samples were ground in their longitudinal axis and further polished with an aluminium oxide suspension (Buehler GmbH). The luminal and abluminal thicknesses of all appearing struts within the longitudinal cross section were determined at 5 positions by means of optical microscopy (Olympus LEXT OLS 3000, Hamburg, Germany) using the objective Plan-Achromat MPlanApo 100 × NA 0.95 (Olympus). The mean layer thickness is determined by averaging all single measurements of each strut and stent. The confidence limit for a confidence level of 95 % is given as statistical error.

2.4.3 Thermal analysis

Differential scanning calorimetry (DSC) was used to analyze the crystallinity of PLLA films at different processing stages (tempering and sterilization). The scans of $n = 5$ PLLA films per subgroup were made on a Mettler-Toledo Thermosystem FP900 (Mettler-Toledo, Schwerzenbach, Switzerland) operating at ambient atmospheric pressure. The specimens were heated from 45 to 225 °C at 5 K/min. The sample weight was in the range of 2–4 mg. The degree of crystallinity was calculated using the specific heat of fusion in relation to the reference value for totally crystalline PLLA of 93.7 J/g [27].

2.4.4 Molecular weight analysis

The molecular weight data of PLLA films at different processing stages (tempering and sterilization) was obtained at 30 °C using a PSS SECcurity SEC system (Polymer Standard Services GmbH, Mainz, Germany) including a RI detector combined with a WGE Dr. Bures η 2010 viscosity detector (WGE Dr. Bures GmbH, Dallgow, Germany). Separation was performed with three PSS SDV columns (10^3, 10^5 and 10^6 Å respectively). Chloroform stabilized with ethanol was used as the eluent at a flow rate of 1 mL/min. The samples were prepared with a concentration of 1.5 mg/mL in chloroform with hexylbenzene as internal standard and the injection volume was 0.1 mL. The molecular weights were calculated by the universal calibration method using twelve polystyrene calibration standards in a range between 376 and 2,570,000 g/mol.

2.4.5 Drug recovery

Recovery of SIR and ATOR was evaluated after tempering and sterilization as well as after completion of the drug release studies. Therefore, stents were extracted two times, first in 10 and then in 4 mL methanol for 30 and 60 min at 23 ± 2 °C respectively. 20 µL of each aliquot were injected into an Eurospher 100 C18 column, 120 mm × 4 mm ID (Wissenschaftlicher Gerätebau Dr.-Ing. Herbert Knauer GmbH, Berlin, Germany). The chromatographic conditions were: column temperature 50 °C; isocratic eluent acetonitrile /water 65/35 (v/v); flow rate 1.0 mL/min and UV detection at 278 nm (SIR) or 248 nm (ATOR) with calibrated measurement range 0.1–10.0 mg/L. Both drugs could be analyzed simultaneously by one HPLC-method. Values are expressed as fraction of the total drug load as determined by weighing (f_r or f_{rr} for recovered fraction and recovered fraction after drug release respectively).

2.5 Drug release

The time course of SIR and ATOR release was determined at 23 ± 2 °C in 0.9 wt% NaCl supplemented with 0.05 wt% Brij 35 and 0.003 wt% butylated hydroxytoluene (BHT), which assured SIR and ATOR stability over a time period of in minimum 4 days as determined in preliminary experiments (data not shown). Individual stents ($n \geq 3$ in each subgroup) were expanded as described above and subsequently immersed in 2 mL elution medium for a total duration of at least 1,000 h. The elution medium was renewed at periodic intervals in order to realize sink conditions and avoid a saturation of the medium with drugs. The drug amount released at each time period was determined by HPLC, applying conditions described above, after 1:1 dilution with methanol. Remaining drug content after the drug release studies was evaluated by extraction of stents as described in the section Drug recovery. Represented drug release profiles result from the accumulation of the measured values per sampling time and are expressed as fraction of the total drug load as determined by weighing. Drug release profiles were approximated by Eq. (1), introduced by Gallagher et al. [24], which combines an initial fast or burst release phase following first-order kinetics and a subsequent slower release phase.

$$f_t = f_1 * \left(1 - e^{k_1 * t}\right) + (f_{tot} - f_1) * \left(\frac{e^{k_2 * t - k_2 * t_{2\,max}}}{1 + e^{k_2 * t - k_2 * t_{2\,max}}}\right) \quad (1)$$

f_t is the fraction of drug released at time t, $f_1$the fraction of drug released during the first release phase (burst release), f_{tot} the maximal fraction of drug released during the whole process, k_1 the first order kinetic constant (h^{-1}) for the first

release phase and k_2 the kinetic constant (h^{-1}) for the second release phase. t_{max} has not been discussed within this study. For the sake of completeness it should be mentioned that it stands for the time needed to maximal drug release rate within the second release phase. t_1, which has been recalculated by the fitted equation, presents the time when f_1 is reached. Origin 8.1G was used for data fitting.

2.5.1 Statistics

If not indicated differently, mean values and standard deviations (SD) were analyzed using IBM SPSS software 20.0. Confidence intervals (CI) and data of cell assays were analyzed using Student′s t test with $p < 0.05$ being defined as significantly different. IC_{50} values were determined calculating sigmoidal dose response curves using the GraphPad Prism© software (LaJolla, CA, USA).

3 Results and discussion

3.1 Applicability of the combination of ATOR and SIR in DDES

The developed DDES foresee the inhibition of ISR without provoking long-term impact on healing and re-endothelialization, by a preferential abluminal location of SIR and a luminal location of ATOR within a biodegradable PLLA coating. Hereby we aim at the combination of the highly potent anti-proliferative effect of SIR on SMC and reduced impact on EC via minimized systemic exposure of SIR with the general observed positive effects of ATOR as the stabilization of atherosclerotic deposits [22] and the stimulation of endothelial function on the clinical outcome of patients treated with DES [23]. However, this combination on DDES does only become applicable when ATOR does not annihilate the anti-proliferative effect of SIR on SMC if both drugs accumulate in the vessel wall or does itself provoke significant anti-proliferative effects on EC in high local concentrations as reached by DES. In this context, we investigated the influence of both substances on proliferation and viability of HCASMC and HCAEC separately and in co-treatment.

3.1.1 Influence of ATOR and SIR on proliferation and viability of HCASMC und HCAEC

In order to assess the impact of ATOR and SIR on HCASMC and HCAEC proliferation a BrdU ELISA was performed revealing significant anti-proliferative effects of both substances on HCASMC. As shown in Fig. 1a, b, ATOR inhibited HCASMC proliferation with an IC_{50} value of 0.77 µM [Confidence Interval (CI) 0.49–1.21 µM], while incubation with SIR supported the previously reported anti-proliferative potency of this clinically used compound with an IC_{50} value of 0.41 nM [CI 0.19–0.88] [28, 29].

While in accordance to literature the inhibitory capacity of SIR in HCAEC (IC_{50} value of 0.15 nM, CI 0.08–0.29) exerted a similar value to that measured in HCASMC [28], the observed IC_{50} value of ATOR (4.1 µM, CI 3.3–5.2) was threefold higher for HCAEC compared to HCASMC (data not shown). The observed anti-proliferative effect of SIR on HCAEC is hence about four magnitudes higher than that measured for ATOR, which lets assume that re-endothelialization will be less impaired by DDES releasing ATOR preferentially to the lumen and SIR preferentially to the vessel wall than by commercial SIR-eluting stents. Interestingly, both substances revealed no significant cytotoxic effects compared to DMSO treated cells within the examined concentration range, as measured by the described resazurin-based cell viability assay (data not shown).

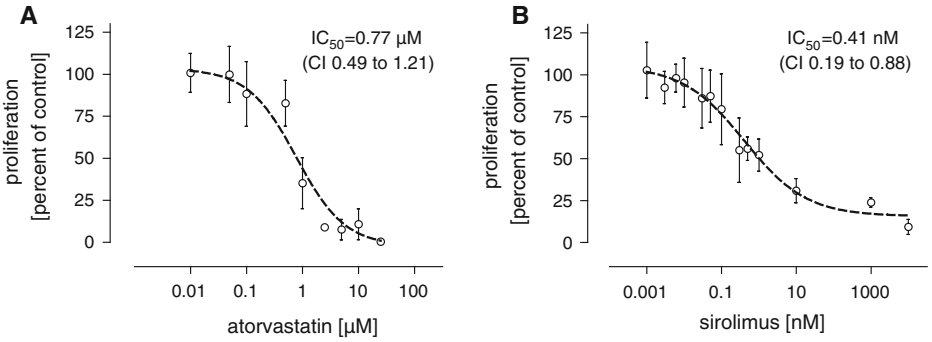


Fig. 1 Anti-proliferative effects of atorvastatin (ATOR) and sirolimus (SIR) on human coronary artery smooth muscle cells (HCASMC). HCASMC were treated with **a** ATOR and **b** SIR for 48 h, respectively. Proliferation was measured by incorporation of BrdU. Data are represented as mean ± SD; $n = 3$. IC50 values, determined by calculation of sigmoidal dose response *curves*, and the 95 % confidence interval (CI) are indicated

3.1.2 Co-treatment with ATOR and SIR

In order to guarantee the maintenance of the anti-proliferative effect of SIR on HCASMC in developed DDES, HCASMC of one donor, which showed high effect to SIR incubation in previous experiments, were treated with SIR (10 nM) in the presence or absence of ATOR (1 μM). As shown in Fig. 2a co-administration of ATOR reduced the anti-proliferative efficacy of SIR only slightly to 28.6 ± 2.26 %; mean % of control ± SD. In order to furthermore obtain insight into morphological changes induced by ATOR or SIR, treated cells were stained with haematoxylin and eosin. Surprisingly, even if significantly reduced cellular proliferation was detected performing the sensitive BrdU ELISA no impact on cell density or morphology was observed in the presence of 10 nM SIR (Fig. 2b). In subsequent experiments a higher concentration of SIR (1 μM) was used. As shown in Fig. 2b, H&E staining revealed lower density of the cells in the presence of SIR. Additionally, treatment with 1 μM ATOR significantly reduced cell density (Fig. S1 in Supplementary material). In the presence of both substances, SMC appeared expanded. This morphological change might be explained by the reduced cell count.

3.2 Coating morphology and distribution of DDES

Several different technological approaches are currently being applied for the coating of DES. While some of them are already used for commercial products, others are still under development. Most widely used are probably dip-coating and 2-component jet or ultrasonic atomization processes. Selective coating of either the abluminal or luminal surface of the stent, however, requires adapted or novel technologies. For example, selective abluminal coating of the Biomatrix stent has been achieved via an advanced spray coating technology, equipped with an imaging system, which allows for exact determination of the stent skeletal elements and defines the traverse path of a coating dispenser head [30]. In this way, the abluminal surface of stent struts can be coated selectively. Moreover, a technique for selective luminal stent coating was proposed by masking the outer stent surface with a sleeve and subsequent insertion of a spraying nozzle inside the stent [31]. However, both processes will potentially lead to coatings with sharp edges, which could result in coating delamination during stent implantation. The here presented spray coating processes provide form-fit of the polymer coatings around the entire cross section of the individual stent struts. Representative stent strut cross sections are shown in Table 1. By reason of the coating set-up, the luminal coating process allows high selectivity with about 10 % of the drug/polymer solution being deposited at the abluminal stent surface. In contrast, the abluminal process is less selective with up to 30 % of the drug/polymer solution being deposited on the luminal stent surface. However, in comparison to the conventional complete spray coating process, which results in average in a coating distribution of 60 % abluminal and 40 % luminal (data not shown), selectivity of the abluminal stent coating was enhanced by the novel process. For production of DDES, the two selective coating processes were combined in both orders. In either case, a smooth and uniform surface without any layer boundaries due to the two-step coating process was observed, as exemplarily shown for DDES, obtained by a luminal/abluminal coating sequence (Fig. 3a–c). Furthermore, it was observed that structural integrity of the coating was maintained after sterilization, crimping and full expansion of DDES prototypes (Fig. 3d–f).

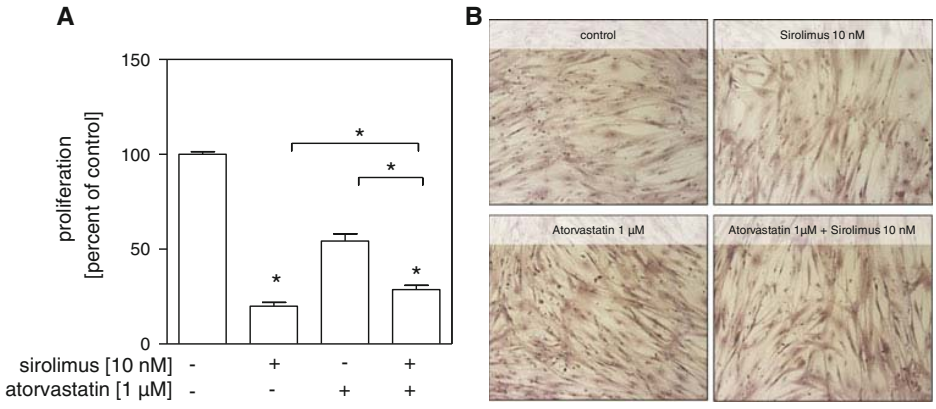
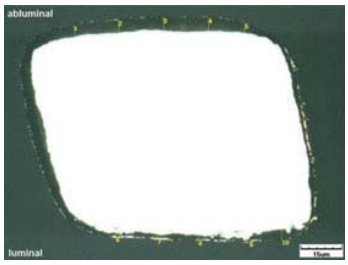
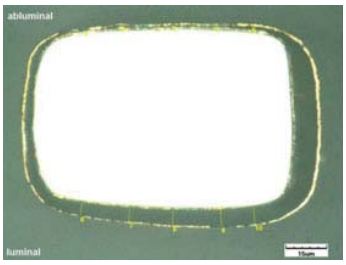


Fig. 2 Influence of atorvastatin (ATOR) in combination with sirolimus (SIR) on proliferation and viability of human coronary artery smooth muscle cells (HCASMC). HCASMC were treated with ATOR in the presence or absence of SIR. (**a**) Proliferation of HCASMC after 48 h incubation with SIR (10 nM) and ATOR (1 μM) was measured by incorporation of BrdU. Data are represented as mean + SD, $n = 3$. (**b**) H&E staining of HCASMC after 48 h incubation with ATOR (1 μM) in presence or absence of SIR (10 nM)

Table 1 Coating distributions of the site-selective spray coating processes. Optical microscopy images of one representative strut cross section of stents containing 250 μg drug/PLLA coating either applied via the abluminal- or the luminal-selective spraying process with indicated measuring positions and measured coating thicknesses

	Abluminal coating						Luminal coating					
Optical microscopy image of one representative strut												
Measuring position abluminal	1	2	3	4	5	Mean	1	2	3	4	5	Mean
Coating thickness (μm)	4.46	4.95	5.19	4.93	3.92	4.69	0.77	0.51	0.76	0.89	1.52	0.38
Measuring position luminal	6	7	8	9	10	Mean	6	7	8	9	10	Mean
Coating thickness (μm)	1.01	1.26	1.64	1.02	3.55	1.70	5.22	5.69	6.20	6.45	6.73	6.06
Average abluminal coating thickness over all appearing struts of 3 stents (μm) (±confidence limit, confidence level 95 %)	4.60 ± 0.16						0.69 ± 0.06					
Average luminal coating thickness over all appearing struts of 3 stents (μm) (±confidence limit, confidence level 95 %)	1.93 ± 0.09						5.78 ± 0.28					

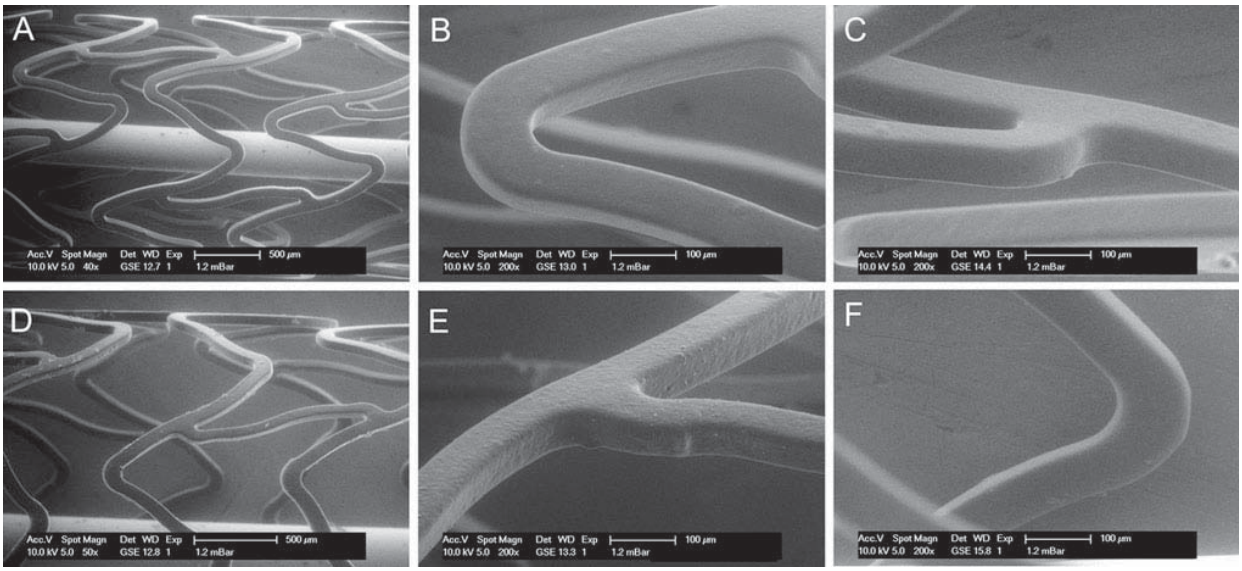


Fig. 3 Representative electron micrographs of at 80 °C tempered DDES, obtained by a luminal–abluminal coating sequence, (**a**–**c**) prior and (**d**–**f**) after sterilization, crimping and expansion to their nominal diameter with 8 *bar* by means of conventional balloon catheter

3.3 Drug recovery and in vitro drug release of DDES

3.3.1 Impact of tempering and sterilization

The tempering and sterilization procedures, which have been chosen due to their applicability for PLLA, as evidenced by absence of solvent (chloroform) and molecular weight maintenance [32], were evaluated in detail with regard to SIR and ATOR stability at the example of DDES, obtained by a luminal–abluminal coating sequence. Performed HPLC analysis of the total content of stents after the different processing steps fortunately revealed in each case more than 97 % of the theoretically calculated total drug load as estimated by the observed coating masses.

Consequently, SIR- and ATOR-containing DDES can be processed using standard conditions, while the tempering process has been applied twice after each of the two coating steps in order to allow for precise weighing of the deposited coating mass. Furthermore, we investigated the impact of tempering and sterilization on the in vitro drug release, exemplarily depicted in Fig. 4 for SIR of DDES, obtained by a luminal–abluminal coating sequence. Independently of the process, a two-phase release profile with an initial burst release phase following first-order kinetics and a subsequent slower release phase, which could be well fitted by Eq. (1) with $r^2 > 0.95$, was observed. Estimated parameters obtained by approximation are shown in Table 2. Striking is the difference in total SIR fraction released, which is more than doubled for sterilized DDES, tempered at 40 °C and even decupled for DDES tempered at 80 °C independently of sterilization in comparison to unsterile DDES, tempered at 40 °C. The tendency is the same for ATOR release (Fig. S2 in Supplementary material). It is well known that drug release rate from polymers is generally accelerated by less crystallinity and lower molecular weight [33]. In this context, we prepared sterile and unsterile PLLA films, which have been tempered at 40 or 80 °C in order to investigate possible changes in crystallinity or molecular weight. While no considerable differences are observed in molecular weight and the degree of crystallinity (Table 3), DSC-curves revealed the complete disappearance of the glass transition for sterilized and non-sterilized PLLA films, tempered at 80 °C (Fig. S3 in Supplementary material). At first sight, this might seem contradictory, but it has been previously reported that tempering of semi-crystalline polymers might lead to the densification of the amorphous phase and the increase of a so-called rigid amorphous fraction without change in the crystalline fraction [34, 35]. Also contradictory appears the enormous increase in release rate with smaller mobile amorphous fraction (as the conventional amorphous phase might be called). In accordance to Hurrell et al. [36], who reported on the release of theophylline from polyglycolide, we assume two factors which could account for the higher release from DDES tempered at 80 °C. First, a non-linear drug distribution through the sample may be formed during the temper process by drug diffusion and partition towards the surface. The second factor which may contribute to enhanced burst drug release is an increased content of drug in the mobile amorphous phase of the polymer due to the exclusion of drug molecules from crystalline and rigid amorphous phases created during tempering. Both mechanisms would lead to drug supersaturation in confined regions and hence drug crystallization. If then water enters the sample, these drug crystals dissolve, causing the polymeric matrix to be more porous and drugs are released more quickly compared to samples with more homogeneous drug distribution. Additionally, remaining solvent in coatings tempered at 40 °C might reduce the water interpenetration within the bulk material leading to a further deceleration of drug release.

In summary, this discussion clearly highlights the importance of tempering stent coatings, not only for

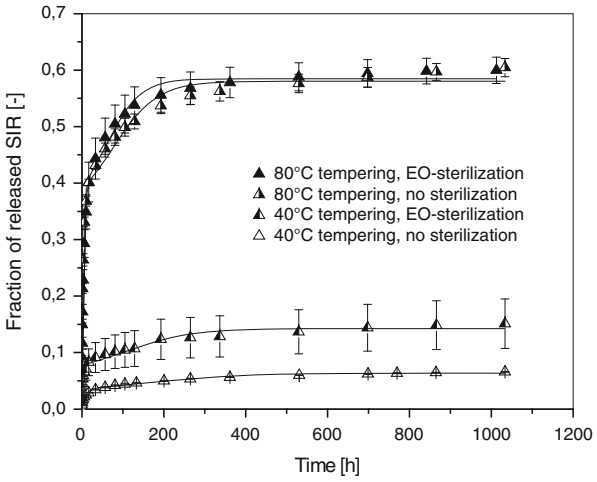


Fig. 4 Sirolimus (SIR) release profiles of non-sterilized and sterilized DDES (obtained by a luminal–abluminal coating sequence and tempered at 40 or 80 °C) at 23 ± 2 °C in 0.9 wt% NaCl supplemented with 0.05 wt% Brij 35 and 0.0003 wt% BHT. Each *curve* represents 3 stents (mean values ± SD), fitting was performed using Eq. (1)

Table 2 Estimated kinetic parameters obtained by approximation using Eq. (1) combining an initial fast phase and a subsequent slower release phase for sirolimus (SIR) release profiles of non-sterilized and sterilized DDES (obtained by a luminal–abluminal coating sequence and tempered at 40 or 80 °C) at 23 ± 2 °C in 0.9 wt% NaCl supplemented with 0.05 wt% Brij 35 and 0.0003 wt% BHT

Processing	f_1 (−)	k_1 (h^{-1})	t_1 (h)	f_{tot} (−)	k_2 (h^{-1})	r^2 (−)	f_{rr} (−)
40 °C tempering no sterilization	0.03 ± 0.00	0.10 ± 0.02	13	0.06 ± 0.00	0.01 ± 0.00	0.9834	0.93 ± 0.01
40 °C tempering EO-sterilization	0.06 ± 0.01	0.55 ± 0.18	2	0.14 ± 0.00	0.01 ± 0.00	0.9583	0.81 ± 0.09
80 °C tempering no sterilization	0.34 ± 0.02	0.36 ± 0.06	5	0.58 ± 0.01	0.02 ± 0.00	0.9853	0.81 ± 0.01
80 °C tempering EO-sterilization	0.36 ± 0.02	0.23 ± 0.03	9	0.59 ± 0.01	0.02 ± 0.00	0.9907	0.77 ± 0.02

f_1 drug fraction released during the burst release, k_1 kinetic constant of the burst release, t_1 time to reach f_1, f_{tot} total drug released during process, k_2 kinetic constant of the second release phase, r^2 coefficient of determination for fitting Eq. (1), f_{rr} recovered drug fraction after completed drug release study (1,000 h), Fractions are calculated in relation to the theoretical drug amount

complete solvent removal but also for the achievement of adequate and sustained drug release.

3.3.2 Impact of DDES design

Besides the stent processing, drug release is also likely influenced by the coating design. In this context, we examined the in vitro drug release of various DDES and DES designs with regard to release profiles of SIR and ATOR. Corresponding drug release profiles are shown in Fig. 5 and estimated kinetic parameters obtained by approximation using Eq. (1) in Table 4.

As the here applied spraying processes provide form-fit polymer coatings at the abluminal and luminal side, the order of their combination will lead to a capping of either the SIR- or the ATOR-containing layer (Table 1). In detail, the SIR-containing coating of DDES prototypes, obtained by an abluminal–luminal coating sequence, is covered by a 1 μm and a 6 μm thick polymeric coating at the outer or the inner surface respectively. In other words, intended SIR eluting from the abluminal side has to pass a small diffusion barrier of 1 μm, while unwanted SIR elution from the luminal side is shielded by a 6 μm thick polymeric layer. Compared to DDES prototypes, obtained by a luminal–abluminal coating sequence, such that SIR elutes without diffusion barrier, a decreased drug release rate of the burst release phase k_1 is observed (luminal–abluminal coating: 0.23 h^{-1}, abluminal–luminal coating: 0.08 h^{-1}, Table 4). However, the rates

Table 3 Molecular weight (M_w) and degree of crystallinity of non-sterilized and sterilized PLLA films tempered at 40 °C or 80 °C

Processing	M_w (g/mol)	Degree of crystallinity (%)
40 °C tempering no sterilization	317,000 ± 3,000	31 ± 2
40 °C tempering EO-sterilization	318,000 ± 6,000	31 ± 3
80 °C tempering no sterilization	319,000 ± 5,000	32 ± 2
80 °C tempering EO-sterilization	316,000 ± 9,000	33 ± 5

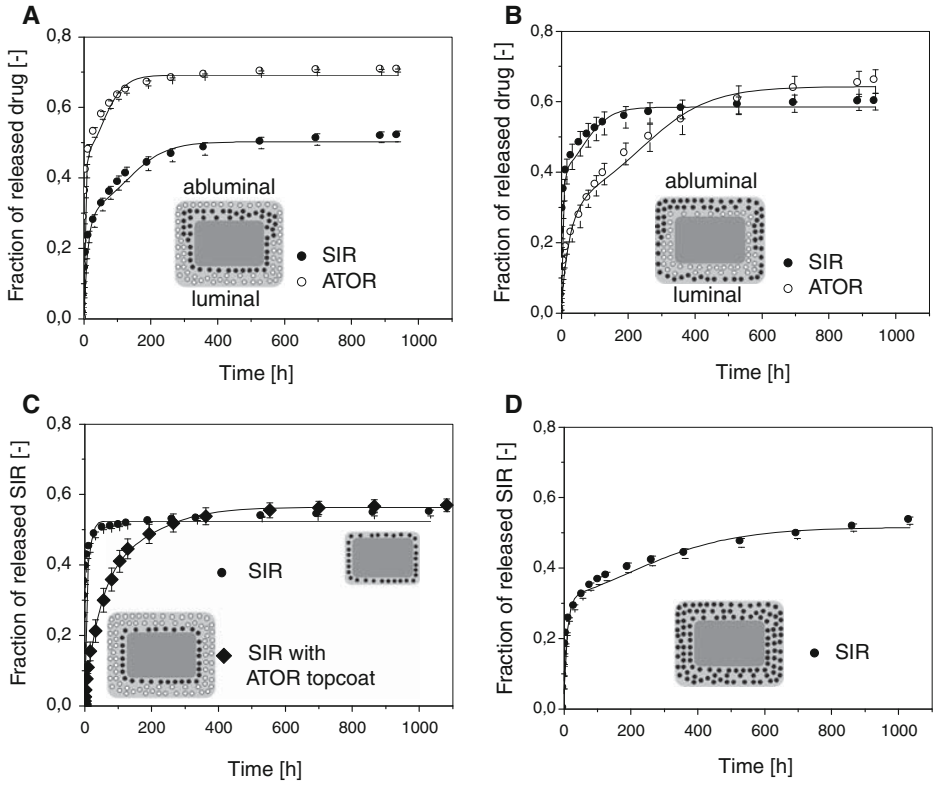


Fig. 5 Sirolimus (SIR) and atorvastatin (ATOR) release profiles of sterilized and at 80 °C tempered DDES and DES prototypes at 23 ± 2 °C in 0.9 wt% NaCl supplemented with 0.05 wt% Brij 35 and 0.0003 wt% BHT. (**a**) SIR and ATOR release profile of DDES obtained by a abluminal–luminal coating sequence, (**b**) SIR and ATOR release profile of DDES obtained by a luminal–abluminal coating sequence, (**c**) SIR release profiles of DES obtained by a complete SIR-containing coating with optional ATOR-containing topcoat for simulation of the luminal coating of DDES obtained by an abluminal–luminal coating sequence. (**d**) SIR release profile of DES obtained by a complete SIR-containing coating. Each *curve* represents 3 stents (mean values ± SD), fitting was performed using Eq. (1)

Table 4 Estimated kinetic parameters obtained by approximation using Eq. (1) combining an initial fast phase and a subsequent slower release phase for sirolimus (SIR) and atorvastatin (ATOR) release profiles of sterilized and at 80 °C tempered DDES and DES prototypes at 23 ± 2 °C in 0.9 wt% NaCl supplemented with 0.05 wt% Brij 35 and 0.0003 wt% BHT

Drug	Layer composition	f_1 (−)	k_1 (h^{-1})	t_1 (h)	f_{tot} (−)	k_2 (h^{-1})	r^2 (−)	f_{rr} (−)
Sirolimus (SIR)	1. SIR abluminal 2. ATOR luminal	0.28 ± 0.02	0.08 ± 0.01	26	0.50 ± 0.01	0.02 ± 0.00	0.9938	0.71 ± 0.01
	1.ATOR luminal 2. SIR abluminal	0.36 ± 0.02	0.23 ± 0.03	9	0.59 ± 0.01	0.02 ± 0.00	0.9907	0.77 ± 0.02
	Simulated luminal coating 1. SIR	0.33 ± 0.03	1.59 ± 0.27	1	0.52 ± 0.01	0.13 ± 0.04	0.9819	0.81 ± 0.01
	Simulated luminal coating 1. SIR 2. ATOR	0.42 ± 0.03	0.02 ± 0.00	116	0.56 ± 0.01	0.01 ± 0.00	0.9976	0.79 ± 0.01
	SIR	0.26 ± 0.02	0.10 ± 0.02	14	0.52 ± 0.02	0.01 ± 0.00	0.9789	0.78 ± 0.02
Atorvastatin (ATOR)	1. SIR abluminal 2. ATOR luminal	0.41 ± 0.02	0.28 ± 0.04	7	0.69 ± 0.01	0.03 ± 0.00	0.9912	0.81 ± 0.02
	1. ATOR luminal 2. SIR abluminal	0.29 ± 0.02	0.04 ± 0.01	51	0.64 ± 0.01	0.01 ± 0.00	0.9923	0.82 ± 0.03

f_1 drug fraction released during the burst release, k_1 kinetic constant of the burst release, t_1 time to reach f_1, f_{tot} total drug released during process, k_2 kinetic constant of the second release phase, r^2 coefficient of determination for fitting Eq. (1), f_{rr} recovered drug fraction after completed drug release study, fractions are calculated in relation to the theoretical drug amount

result from the combination of both, drug release from the inner and the outer surface, raising the question whether unwanted SIR elution rate from the luminal side of DDES obtained by an abluminal–luminal coating sequence is efficiently reduced, while maintaining an effective SIR release at the abluminal side. In order to solve this question we simulated the luminal coating of a DDES, obtained by an abluminal–luminal coating sequence. According to Table 1 the luminal coating of this DDES is build up by a 2 μm thick SIR-containing basecoat and an ATOR-containing topcoat of 6 μm in thickness. As this correlates to 30 % of the first and 90 % of the second coating having both a total mass of 250 μg for each stent side, we generated a DDES with a SIR-containing basecoat of 150 μg and an ATOR-containing topcoat of 450 μg via the complete spraying process, detailed in the experimental section. In vitro release studies in comparison to DES, containing only the SIR-containing basecoat, demonstrate a decrease of the burst release rate constant k_1 from 1.59 to 0.02 h^{-1} (Table 4; Fig. 5c). This indicates on one hand an effective shielding of the luminal SIR elution and also suggests that the abluminal SIR elution of DDES, obtained by an abluminal–luminal coating sequence, is not considerably influenced. The in vitro release profile of DES with a complete 480 μg SIR-containing coating, evidencing similar drug fraction released during burst phase and in total as well as release rate constants as DDES obtained by an abluminal–luminal coating sequence (Table 4; Fig. 5d), supports this hypothesis.

The total fraction of released ATOR is for both DDES prototypes higher than for SIR (Figure 5a, b), which might be dedicated in accordance to Pan et al. [37] to its lower molar mass (ATOR 558.65 g/mol, SIR 914.17 g/mol) resulting in higher diffusion coefficient at similar solubility in aqueous media. Its abluminal elution and accumulation in the vessel wall, which is in particular expected for DDES, obtained by an abluminal–luminal coating sequence, is not considered to reduce the DDES performance, as in vitro cell assays evidenced the maintenance of the potent anti-proliferative effect of SIR in the presence of ATOR.

Summarizing these in vitro data, the coating sequence for DDES should be rather abluminal–luminal than luminal–abluminal. The achieved reduction of the amount of SIR eluted luminally could then potentially minimize the induction of endothelial dysfunction, while efficacy concerning the inhibition of neointima proliferation is assumed due to the SIR release profile being similar to completely coated SIR-eluting stents. The luminal–abluminal coating sequence would in contrast lead to higher local SIR concentrations due to the increased release rate in the first release phase, which might in the worst case result in an even enhanced endothelial dysfunction.

4 Conclusions

Novel DES concepts often either foresee an abluminal location of anti-proliferative drugs in order to ensure more targeted release and reduced systemic exposure or the use of drug combinations for simultaneous inhibition of ISR as a result of disproportionally increased smooth muscle cell

proliferation and migration as well as thrombosis due to failed or incomplete endothelialization. In the present study, we combined both approaches by the establishment of an abluminally focused release of the potent anti-proliferative drug SIR and a luminally focused release of ATOR with stabilizing effect on atherosclerotic deposits and stimulating impact on endothelial function from biodegradable PLLA-based stent coatings. In performed cell culture assays, we were able to evidence that ATOR does not annihilate the anti-proliferative effect of SIR on SMC and provoke any harmful effects on EC at concentrations below 1 μM. The here applied spraying processes provide form-fit polymer coatings at the abluminal and luminal side with 70 and 90 % of the drug/polymer solution being deposited at the intended stent surface. In either case, a smooth and uniform surface with structural integrity after sterilization, crimping and full expansion was observed, evidencing the good applicability and performance of the especially adapted spraying process for the generation of DDES. As stability of both drugs after tempering and sterilization was demonstrated, SIR and ATOR-containing DDES can be processed using standard conditions: 80 °C tempering and a common ethylene oxide sterilization process. In vitro drug release studies revealed for both drugs a two-phase release profile with an initial burst release phase following first-order kinetics and a subsequent slower release phase, which could be well fitted by a semi-empirical model introduced by Gallagher et al. [24]. From those data we conclude that tempering of the sprayed coatings at elevated temperatures is not only important for complete solvent removal but also for the achievement of adequate and sustained drug release, a temperature increase from 40 to 80 °C during drying decupled the total drug fraction released during a defined time period. Importantly, based on our data from the in vitro release the coating sequence for DDES should be rather abluminal–luminal than luminal–abluminal. It might be hypothesized that the thereby achieved reduction of SIR eluted luminally would minimize the induction of an endothelial dysfunction, while exerting similar inhibitory efficacy concerning the neointimal proliferation due to the similar SIR release profile as shown for the completely coated SIR-eluting stents. In summary, in vitro data of the developed DDES concerning effects on smooth muscle cell and endothelial cell proliferation and viability, coating morphology, layer thickness and distribution as well as in vitro release of SIR and ATOR are promising. Their safety and efficacy also with regard to healing however warrants further research using in vivo models.

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Supplementary data

In vitro study of dual drug-eluting stents with locally focused sirolimus and atorvastatin release

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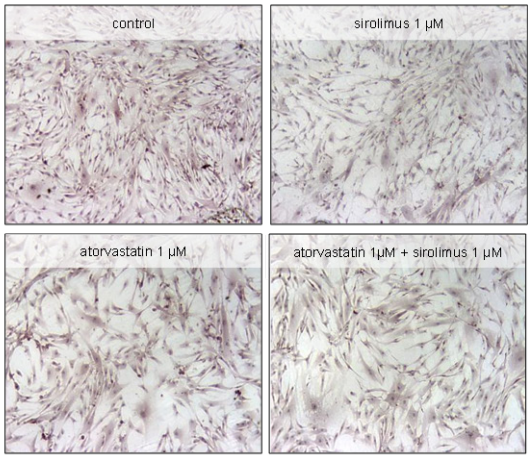

Figure S1. Influence of atorvastatin plus sirolimus on morphology of human coronary artery smooth muscle cells (HCASMC). HCASMC were treated with atorvastatin (1 µM) in the presence or absence of sirolimus (1 µM) followed by H&E-staining of HCASMC after 48hr incubation.

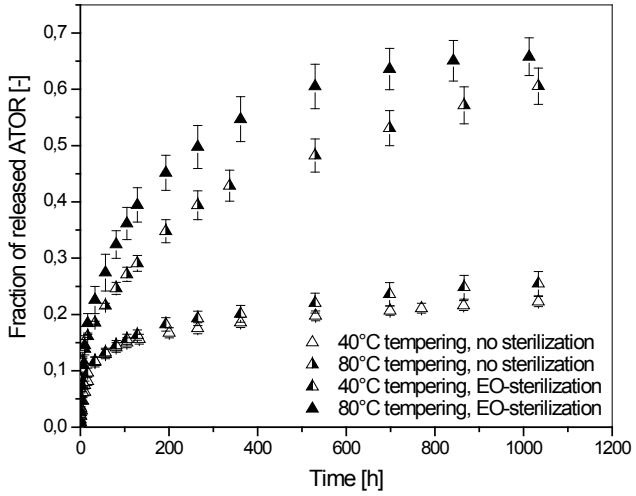

Figure S2. Atorvastatin release profiles of unsterile and sterile DDES tempered at 40 °C or 80 °C at 23 ± 2 °C in 0.9% w/w NaCl supplemented with 0.05 w% Brij 35 and 0.0003 w% BHT. Each curve represents 3 stents (mean values ± SD).

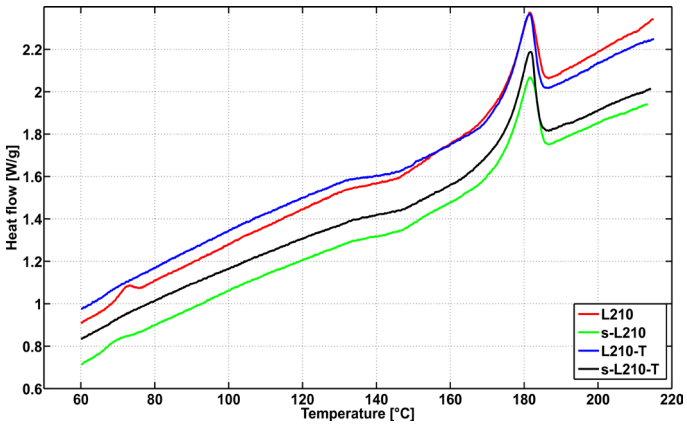

Figure S3. Differential scanning calorimetry – curves for unsterile and sterile PLLA (Resomer L210, Boehringer Ingelheim, Germany) films tempered at 40 °C (L210, s-L210) or 80 °C (L210-T, s-L210-T). Each curve represents the mean of 5 PLLA films.

3.2. Cell-specific expression of uptake transporters – A potential approach for cardiovascular drug delivery devices

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Contribution J. Hussner: experimental part

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Cell-Specific Expression of Uptake Transporters—A Potential Approach for Cardiovascular Drug Delivery Devices

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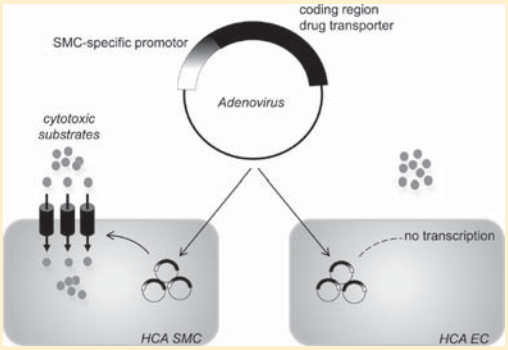
ABSTRACT: Enhanced proliferation of human coronary artery smooth muscle cells (HCASMCs) and thereby formation of neointima is one of the factors contributing to failure of coronary stents. Even if the use of drug eluting stents (DES) and thereby the local delivery of cytotoxic compounds has significantly improved the clinical outcome, unselective cytotoxic effects are assumed to hamper clinical success. Novel pharmacological approaches are required to enhance cellular selectivity of locally delivered drugs. Cell specific overexpression of a drug transporter could be used to enhance cellular accumulation and therefore cell specificity. In the herein reported study we tested the possibility of cell specific transporter expression to enhance drug effects in HCASMCs. We generated adenoviral constructs to overexpress the organic cation transporter 1 (OCT1) under control of the promoter of SM22α, which had been previously reported as muscle cell specific gene. First the activity of the SM22α-promoter was assessed in various cell types supporting the notion of muscle cell specificity. Subsequently, the activity of the transporter was compared in infected HCAECs and HCASMCs revealing enhanced accumulation of substrate drugs in HCASMCs in presence of the SM22α-promoter. Testing the hypothesis that this kind of targeting might serve as a mechanism for cell-specific drug effects, we investigated the impact on paclitaxel treatment in HCASMC and HCAECs, showing significantly increased antiproliferative activity of this substrate drug on muscle cells. Taken together, our findings suggest that cell-specific expression of transport proteins serves as mechanism governing the uptake of cytotoxic compounds for a selective impact on targeted cells.



KEYWORDS: *drug delivery, pharmacokinetics, vascular smooth muscle cells, paclitaxel, organic cation transporter1 (OCT1), SM22*

INTRODUCTION

Drug-eluting stents (DES) have been an outstanding improvement in interventional therapy of coronary atherosclerosis. Indeed, the first generation DES, which were coated with substances such as the mTOR-antagonist sirolimus (rapamycin) (Cypher, Johnson & Johnson) or the mitotic inhibitor paclitaxel (Taxus, Boston Scientific), have been shown to significantly improve clinical outcome of patients after angioplasty with stent implantation (summarized in ref 1). It is assumed that one of the mechanisms involved in this improvement is the reduction of neointima formation due to the antiproliferative drugs. The histopathological correlate of neointima formation is the proliferative activation of vascular smooth muscle cells, which is significantly reduced in the presence of sirolimus and paclitaxel.[2] However, even if neointima formation is decreased, there is still the issue of delayed arterial healing, characterized by incomplete stent strut endothelialization in presence of cytotoxic compounds. Delayed arterial healing is assumed to contribute to the occurrence of in-stent thrombosis.[3]

In accordance, it is aim of the field to identify agents with cell-specific efficacy. The perfect drug should enhance re-endothelialization of the stent struts, while inhibiting proliferation and migration of smooth muscle cells.[1] From a pharmacological point of view, there are two different approaches to realize cell-specific effects of drugs: the first would be to identify cell-specific drug targets; the second would be to improve pharmacokinetics of candidate drugs. The latter concept is the subject of the herein reported study. One of the mechanisms contributing to pharmacokinetics of xenobiotics is the expression of drug transporters, which facilitate transmembrane movement of substrates.[4] The first transporters identified were members of the ATP-binding cassette superfamily mediating ATP-dependent cellular efflux of their substrates. In accordance, overexpression of those transporters has been associated with the incidence of multidrug resistance

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in cancer cells.[5] Especially P-glycoprotein (P-gp, MDR1, ABCB1) has been focus of several studies, aiming to inhibit transport activity to overcome chemoresistance.[6] However, MDR1 is also expressed in non-malignant transformed tissues, taking part in the elimination and distribution of its substrates in the organism, thereby hampering the success of transporter inhibition to improve cancer treatment.[7] With the identification of drug transporters facilitating cellular uptake the concept of renal or hepatic elimination of drugs has changed. Indeed, nowadays we know that members of the solute carrier (SLC) protein superfamily are mediating cellular entry of a variety of compounds prior to metabolism and/or efflux, thereby representing one mechanism limiting hepatic or renal elimination.[8]

Organic cation transporters (OCT) are one subfamily within the superfamily of SLC transporters, mediating sodium independent uptake of exogenous and endogenous cations.[9] In this study we used the hepatic uptake transporter OCT1 (SLC22A1),[10] which has been shown to transport a variety of drugs, including the clinically established DES-drug paclitaxel.[11] To drive specific expression of the drug transporter in the targeted cells the promoter of transgelin (SM22α) a commonly used marker of smooth muscle cells was used. This promoter has been previously reported as a mechanistic tool to perform cell type specific deletion and/or overexpression in mouse models.[12,13]

The herein reported data provide evidence that cell-specific overexpression of a drug transporter can be used to enhance cellular accumulation and therefore efficacy of compounds with intracellular drug targets.

EXPERIMENTAL SECTION

Cell Lines. MDCKII (ATCC no. CRL-2936) and A549 (ATCC no. CCL-185) were obtained from ATCC. HL-1 cells are murine cardiomyocytes and have been previously described by Claycomb et al.[14] Primary isolated human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (HCASMCs) were purchased from PromoCell GmbH (Heidelberg, Germany).

Real-Time PCR. For quantification of mRNA expression of SM22α (Transgelin) and the housekeeping gene 18S rRNA total mRNA was isolated from HCAECs and HCASMCs using peqGOLD RNAPure as described by the manufacturer. After quantification of mRNA content using the NanoDrop 2000 (peqlab, Erlangen, Germany) cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany). A portion of 20 ng of cDNA was used for real-time PCR using predeveloped TaqManAssays Hs01038777_g1, and Hs99999901_s1 for detection of SM22α (transgelin) and 18S rRNA, respectively, the TaqManGene Expression Master Mix, and the 7900HT Fast Real-Time PCR System (Life Technologies).

Cloning of the Promoter Region of SM22α. A 1269 bp fragment of the 5′-UTR of human SM22α was amplified from human genomic DNA using the following primers SM22prom_for 5′-ACGTGGTACCCCCCAAGATCCC-TGAAGCCAGGTA-3′ and SM22prom_rev 5′-ACGT-CTCGAGGCCTGCCTGAAATGCACCCACT-3′. The resulting PCR product was first subcloned into pGL3-basic (Promega, Mannheim, Germany) where the luciferase coding region was replaced by eGFP using the restriction enzymes *Kpn*I and *Xho*I. After sequencing the insert, SM22-eGFP-polyA was subcloned into pENTR1A Dual Selection (Life Technologies), which was then used in an LR Clonase mediated recombination reaction for transfer into pAd/PL-DEST a replication deficient variant of human Adenovirus subtype 5 (Life Technologies). After amplification in *E. coli* the isolated adenoviral vector was digested with *Pac*I and transfected into HEK293A cells (Life Technologies) using Lipofectamin 2000 (Life Technologies) for virus production. The virus was isolated and tested for plaque forming units as described in the manufacturer's instructions.

eGFP Fluorescence and Immunofluorescent Staining. Efficacy of infection and expression was determined using enhanced green fluorescence protein (eGFP) fluorescence. Twenty-four hours after seeding 2×10^5 cells/well on coverslips, cells were infected with 50 pfu/cell of the respective pAD-promoter-eGFP construct. Cytomegalivirus (CMV)-promoter driven expression served as control for the SM22α-promoter. Forty-eight hours after infection cells were fixed with 4%-paraformaldehyde, washed twice with PBS, and incubated for 15 min with 4′,6-diamidino-2-phenylindole (DAPI) diluted 1:1000 in PBS for nuclei stain. After that the coverslips were mounted onto slides and green fluorescence was determined using the fluorescence microscope Axio Observer D1 (Zeiss GmbH, Jena, Germany). Immunofluorescent staining was performed 48 h after adenoviral infection (50 pfu/cell) or 48 h in culture in virus naïve cells, respectively. In detail, after fixation with methanol−ethanol (1:1), cells were washed twice with PBS and blocked for 1 h with PBS containing 5% FCS and 0.01% Triton-X 100. Subsequently, the cells were incubated with the respective antibody, ab123128 (abcam, Cambridge, UK) for OCT1 diluted 1:25, and ab14106 (abcam) for SM22α diluted 1:250, at 4 °C overnight in a humidified atmosphere. After that, cells were washed several times with PBS and incubated with a fluorescence labeled secondary antibody (Alexa Fluor 568 or Alexa Fluor 488, Life Technologies). After staining with DAPI and mounting on slides, fluorescence was visualized using the Axio Observer.D1 microscope (Zeiss GmbH).

HE Staining of Cultured Cells. To determine the influence of viral infection on cell morphology, cells were infected with 50 pfu/cell of the control virus pAD-CMV-lacZ (Life Technologies). After 48 h of infection cells were fixed with ice-cold methanol−aceton (1:1) for 15 min. Then Hematoxylin Eosin staining was performed as follows. First, cells were incubated with Hematoxylin solution, Gill No. 3 for 15 min. After washing several times with distilled water and blueing with tap water eosinophilic structures were counterstained with an alcoholic eosin solution. Staining was visualized using the Axio Observer.D1 microscope (Zeiss GmbH).

Transport Assay. The transport activity of OCT1 was determined 48 h after adenoviral infection with 50 pfu/cell. Briefly, cells were washed twice with incubation buffer (140 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM Hepes, pH 7.3), then radiolabeled substances [^{3}H]-1-methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$) or [^{14}C]-Tetraethylammonium bromide ([^{14}C]-TEA) (100 000 dpm/well) were added and incubated for the indicated times. After incubation cells were washed two times with ice-cold phosphate buffered saline (PBS). Cellular accumulation of the radiolabel was determined after cell lysis in 400 μL of SDS-EDTA buffer (0.2% SDS, 5 mM EDTA) using a liquid scintillation counter (type 1409, LKB-Wallac, Turku, Finland).

Western Blot Analysis. For protein analysis cells were harvested in 5 mM Tris-HCl supplemented with aprotinin (1

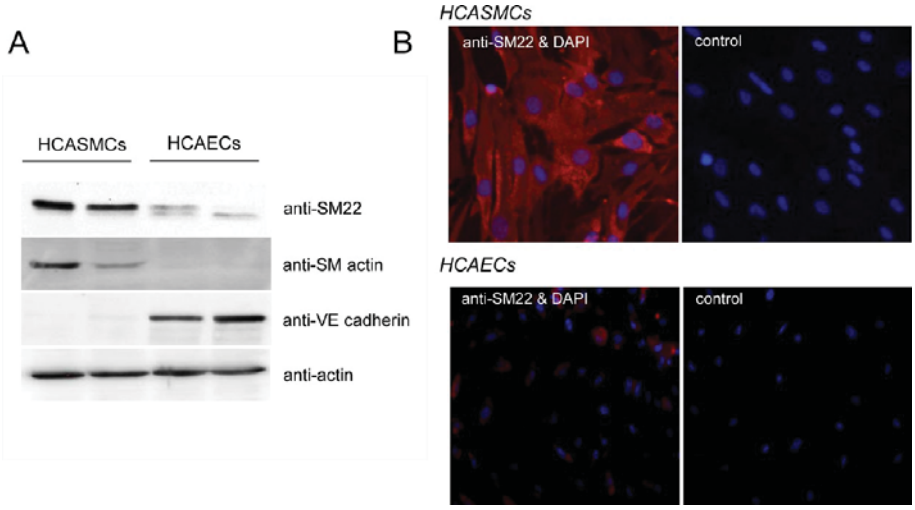

Figure 1. Detection of SM22α (transgelin) in human coronary artery smooth muscle cells (HCASMCs) and endothelial cells (HCAECs). (A) Protein lysates of cultured primary HCASMCs and HCAECs obtained from two different individuals were used for Western blot analysis of SM22α (transgelin) expression; smooth muscle actin (SM-actin) was used as a smooth muscle cell marker, while vascular endothelial cadherin (VE-cadherin) served as a endothelial marker. (B) SM22alpha was also detected by immunofluorescent staining of cultured HCASMCs and HCAECs. In the case of the control staining the primary antibody was omitted.

mM), leupeptin (1 mM), and phenylmethylsulfonyl fluoride (0.1 μM). Afterward the cells were lysed by 5 cycles of freezing in liquid nitrogen and thawing at 37 °C. The protein content was determined performing bicinchoninic acid (BCA) assays (Thermo Fisher Scientific, Bonn, Germany). In total 25 μg of the lysate were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane using a TANK blotting system (Bio-Rad, Munich, Germany). After blocking with 5% FCS in a mixture of Tris-buffered saline and 0.05% Tween 20 (TBS-T), the membranes were incubated with the primary antibody. The anti-SM22α antibody (ab14106, abcam) was diluted 1:2000, anti-GFP antibody (Santa Cruz) was diluted 1:2500, the anti-Actin-antibody (Santa Cruz) was diluted 1:2500, and the antivascular endothelial (VE) cadherin-antibody (ab33168, abcam) was diluted 1:1000 for detection of SM22α, eGFP, actin, and VE cadherin (CD144). After several washing steps with TBS-T, binding of the primary antibody was visualized using a HRP-coupled secondary antibody (Bio-Rad) and ECL Plus reagent (GE Healthcare, Munich, Germany). Chemiluminescence was visualized using the molecular imager Chemi Doc XRS from Bio-Rad.

BrdU Proliferation and Resazurin Viability Assay. Proliferation and viability of cells were assessed performing BrdU ELISA (Cell Proliferation ELISA, Roche, Basel, Switzerland) and resazurin viability assays (Fluorometric Cell Viability Kit I from PromoKine GmbH, Heidelberg, Germany), respectively. Briefly, after seeding in 96-well plates and culture for 24 h cells were treated with 100 nM paclitaxel (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 5 h in basal media. Afterward the cells were washed with PBS, and the medium was replaced with corresponding growth medium (basal medium containing culture supplements). After incubation for 24 h at 37 °C the BrdU-labeling solution was added. For detection of cell viability, the medium was replaced by resazurin containing growth medium after 48 h treatment, then the cells were incubated for additional 2 h at 37 °C. Subsequently, the formed resorufin was quantified using the Infinite200 microplate reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Cells treated with solvent served as the control. After washing the cells with PBS the amount of incorporated BrdU was quantified according to the manufacturer's instructions. Chemiluminescence was determined using the Infinite 200 microplate reader.

RESULTS

Expression of SM22α in Human Vascular Cells. To verify the cell-specific activity of the promoter chosen as an expression driving force in the herein proposed adenoviral construct, SM22α (transgelin) expression was determined comparing HCAECs and HCASMCs isolated from three different individuals. Quantitative real-time PCR revealed significantly higher mRNA levels of transgelin in HCASMCs (data not shown). In accordance, enhanced protein expression of SM22α was observed in HCASMCs, which were also tested for expression of smooth muscle actin as an additional marker protein and expression of VE-cadherin an endothelial marker, performing Western blot analysis (Figure 1A). Similar results were obtained by immunofluorescent staining of SM22α comparing HCASMCs and HCAECs (Figure 1B).

Comparing Adenoviral Gene Transfer in Different Cell Types. The efficacy of gene transfer was tested in different cellular models. Therefore expression of eGFP was assessed after infection with adenoviruses containing the coding region under control of the SM22α-promoter fragment (pAD-SM22-eGFP) or the commonly in molecular biology used CMV-promoter (pAD-CMV-eGFP), respectively. As shown in Figure 2A gene transfer was most efficient in HCASMCs. Especially, murine cardiomyocytes (HL-1) and endothelial cells HCAECs exhibited very low infection efficacy compared to HCASMCs even if using CMV-promoter driven eGFP expression. In contrast, canine MDCKII and human A549 cells were well responsive to adenoviral gene transfer. Importantly, after infection with pAD-SM22-eGFP only little fluorescence was observed in all cells but HCASMCs. As shown in Figure 2B, similar results were obtained performing Western blot analysis. In detail, after infection with 50 pfu/cell HCASMCs exhibited

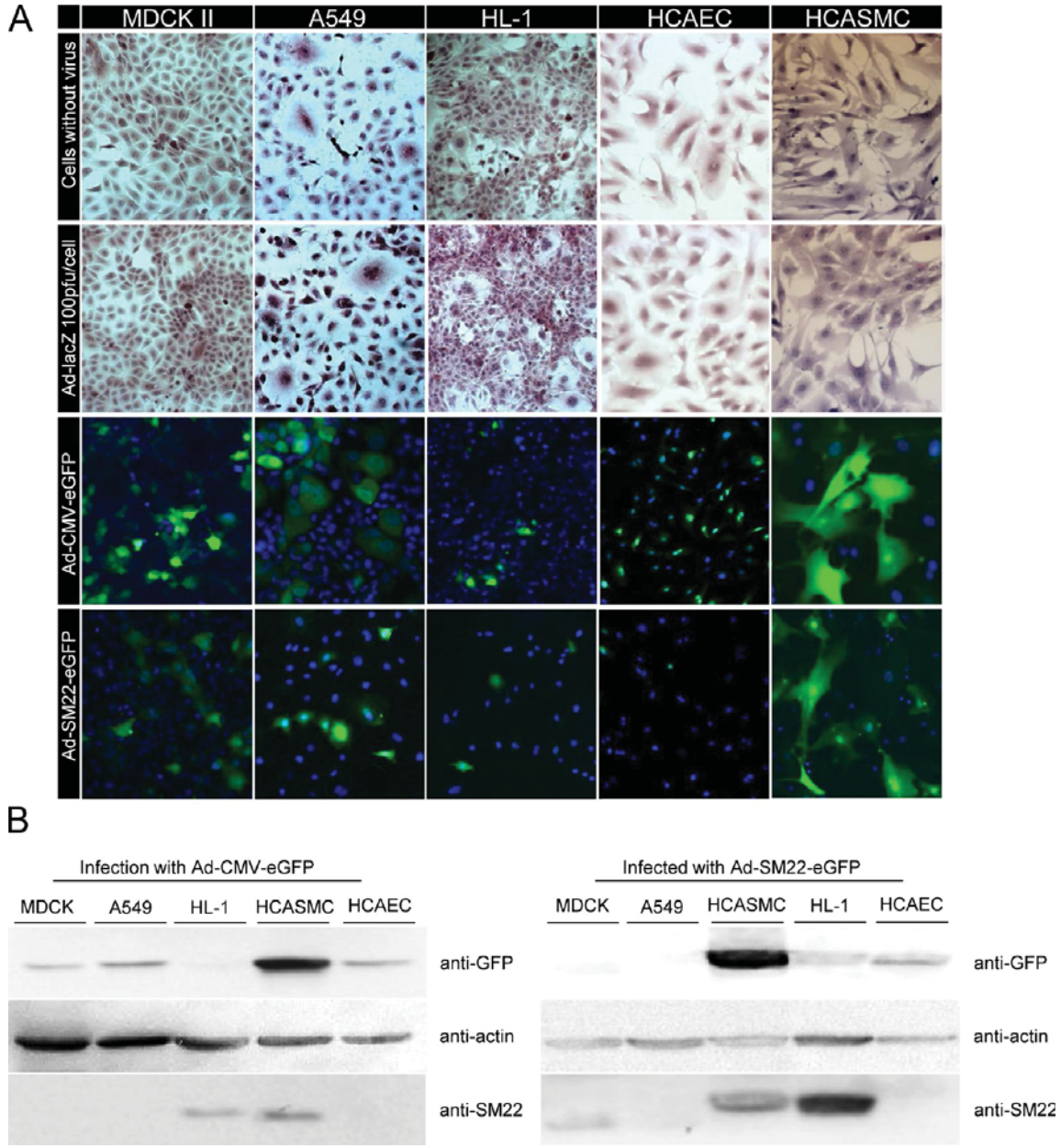

Figure 2. Adenovirus mediated gene transfer comparing different cellular models. Cells were cultured on coverslips, after treatment with adenovirus cells were fixed and cellular morphology was determined by HE-staining (upper panel). No significant impact on cellular morphology was observed after adenoviral infection with 50 pfu/cell; in control cells the virus was omitted. Expression of GFP was visualized by fluorenscence microscope 48 h after infection with 50 pfu/cell of pAD-SM22-eGFP or pAD-CMV-eGFP, respectively.

highest expression of eGFP, while HCAECs exhibited only modest levels of the protein even if driven by the CMV-promoter. Importantly, in the presence of the SM22α-promoter eGFP expression was nearly abolished in MDCKII and A549. However, there was some eGFP detectable in pAD-SM22-eGFP infected endothelial cells.

OCT1 Activity after Adenoviral Transfer in MDCKII Cells. MDCKII cells are often used to characterize transporters *in vitro*;[15] therefore we tested the expression and activity of the organic cation transporter OCT1 in those cells after adenoviral gene transfer. At first, expression of the transporter was determined by Western blot analysis after infection with different viral concentrations (ranging from 5 to 50 pfu/cell) revealing intensified expression in the presence of increasing virus concentrations (Figure 3A). In addition the transporter was located in the plasma membrane of infected MDCKII cells as determined by immunofluorescent staining. Importantly, no signal was observed in MDCKII cells infected with pAD-SM22-OCT1 (Figure 3B). This is in accordance with following findings on the enhanced uptake of the prototypical OCT1-substrate MPP^+ in MDCKII cells infected with the virus encoding the transporter under the control of the CMV promoter (Figure 3C).

Transport Activity in Different Cellular Models. It was aim of the study to determine whether adenoviral gene transfer could be used to enhance cellular accumulation and therefore

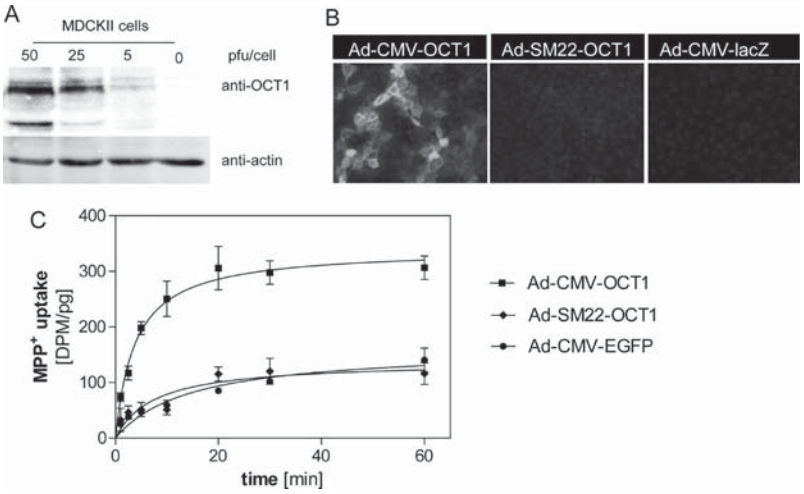

Figure 3. Detection and functional characterization of OCT1 in MDCKII cells. Expression of OCT1 was determined by Western blot analysis 48 h after infection with different amounts of pAD-CMV-OCT1 (ranging from 5 to 50 pfu/cell). Localization of the transporter in the basolateral membrane of polarized MDCKII-cells was observed performing immunofluorescent staining with an OCT1-specific antibody; no significant membrane staining was observed in cells infected with 50 pfu/cell of pAD-SM22-OCT1 or the control vector pAD-CMV-lacZ. Significant uptake of MPP^+ was observed in cells infected with OCT1 driven by a CMV promoter.

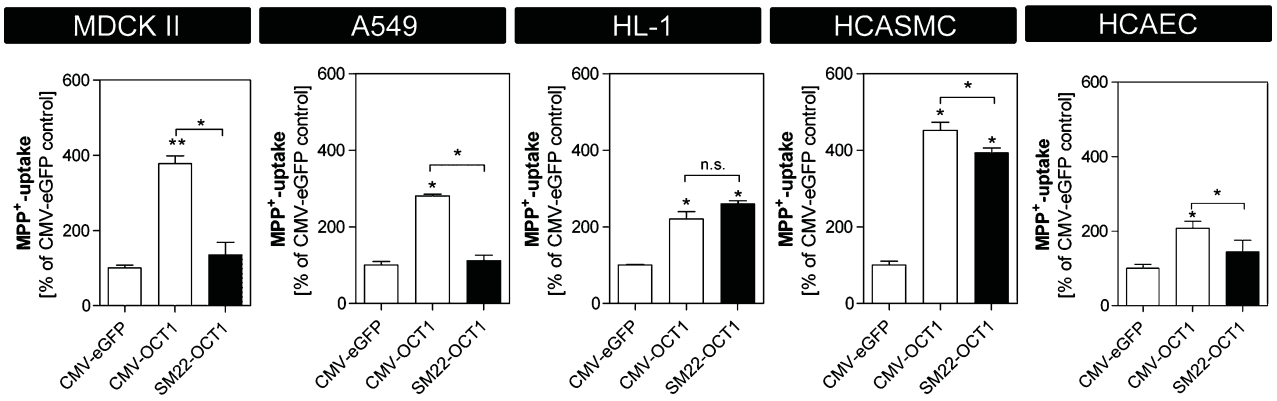

Figure 4. Uptake of substrate drugs after adenoviral transfer of the organic cation transporter OCT1. Different cell lines were infected with pAD-CMV-OCT1 or pAD-SM22-OCT1 50 pfu/cell. Forty-eight hours after infection transport activity was determined measuring the uptake radiolabeled MPP^+ after 5 min incubation (**$p < 0.005$, *$p < 0.05$, Student's t-test, $n = 3$).

efficacy of substrate drugs in a cell type specific manner. To test cellular accumulation in presence of OCT1, cells were infected with adenoviruses containing the coding sequence of this transporter either under the control of the CMV-promoter or the control of the SM22-promoter, followed by transport studies with two different prototypical substrates of OCT1, namely, [^{3}H]-MPP^+ and [^{14}C]-TEA. As shown in Figure 4 all cell types tested exhibited enhanced uptake of MPP^+ in the presence of pAD-CMV-OCT1, with the highest increase in HCASMCs (% uptake of pAD-CMV-eGFP, mean ± SD; 451.7 ± 20.9%), even if significantly lower compared to the CMV promoter those cells also exhibited enhanced intracellular accumulation when infected with pAD-SM22-OCT1 (392.7 ± 13.2%). Similar results were obtained in murine cardiomyocytes (pAD-CMV-OCT1 infected 220.5 ± 33.0; pAD-SM22-OCT1 infected 259.6 ± 14.1%). However, in HCAECs infected with pAD-CMV-OCT1 uptake of MPP^+ was increased by about 2-fold (206.9 ± 19.9%), whereas no statistically significant uptake was observed after infection with pAD-SM22-OCT1 (144.0 ± 31.2%). Similar results were obtained for TEA (data not shown).

OCT1 Enhances Antiproliferative Activity in HCASMCs of the Substrate Drug Paclitaxel. Paclitaxel is one of the previously reported cytotoxic OCT1 substrates.[11] To determine the impact of adenoviral-mediated OCT1-expression on the antiproliferative efficacy of this compound, HCASMCs and HCAECs were infected with pAD-CMV-OCT1 or pAD-SM22-OCT1 and treated with 100 nM paclitaxel. As shown in Figure 5 in the presence of the CMV-promoter driven OCT1 both cell types exhibited enhanced efficacy of the mitotic inhibitor compared to cells infected with the control virus pAD-CMV-lacZ (proliferation fold of control cells; mean ± SD; HCASMCs 0.54 ± 0.08, HCAECs 0.76 ± 0.11). Importantly, the influence of OCT1 was not present in HCAECs infected with pAD-SM22-OCT1 (0.9 ± 0.1), while HCASMCs exhibited enhanced antiproliferative activity of paclitaxel (0.56 ± 0.13).

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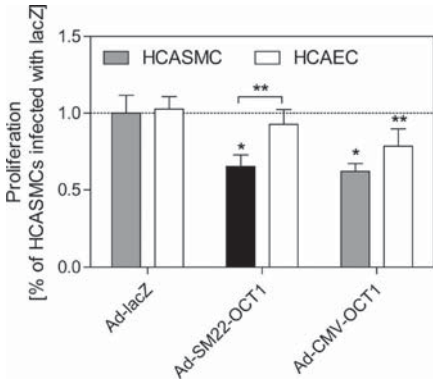

Figure 5. Impact of OCT1 on antiproliferative activity of paclitaxel. Proliferation of HCASMCs and HCAECs was determined after viral infection. 48 hrs after infection cells were treated 5 h with 100 nM paclitaxel. 48 h after treatment the proliferation rate was determined performing a BrdU-ELISA (**$p < 0.005$, *$p < 0.05$, Student's *t*-test, $n = 6$).

DISCUSSION

The use of drug delivery devices, such as stents, in the treatment of coronary atherosclerosis is clinically well-established and is the basis of the herein tested concept of a transporter/substrate combination to target drug effects. It was aim of the study to provide a proof of concept that changing the pharmacokinetics of a drug by the means of adenoviral gene expression can be used to enhance cellular specificity. In accordance, we were able to show *in vitro* that adenoviral expression of the human organic cation transporter 1 (hOCT1) significantly increases uptake and thereby antiproliferative activity of the substrate paclitaxel in smooth muscle cells. The interaction of paclitaxel with this particular transporter has been previously reported by Gupta et al. showing that OCT1-expressing cell lines and OCT1-transfectants exhibit higher susceptibility to cytotoxic effects of paclitaxel compared to OCT1-negative cells.[11] In the case of the vascular system, where target and nontarget cells, namely, smooth muscle and endothelial cells, respectively, of a cytotoxic compound are in very close vicinity, a cell-specific driving force for gene expression is needed to realize the concept of the transporter−drug combination strategy. The SM22α promoter is a widely accepted tool for deletion or overexpression of genes in smooth muscle cells.[16−18] We also observed muscle cell specific activity when quantifying the OCT1 transport function. However, SM22α-driven expression was not as restricted to HCASMCs, when assessing for eGFP expression, suggesting at least some activity of the SM22α promoter in cell lines other than HCASMCs *in vitro*. The specificity and potency of SM22α-promoter driven gene expression after balloon catheter based delivery of an adenovirus has recently been tested *in vivo*, showing GFP expression in smooth muscle cells after treatment of swine. In this particular study Pankajakshan et al. observed muscle cell specific expression after short-term exposure with a virus coated balloon.[19] A different approach to enhance cellular selectivity of an adenoviral based transfection system would be the use of transductional targeting. The clinical use of adenovirus type 5 as a gene therapeutic tool at least for systemic administration is hampered by the liver tropism of the virus, which is associated with significant hepatotoxicity. The tropism is mediated by higher expression of the coxsackie and adenovirus receptor (CAR) and other membrane proteins assumed to be responsible for cellular entry of the virus in hepatocytes compared to other tissues.[20] Previous studies have tested modifications in the knob domain of the adenoviral fiber the region assumed to bind to the CAR receptor, which have resulted in different transduction efficacy of the adenovirus.[21] In uterine leiomyoma, a tumor entity deriving from smooth muscle cells, enhanced transduction efficacy was observed using an adenovirus with a modified RGD sequence in the adenoviral fiber.[22] The integration of fiber modified adenovirus with restricted promoter activity into a local drug delivery device seems of high potential for future developments in gene therapy, which should not be limited to the vascular system.

Several studies have previously tested the combination of adenovirus with local delivery devices *in vivo*. Ohno et al. first provided evidence that changing pharmacokinetics of a cytotoxic compound could be used to target drug effects after angioplasty. Indeed, the authors combined angioplasty mediated adenoviral gene transfer of the herpes virus thymidine kinase gene, an enzyme converting the purine analogue ganciclovir to its biologically active metabolite with the systemic administration of this compound, and showed significantly reduced vascular smooth muscle cell proliferation after adenoviral infection.[23] Importantly, metabolically activated ganciclovir has no impact on nondividing cells, thereby restricting the pharmacodynamic effect to thymidine kinase expressing and proliferating cells. However, the authors did not clearly report any data on the impact of ganciclovir treatment on endothelial cells in their animal study. Importantly, in the same study the authors observed no significant accumulation of the virus in other tissues, such as liver, suggesting that viral effects are limited to the vascular system.

It seems noteworthy, that in the above-mentioned study the authors used the two-balloon catheter technique which had been previously reported by Willard et al. as the most efficient catheter based local delivery device for gene transfer.[24] Performing this procedure, the virus solution is injected in a chamber between an inflated proximal and distal compartment of the balloon. Using a similar technique Laitinen et al. were able to show adenovirus-mediated gene transfer to the vascular wall in humans *in vivo*. Even if rather low gene transfer efficacy (0.04−5% of all arterial cells) was reported, expression of the transgene was observed in all cell types including smooth muscle and endothelial cells.[25] However, our *in vitro* data suggest very low adenovirus-mediated gene expression in HCAECs compared to HCASMCs even if using a CMV-promoter as driving force. This low infection efficacy is in accordance with findings by Lenaerts et al. showing lower virus binding to endothelial cells,[26] thereby explaining lower gene expression after adenoviral infection. In the following years the therapeutic efficacy of different transgenes including growth factors (e.g., VEGF), synthetic peptides,[27] and other proteins functional in cell proliferation or migration[28] have been tested. However, most of the studies were performed exposing the vasculature directly after balloon injury to the virus, although it had been reported that gene delivery is most efficient 7−8 days after balloon injury, thereby suggesting that a long-term exposure with the a gene delivery device coated with adenovirus would be most efficient.[27,29] However, it cannot be excluded that this observed rather short period of gene expression after viral delivery would also be sufficient for a transporter-mediated cell specific drug targeting. Particularly when considering the drug release profile of the first generation paclitaxel stent TAXUS Express,[30] where the drug release profile consisted of a

48 h burst followed by a 10 day period of slow drug release, which despite this rather short-term drug release profile resulted in poor endothelialization.[31]

Since the study of Laitinen et al. in 1998, the field of biomedical engineering has significantly evolved, now providing other solutions for adenoviral gene transfer including bioabsorbable poly(L-lactide) (PLLA)/poly(ε-caprolactone) (PCL) stents,[32] nanobots (nanoparticles based on the biodegradable poly(lactide-*co*-glycolide) (PLGA)),[33] that have been shown to serve as potent delivery devices for adenovirus. An alternative to the use of the adenovirus-based technology would be the use of plasmid/liposome solutions. It has been previously reported that both methods resulted in expression of the transgene in humans. Importantly, Makinen et al. even reported results on a clinical study in humans where VEGF was expressed by both methods.[34] In the eight year follow up, no significant difference was observed for the two techniques of VEGF gene delivery determining the clinical outcome.[35]

It might be hypothesized that the transporter used in a transporter/drug concept based drug delivery devices is exchangeable. Indeed, there are several members of the superfamily of the SLC transporter that have been shown to transport cytotoxic compounds including organic anion transporters such as OATP1B3 which has been reported to facilitate uptake SN-38 or docetaxel,[36,37] or such as OAT2 which increases cellular accumulation of 6-fluouracil or paclitaxel (summarized in ref 38).

In conclusion, adenovirus-mediated expression of uptake transporters to enhance accumulation and thereby pharmacodynamics of substrate drugs as shown for the SMC-specific expression of OCT1 and the enhanced activity of paclitaxel in those cells is not limited to cationic substrates. In current approaches to identify potential novel candidates for drug eluting stents primary human smooth muscle and endothelial cells are screened. With the transporter/drug-concept, the specificity of cytotoxic compounds could be enhanced by specifically increasing the mechanism of uptake. It seems noteworthy that the combination of delivery device and adenovirus-mediated transporter expression even seems of potential for other therapeutic strategies where cellular enrichment of a compound would improve the outcome or reduce unwanted side effects.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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3.3. Expression of OATP2B1 as determinant of drug effects in the microcompartment of the coronary artery

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Expression of OATP2B1 as determinant of drug effects in the microcompartment of the coronary artery

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ABSTRACT

Clinical success of coronary drug-eluting stents (DES) is hampered by simultaneous reduction of smooth muscle cell (HCASMC) and endothelial cell proliferation due to unspecific cytotoxicity of currently used compounds. Previous in vitro data showing SMC-specific inhibition of proliferation suggested that statins may be suitable candidates for DES. It was aim of this study to further investigate statins as DES drug candidates to identify mechanisms contributing to their cell-selectivity. In vitro proliferation assays comparing the influence of various statins on HCASMC and endothelial cells confirmed that atorvastatin exhibits HCASMC-specificity. Due to similar expression levels of the drug target HMG-CoA reductase in both cell types, cellular accumulation of atorvastatin was assessed, revealing enhanced uptake in HCASMC most likely driven by significant expression of OATP2B1, a known uptake transporter for atorvastatin. In accordance with the finding that endogenous OATP2B1 influenced cellular accumulation in HCASMC we used this transporter as a tool to identify teniposide as new DES candidate drug with HCASMC-specific effects. We describe OATP2B1 as a determinant of pharmacokinetics in the coronary artery. Indeed, endogenously expressed OATP2B1 significantly influences the uptake of substrate drugs, thereby governing cell specificity. Screening of candidate drugs for interaction with OATP2B1 may be used to promote SMC-specificity.

1. Introduction

Atherosclerosis is a major risk factor of coronary artery disease which finally may lead to myocardial infarction [1]. In order to restore the blood flow in obstructed arteries the percutaneous coronary transluminal angioplasty (PTCA) is commonly used in clinics. However, after dilatation a variety of cellular mechanisms take place including aggregation of platelets, release of growth factors, infiltration of inflammatory cells, and proliferation and migration of smooth muscle cells [2,3] finally resulting in restenosis of the vessel often with fatal impact on the outcome. In order to prevent restenosis after transluminal artery dilatation the implantation of bare metal stents (BMS) was introduced in this clinical procedure. Although prospective multicenter studies demonstrated an improved clinical outcome with reduced rates of restenosis after stenting [4,5], success of BMS was significantly hampered by the manifestation of subacute thrombosis and in-stent restenosis [6]. Indeed, it is assumed that the injury of the vessel wall during stent deployment triggers an inflammatory process resulting in production and secretion of various growth factors that stimulate smooth muscle cell proliferation resulting in the pathological phenomenon of neointima formation [2,7–9]. The first-generation drug-eluting stents (DES), loaded with sirolimus (Cypher, Cordis, Warren, New Jersey, USA) or paclitaxel (Taxus, Boston Scientific, Natick, USA) were introduced with the idea that cytotoxic compounds inhibit the formation of a neointima due to their antiproliferative activity, thereby reducing the adverse event of in-stent restenosis [10,11]. In accordance with this notion are findings of controlled clinical trials reporting reduced in-stent late lumen loss and in-stent angiographic restenosis comparing DES and BMS [12]. However, the adverse event of stent thrombosis remains a major safety problem, where late stent thrombosis (>1 year after stenting) is more likely after implantation of DES [13,14]. Numerous factors have been associated with the risk of thrombotic events in DES-treated arteries, including reduced endothelialization of the stent strut due to inhibition of migration and proliferation of endothelial cells. Subsequent developments of DES focused on agents demonstrating not only antiproliferative, but also enhanced anti-inflammatory activity as observed with everolimus or zotarolimus, which are both currently used on second-generation DES. However, even if there is a lower risk of late stent thrombosis in patients treated with second-generation DES the incidence of this adverse event has not been abolished [15]. Despite the fact that limus derivatives reduce inflammation after stenting, from a pharmacological perspective there is still the need for

Abbreviations: HCASMC, human coronary artery smooth muscle cells; HCAEC, human coronary artery endothelial cells; HMGCR, HMG-CoA reductase.

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compounds which specifically inhibit the migration and proliferation of smooth muscle cells without affecting endothelial cells in order to enhance healing of vascular implants.

One approach to improve the outcome of DES may be the use of drugs showing benefits in therapy of atherosclerosis. Several studies have previously reported an improved clinical outcome of patients orally treated with statins during the time of DES deployment. Especially the treatment with atorvastatin or fluvastatin has been associated with a reduced risk of adverse cardiac events and other ischemia-related outcomes in patients undergoing PTCA [16,17], thereby being the basis for previous reports suggesting that statin-coated stents may be an option for improved clinical outcome [18,19]. In this context, it seems noteworthy that several in vitro and in vivo studies have reported a positive impact of delivery devices coated with statins. Particularly, for cerivastatin a pronounced effect on smooth muscle cell proliferation and migration [20] without impeding endothelial cell proliferation has been shown [21], supported by in vivo findings in a porcine coronary model [22]. Even if statins are all primarily targeting HMG-CoA reductase thereby modulating cholesterol levels, there are significant differences in their physicochemical and pharmacokinetic properties, which include passive permeability and active transport [23]. Assuming that distribution in the area of the coronary artery, defined as microcompartment, surrounding the drug-delivery devices also depends on passive diffusion or active transport those mechanisms may significantly influence cell-type specificity of drugs used on DES. In order to test this hypothesis we compared the impact of different statins on cellular proliferation, showing pronounced differences comparing smooth muscle and endothelial cells.

The aim of the study was to investigate whether cellular uptake may be a determinant of pharmacodynamics of statins in the micro compartment of the vessel wall, and whether transport by a membrane transporter might be predictive for cell specificity of a compound used on coronary drug delivery devices.

2. Methods

2.1. Materials

[^{3}H]-atorvastatin (specific activity 10 Ci/mmol), [^{3}H]-atorvastatin lactone (specific activity 10 Ci/mmol) and [^{3}H]-estrone 3-sulfate (specific activity 50 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany). Mevastatin, pravastatin, and simvastatin were purchased from Sigma-Aldrich (Deisenhofen, Germany) and cerivastatin from Bayer (Leverkusen, Germany). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and atorvastatin from Pfizer Pharma (Karlsruhe, Germany). All other chemicals were obtained from Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

2.2. Cell culture

Human coronary artery smooth muscle cells (HCASMC) and endothelial cells (HCAEC), isolated from different human individuals and genders, and their optimized media i.e., Smooth Muscle Cell Growth Medium 2 and Endothelial Cell Growth Medium MV were obtained from PromoCell GmbH (Heidelberg, Germany). Cells were cultivated in a humidified atmosphere at 37 °C and 5% CO_2 from passage four to ten. For proliferation assays, cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h in growth medium, HCASMC or HCAEC were maintained for further 24 h in basal medium (PromoCell GmbH) supplemented with only 0.05% or 0.5% FCS, respectively. To simulate cellular stimulation after stent implantation, cells were then treated with growth medium in presence or absence of the respective drug for 48 h. For transport studies and expression analysis cells were seeded in respective well plates at a density of 1.2×10^4 cells/cm^2. Madin–Darby canine kidney II (MDCKII) cells overexpressing OATP2B1 as previously reported [24] were cultivated in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 1% GlutaMax and 250 μg/ml Hygromycin B, both obtained from Life Technologies (Carlsbad, USA), at 37 °C and 5% CO_2.

2.3. Bromodesoxyuridine (BrdU) cell proliferation assay

Cell proliferation of HCASMC and HCAEC in the presence of statins was determined using the commercially available BrdU Cell Proliferation Kit (Roche, Basel, Switzerland). In brief, BrdU labeling solution was added to the medium after 24 h treatment followed by incubation for 24 h. Subsequently, BrdU ELISA was performed according to the manufacturer's instructions and luminescence was measured with the microplate reader (Infinite® 200 Pro-Tecan, Männedorf, Switzerland). Cells treated with the solvent served as control. Data are presented as percent of control.

2.4. Adenoviral infection

For adenoviral infection the previously reported Ad-OATP2B1 [25] was used. To study the influence on cell proliferation, HCASMC were seeded in 96-well plates 24 h prior to infection with 50 pfu/cell Ad-OATP2B1 followed by incubation with atorvastatin for 48 h. For short-time incubation with teniposide, cells were treated for 10 min, washed with PBS and cultivated in growth medium for 48 h. Transport activity in HCASMC and HCAEC was assessed 48 h after adenoviral infection. Cells infected with Ad-lacZ served as reference for all experiments if not otherwise stated.

2.5. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from six different donors of both naïve HCASMC and HCAEC was isolated using NucleoSpin® RNA (Machery-Nagel, Düren, Germany) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. For qRT-PCR, we used TaqMan® gene expression assays from Life Technologies detecting HMGCR (Hs00168352_m1), 18S rRNA (Hs99999901_s1), or GAPDH (4326317E). PCR reactions were carried out using the Viia 7™ (Life Technologies). Expression relative to human liver was calculated using the ΔΔCt-method.

2.6. Immunoblot analysis

For Western blot analysis cells were harvested in 100 μl of the commercially available RIPA buffer supplemented with 10 μg/ml aprotinin and 10 μg/ml leupeptin. Protein content in cell lysate was quantified by a bicinchoninic acid assay (Pierce, Thermo Fisher Scientific Inc., Rockford, USA). Protein samples were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes using a Tank blotting system (Bio-Rad Laboratories, Berkeley, USA). After protein transfer, membranes were placed in 5%-FCS in TBS-T (0.25 M Tris–base, 0.026 M KCl, 0.04% Tween 200) for 1 h prior to incubation with the primary antibody. The antibodies used were anti-HMGCR (sc-27578, 1:2000 Santa Cruz Biotechnology, Inc.), anti-β-actin (sc-1616, 1:2000 Santa Cruz Biotechnology, Inc.), anti-van Willebrand factor (vWF, ab6994, 1:5000 Abcam® Cambridge, UK) and anti-α-smooth muscle actin (α-SM-actin, ab7817, 1:2000 Abcam®) diluted in TBS-T supplemented with 0.5% BSA. The previously reported anti-OATP2B1 serum [24] was used in a dilution of 1:5000. Incubation was performed at 4 °C overnight followed by several washing steps with TBS-T and incubation with the respective HRP-labeled secondary antibody for 1 h at room temperature. Luminescence was visualized using the ECL Western blotting substrate (Pierce, Thermo Fisher Scientific Inc.) and digitalized with the ChemiDoc XRS system (Bio-Rad Laboratories). Densitometry was conducted using Image Lab 4.1 software from Bio-Rad Laboratories.

2.7. Transport studies

For uptake studies confluent layers of naïve HCASMC and HCAEC, cells were incubated at 37 °C for 5 min with 1 μM atorvastatin or 1 μM atorvastatin lactone supplemented with [^{3}H]-atorvastatin (100,000 dpm/well) or [^{3}H]-atorvastatin lactone (100,000 dpm/well), respectively, solved in incubation buffer (0.14 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 1.5 mM $CaCl_2$, 5 mM glucose, 12.5 mM HEPES, pH 7.3). After incubation cells were washed three times with ice cold PBS and lysed in 0.2% SDS–5 mM EDTA solution. Intracellular accumulation of radioactivity was quantified using a liquid scintillation counter (type 1409, LKBWallac, Turku, Finland). The amount of atorvastatin and atorvastatin lactone was related to the protein content of the respective sample and is presented as fmol/μg protein. For functional characterization of OATP2B1 after adenoviral infection, HCASMC were incubated with 1 μM estrone 3-sulfate (E_1S) for 5 min in the presence of [^{3}H]-E_1S (100,000 dpm/well) 48 h after infection with 50 pfu/cell Ad-OATP2B1 or Ad-lacZ. Inhibitors of OATP2B1 were identified in uptake studies using MDCKII-OATP2B1 cells. Therefore, cells were exposed to the cytotoxic drugs (each 10 μM) or increasing concentrations of statins in the presence of [^{3}H]-E_1S for 10 min. The inhibition of E_1S uptake by incubation with teniposide (1, 10, and 100 nM) after adenoviral infection with Ad-OATP2B1 in HCASMC was detected as described above. Inhibition of E_1S uptake is presented as the percentage of the values obtained for control cells.

2.8. Statistical analysis

Data are represented as means ± standard deviations (SD), if not otherwise stated of at least three independent experiments performed in triplicates and analyzed using Student's *t*-test or one-way ANOVA comparing more than two groups. Statistical significance was defined as p-value < 0.05. Graphs and calculations (IC_{50}) were performed using GraphPad Prism© software (GraphPad Software, Inc., La Jolla, USA).

3. Results

3.1. Impact of statins on cell proliferation

In order to test the idea of pharmacokinetic mechanisms influencing cell specificity of DES drugs, we searched for a statin exerting antiproliferative activity in smooth muscle cells, which would translate into inhibition of neointima formation, with less activity in endothelial cells. Accordingly, the impact of different statins on proliferation of endothelial (HCAEC) and smooth muscle cells (HCASMC) was compared, treating primary cells for 48 h and assessing the status of proliferation by BrdU ELISA. Incubation of both HCAEC and HCASMC with simvastatin, pravastatin, cerivastatin, mevastatin, or atorvastatin revealed variable effects on proliferation (in % of DMSO treated cells; mean ± SD, [Fig. 1A]). In detail, cerivastatin efficiently inhibited proliferation of both cell types (HCASMC 12.3 ± 10.5% vs. HCAEC 19.8 ± 19.7%). A much lower, but also non-cell-type-specific impact on proliferation was observed for simvastatin (HCASMC 66.8 ± 41.4% vs. HCAEC 61.7 ± 48.7%), mevastatin (HCASMC 69.8 ± 4.8% vs. HCAEC 65.3 ± 22.5%), and finally pravastatin (HCASMC 106.0 ± 22.1% vs. HCAEC 93.9 ± 31.6%). For atorvastatin however, we observed cell specificity, with significantly higher inhibition of HCASMC proliferation (45.6 ± 25.2%) compared to endothelial cells (82.5 ± 3.3%; $^*p < 0.05$, Student's *t*-test). Those findings are in accordance with IC_{50} values of atorvastatin determined incubating HCASMC and HCAEC. As shown in Fig. 1B, the calculated IC_{50} value was about 7-fold lower in HCASMC (0.62 μM; CI 95% 0.43–0.89 μM) compared to HCAEC (4.45 μM; CI 95% 2.19–9.12 μM).

3.2. The involvement of HMC-CoA reductase in atorvastatin-induced cellular effects

Statins exert so called pleiotropic activity mediated by intermediate products of the cholesterol pathway (compare Fig. 2A), where the primary target of statins, the HMG-CoA reductase (HMGCR) is a key enzyme. It may therefore be assumed that differences in cellular expression of this enzyme contribute to the observed cell specificity of atorvastatin. However, quantification of expression in HCASMC and HCAEC performing qRT-PCR as shown in Fig. 2C revealed comparable levels of HMGCR-mRNA in both cell types (HCASMC vs HCAEC as % of expression in human liver; mean ± SD; 237.7 ± 73.1% vs. 273.3 ± 60.8%). Similar results were obtained for protein expression of HMGCR performing Western blot analysis comparing whole protein lysates of HCASMC and HCAEC isolated from three individuals (Fig. 2D). In addition, the influence of intermediate products of the cholesterol pathway, namely geranylgeranyl pyrophosphate (GGPP), farnseyl pyrophosphate (FPP), and mevalonate was assessed in order to examine whether the observed atorvastatin induced antiproliferative effect is mediated by changes in this pathway. HCAEC and HCASMC were incubated with 1 μM or 5 μM atorvastatin, respectively; those concentrations were chosen to obtain a comparable effect in both cell types, thereby accounting for the observed differences in IC_{50} values (Fig. 1B). As shown in Fig. 2B, the antiproliferative activity of 1 μM atorvastatin in HCASMC (proliferation % of DMSO treated cells, mean ± SD; 51.6 ± 12.5%) was significantly reduced in presence of GGPP (87.6 ± 6.5%; $^*p < 0.05$, one-way ANOVA). However, no statistically significant impact was observed for FPP (51.3 ± 22.8%), mevalonate (61.0 ± 23.4%), or cholesterol (52.6 ± 16.5%). Similar results were observed in HCAEC treated with 5 μM of atorvastatin to also induce cytostatic effects in those cells.

3.3. Uptake of atorvastatin in HCASMC and HCAEC

To test whether the observed cell specificity of atorvastatin involves differences in cellular accumulation, the uptake of [^{3}H]-atorvastatin or its pharmacological inactive metabolite [^{3}H]-atorvastatin lactone in HCASMC and HCAEC was compared.

As shown in Fig. 3A, HCASMC exert a 2-fold higher cellular uptake of atorvastatin (mean ± SD uptake in fmol/μg protein; 1.04 ± 0.08; $^*p < 0.05$, Student's *t*-test) compared to HCAEC (0.49 ± 0.23), supporting the notion that pharmacokinetic properties might be a determinant of cell specificity. No such effect was observed for the atorvastatin metabolite. Indeed, even if the accumulating total amount of atorvastatin lactone was higher no difference was observed between both cell types (HCASMC 3.48 ± 0.76; HCAEC 2.27 ± 1.74).

3.4. Expression of OATP2B1 in HCASMC and HCAEC

One mechanism contributing to changes in drug distribution is the expression of drug transporters. One uptake transporter previously reported to transport atorvastatin is the organic anion transporting polypeptide OATP2B1 [24]. In accordance with a potential role of OATP2B1 in atorvastatin's cell specificity are our findings obtained using MDCKII-OATP2B1 cells, where uptake of [^{3}H]-estrone 3-sulfate (E_1S) in presence of 10 μM atorvastatin was significantly inhibited (4.5 ± 1.4%). Importantly, inhibition by cerivastatin (69.1 ± 12.0%) or simvastatin (56.7 ± 4.3%) was much lower, while no inhibitory potency was observed for mevastatin or pravastatin (Fig. 5A). In addition, densitometry of Western blot signals detected for OATP2B1 using protein lysates isolated from four individuals of both HCASMC and HCAEC revealed significantly higher expression in HCASMC compared to HCAEC (mean ± SD OATP2B1/β-actin; HCASMC 0.92 ± 0.29 vs. HCAEC 0.40 ± 0.25,$^*p < 0.05$, Student's *t*-test) (Fig. 3B and C).

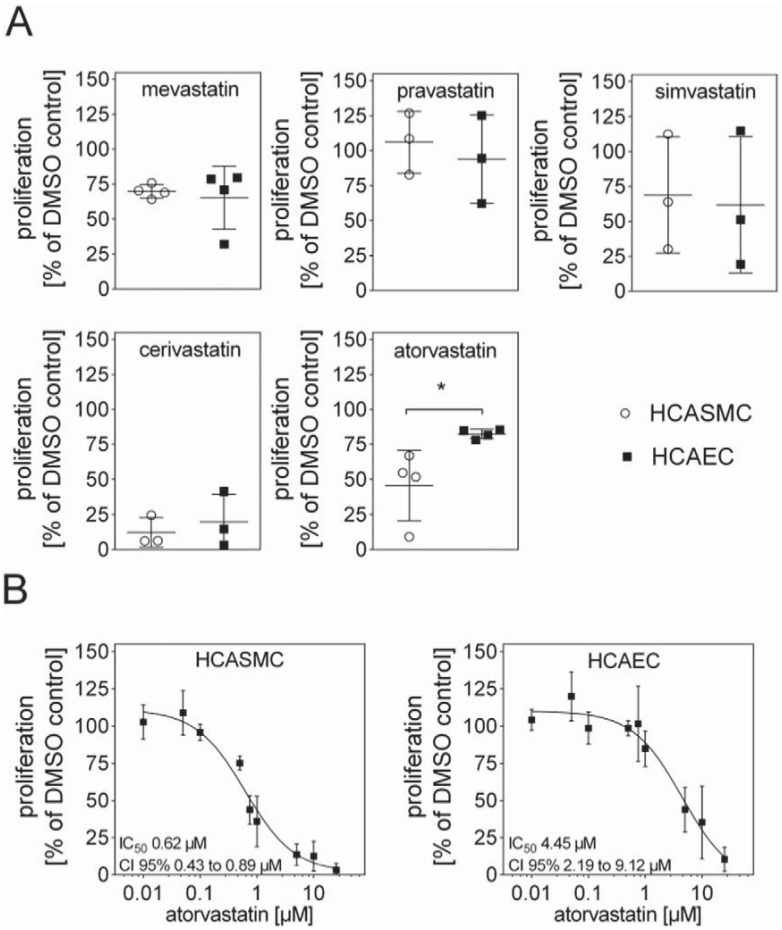

Fig. 1. Impact of statins on cell proliferation. (A) HCASMC and HCAEC were incubated with statins (each 1 µM) for 48 h and proliferation was determined by colorimetric BrdU ELISA. Mean ± SD, n = at least 3 experiments in triplicates, *p < 0.05, Student's *t*-test. (B) HCASMC and HCAEC were incubated with increasing concentration of atorvastatin (0.01–25 µM) 48 h prior to the determination of cellular proliferation status. IC_{50} values were calculated using GraphPad Prism Software. Mean ± SD in a semi logarithmic plot; n = 5.

3.5. Adenoviral-induced overexpression of OATP2B1 and its effect on atorvastatin-induced antiproliferation

Hitherto our data support the idea that OATP2B1 may be involved in differences in cellular accumulation and therefore pharmacological activity of atorvastatin. To support this hypothesis we tested whether enhanced expression of the transporter mediated by adenoviral infection may result in increased antiproliferative activity of this compound. Functionality of the transporter after infection of HCASMC was verified showing higher uptake of the well-known OATP2B1 substrate [24], estrone-3-sulfate (E_1S) as shown in Fig. 4A. In addition, proliferation assays demonstrated higher antiproliferative activity of atorvastatin in HCASMC overexpressing OATP2B1, resulting in a 10-fold lower IC_{50} value comparing cells infected with Ad-OATP2B1 (0.14 µM; CI 95% 0.06–0.28 µM) or the control virus Ad-lacZ (1.44 µM; CI 95% 0.58–3.56 µM). Similar results were obtained infecting endothelial cells with Ad-OATP2B1 (data not shown).

3.6. Teniposide as a candidate for cell-specific antiproliferative activity in HCASMC

Assuming that endogenous expression of OATP2B1 is a determinant of drug effects in HCASMC substrates of this transporter should exert enhanced cell specificity. One possibility to identify potential substrates is the screening for inhibition of uptake of a model substrate using overexpressing cell lines. Accordingly we used MDCK II cells overexpressing OATP2B1 to test the impact of a variety of clinically used cytostatic agents on the uptake of the model substrate estrone-3-sulfate (E_1S). The screening revealed melphalan (1.90-fold), paclitaxel (1.85-fold), and teniposide (5.63-fold) as inhibitors of OATP2B1 mediated E_1S-uptake (compare Fig. 5C).

In evidence that teniposide may be a substrate of OATP2B1 we assessed the influence of this compound on arterial cells, demonstrating that teniposide is a potent inhibitor of HCASMC proliferation (mean ± SD; 66.1 ± 10.8%) with little effect on HCAEC (93.9 ± 8.6%; p < 0.05, Student's *t*-test) as shown in Fig. 6B. The role of OATP2B1 in pharmacokinetics of teniposide was further supported testing the influence of heterologous expressed OATP2B1. As shown in Fig. 6D, the incubation with 100 nM teniposide in presence of OATP2B1 led to significantly enhanced effects on proliferation compared to control infected cells (Ad-lacZ vs. Ad-OATP2B1; mean ± SD in %; 91.78 ± 9.12% vs. 71.99 ± 13.57%, *p < 0.05, Student's *t*-test; n = 5) that is in accordance with data from uptake studies showing a significant inhibition of E_1S by teniposide in presence of OATP2B1 (73.11 ± 7.23% compared to DMSO treated cells, *p < 0.05, one-way ANOVA).

4. Discussion

In this study we provide evidence that endogenous expression of the uptake transporter OATP2B1 in human coronary smooth muscle cells

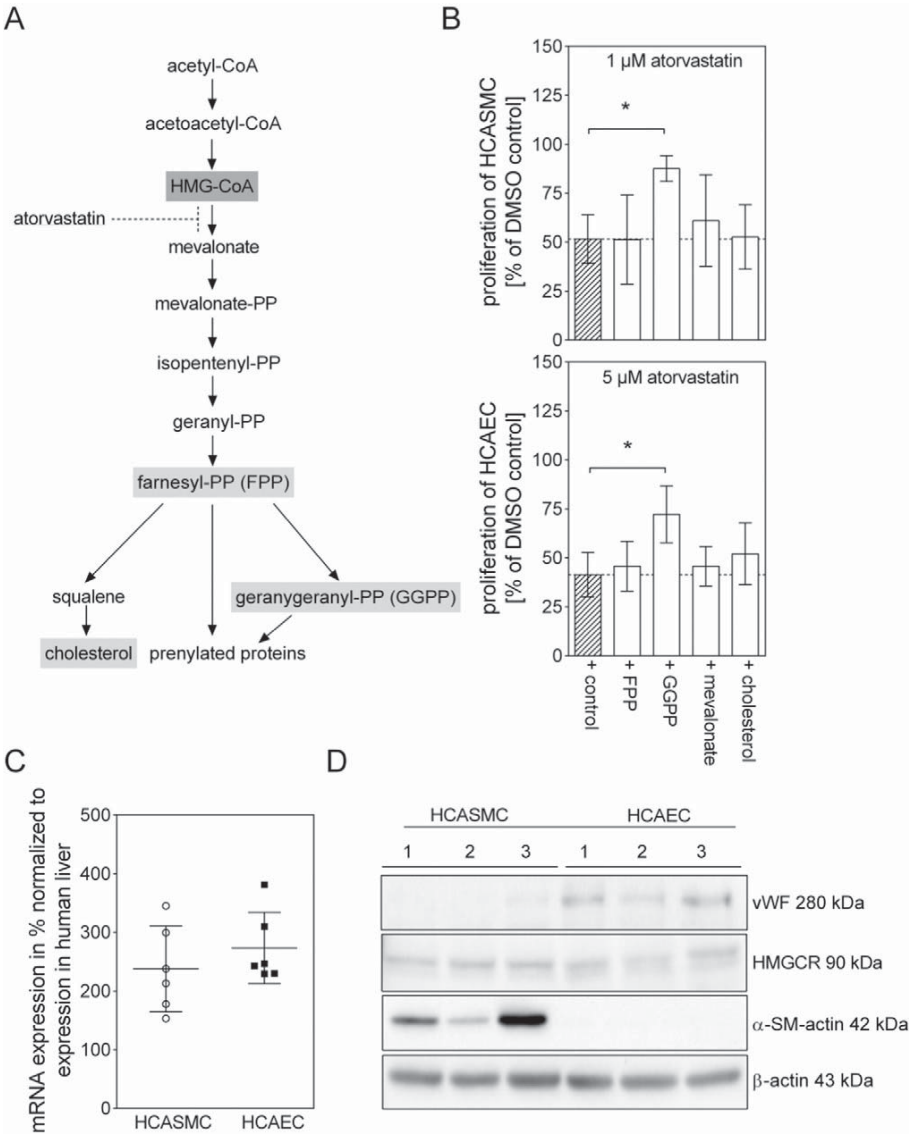

Fig. 2. Inhibition of HMG-CoA reductase (HMGCR) by atorvastatin. (A) Mammalian cell mevalonate pathway ending in cholesterol biosynthesis adapted from Corsini et al. [26]. (B) HCASMC or HCAEC of three individuals were incubated with 1 μM or 5 μM atorvastatin, respectively, in presence of FPP, GGPP, mevalonate, and cholesterol (each 10 μM) and proliferation was determined by BrdU ELISA. Mean ± SD, n = 5, *p < 0.01, one-way ANOVA with Dunnett's post-test compared to atorvastatin treated cells only. (C) Expression of HMGCR in HCASMC and HCAEC was detected using TaqMan® gene expression assays. Data are shown as 2ΔΔCt normalized to 18S rRNA and GAPDH in % compared to expression in human liver. (D) Protein expression of HMGCR, vWF, α-SM-actin, and β-actin was determined using whole cell lysate each (15 μg) from three different individuals of both HCASMC and HCAEC. Luminescence was digitalized and densitometrically analyzed.

(HCASMC) is a determinant of pharmacokinetics in the microcompartment of the vessel wall, resulting in enhanced cell-selectivity thereby influencing efficacy of substrate drugs. Indeed, testing the antiproliferative activity of atorvastatin revealed a significantly higher impact on smooth muscle cell proliferation compared to that of endothelial cells. In accordance with previous findings where atorvastatin was described as a high-affinity substrate of OATP2B1 [24], we show that the cell-specific activity of atorvastatin is associated with enhanced cellular accumulation of this compound, which can be explained in part by a higher expression of OATP2B1 in smooth muscle cells. Adenovirus-driven overexpression of OATP2B1 in HCASMC led to even higher sensitivity to atorvastatin; thereby further supporting the notion that cellular uptake influences pharmacodynamics in this microcompartment. Using OATP2B1 transport activity as a screening approach helped to identify compounds with smooth muscle cell specific activity in vitro, thereby introducing a preclinical tool for the identification of compounds with smooth muscle cell enhanced activity.

From a pharmacological point of few it is one of the major obstacles for the development of DES to identify compounds specifically targeting smooth muscle cells without influencing endothelialization of the stent struts. This is in part based on the localization of target and non-target cells in immediate vicinity of the drug delivery device and the use of compounds with high passive permeability. One of the approaches to enhance cell targeting may be the restricted loading on the drug delivery device, as previously reported, showing dual loading of stents with atorvastatin on the luminal side and sirolimus on the abluminal side [27]. In addition, derivatives of the lead compound sirolimus and their influence on smooth muscle cell proliferation and viability have been tested, showing a pronounced impact on proliferation without affecting cell viability compared to cells treated with

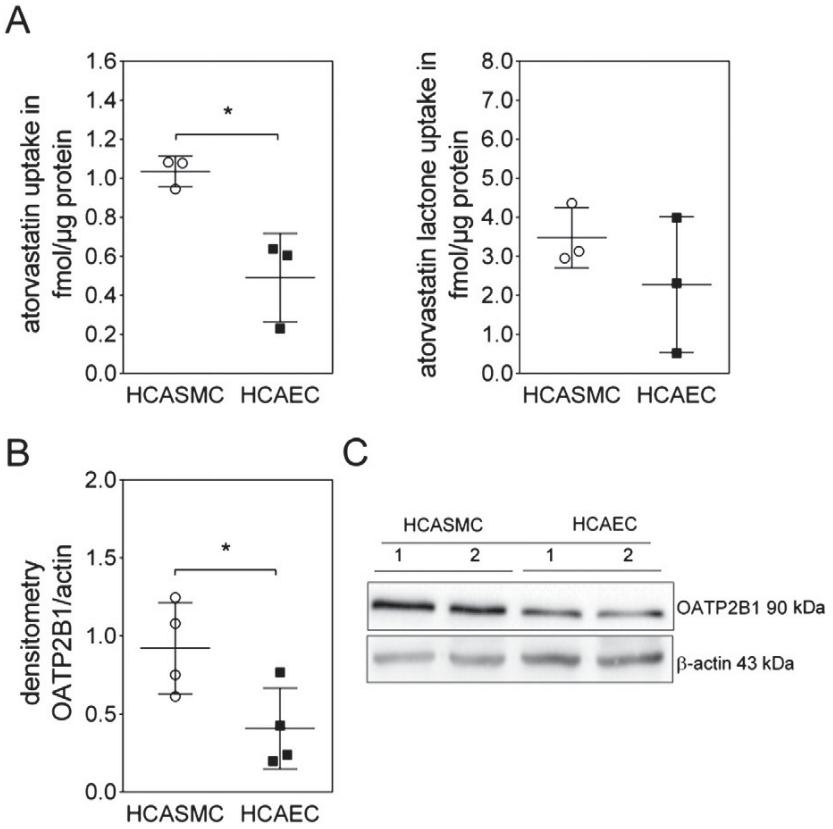

Fig. 3. Uptake of atorvastatin and atorvastatin lactone and expression of OATP2B1. (A) Uptake of [^{3}H]-atorvastatin acid and [^{3}H]-atorvastatin lactone in HCASMC and HCAEC was determined after 5 min incubation in naïve cells. Data are expressed as fmol/µg protein. Mean ± SD, n = 3 in triplicates, *$p < 0.05$, Student's *t*-test. (B, C) Protein expression of OATP2B1 and β-actin as loading control in HCASMC and HCAEC was determined by Western blot analysis (B) using whole cell lysates of two individuals (each 10 µg) and densitometry (C) was conducted using Western blot signals from four different donors of both HCASMC and HCAEC.

paclitaxel, a compound demonstrating high cytotoxic effects [28,29]. Particularly, everolimus and zotarolimus have been proved to be potent inhibitors of neointimal formation while supporting endothelial integrity, thereby translating in a reduction of in-stent restenosis and better re-endothelialization compared to first generation DES or BMS in vivo [30–33].

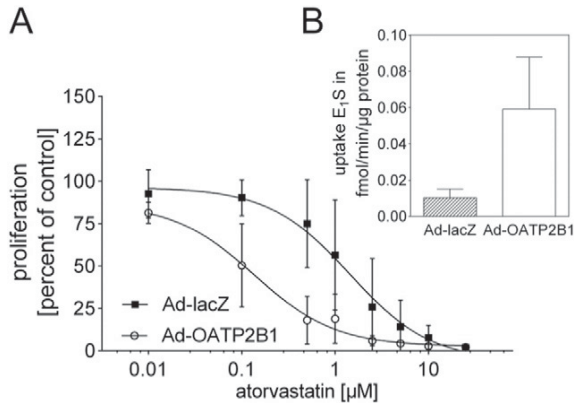

Fig. 4. Impact of adenoviral-driven overexpression of OATP2B1 in HCASMC. (A) HCASMC were incubated with increasing concentration of atorvastatin (0.01–25 µM) 48 h after infection with 50 pfu/cell Ad-OATP2B1 or Ad-lacZ. Proliferation status was determined by BrdU ELISA 48 h after treatment. Mean ± SD, n = 3 conducted in triplicates from one individual. (B) Uptake of 1 µM estrone-3-sulfate supplemented with [^{3}H]-E_1S in HCASMC was measured 48 h after infection with 50 pfu/cell Ad-OATP2B1 or Ad-lacZ. Data are presented as fmol/min/µg protein. Mean ± SD, n = 3 conducted in triplicates from one individual.

Understanding cellular mechanisms influencing pharmacodynamics and -kinetics in the microcompartment of the vessel wall may significantly contribute to the identification of novel compounds with improved clinical outcome. One class of substances which has been reported to influence the clinical outcome of patients undergoing percutaneous intervention with stent deployment is the class of statins. The observation that statins orally administered [17,34] reduce the risk of restenosis was basis for subsequent preclinical studies finally demonstrating that cerivastatin-eluting stents implanted in arteries of pigs reduced neointimal hyperplasia without impairing endothelial cell integrity [35]. Even if cerivastatin had been reported to exert a higher effect on smooth muscle cell proliferation in nanomolar concentrations [21] our experiments failed to show this effect in presence of higher concentrations using human cells. However, in our hands especially atorvastatin exerted enhanced antiproliferative potency in HCASMC compared to HCAEC. Those findings are supported by a previous report of Korybalska et al. showing that atorvastatin did not impair endothelial wound healing in an in vitro model of vascular injury [36]. Considering that there was no difference in expression of the primary drug target HMG-CoA reductase comparing HCASMC and HCAEC we first focused on differences resulting from the pleiotropic activity of statins. Indeed, beside their lipid-lowering effects, this drug class exhibits anti-inflammatory, antiproliferative and anti-thrombotic activity [37,38]. It is well known that HMG-CoA reductase activity is essential for proliferation of growth-stimulated cells [39]. Accordingly, statins inhibit the formation of the isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)

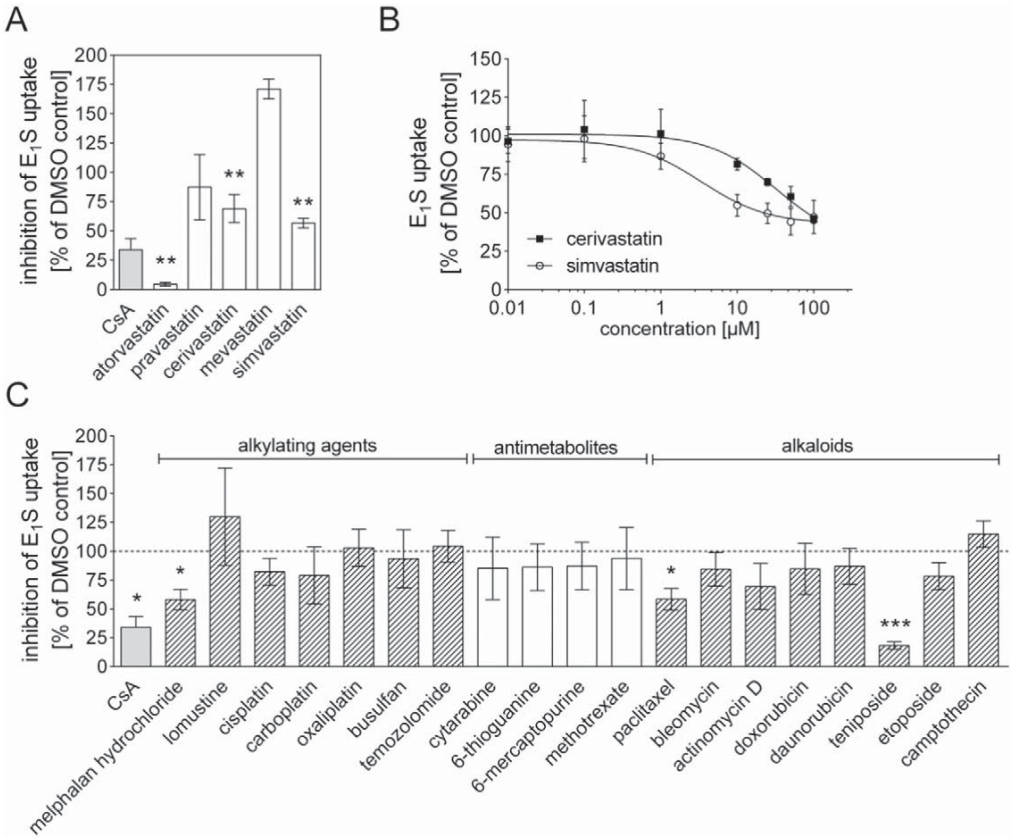

Fig. 5. Screening of cytotoxic drugs on OATP2B1-overexpressing MDCK II cells. (A, C) Cells were incubated for 10 min with [^{3}H]-E_1S in the presence or absence of cyclosporin A (CsA), or different cytostatic drugs (each 10 μM). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way ANOVA). (B) Cells were incubated for 10 min with [^{3}H]-E_1S in the presence of simvastatin or cerivastatin (0.01–100 μM). Graphs are presented as nonlinear regression. Data are presented as mean ± SD, n = 3 experiments conducted in triplicates.

thereby modulating the GTPase activity of members of the Ras and Rho family [40,41]. Particularly, inhibition of the RhoA geranylgeranylation has been shown to result in an accumulation of inactive RhoA in the cytoplasm, which in turn increases the expression of the cell-cycle inhibitor $p27^{kip1}$ resulting in a cell-cycle arrest in smooth muscle cells [37,42]. In our study we observed a reversal of the atorvastatin-induced antiproliferation by co-incubation with GGPP in HCASMC and HCAEC, whereas no such effect was observed by incubation with FPP which has been demonstrated before in studies showing that FPP did not influence the statin-induced cellular effects [43–45]. Farnesyl pyrophosphate is a precursor for synthesis of dolichols, cholesterol, or GGPP and it mediates the farnesylation of proteins such as Ras, the latter not being associated with cell proliferation and cell cycle progression. Due to this it is obviously that FPP in a concentration of 10 μM will not inhibit the atorvastatin-induced antiproliferation. It also explains why mevalonate did not diminish the effect of atorvastatin as mevalonate is an upstream molecule of FPP. The antiproliferative action of atorvastatin was furthermore not inhibited by cholesterol as demonstrated by Porter et al. showing that proliferation of cells is independently of cholesterol-synthesis [46]. However, our data are in line with different studies comparing the influence of statins on endothelial and smooth muscle cell viability, proliferation, and migration [21,36,47].

Although the influence of statins on cell-cycle control and modulation of signal cascades has been demonstrated, the question whether the activity of statins is mediated by pharmacokinetics in the microcompartment of the vessel wall remained unanswered. It is known that cellular processes such as the uptake and metabolism of compounds modulate intracellular drug effects. The major mechanism of cellular uptake is passive diffusion. This mechanism is modulated by compound properties such as lipophilicity, molecular weight, and H-bond acceptance to mention some of them [48]. Comparing the ability of statins passing membranes under physiological conditions, it has been shown that simvastatin and cerivastatin have the highest lipophilicity, while pravastatin exhibits the lowest of the herein tested statins [49]. However, considering that lipophilicity translates in high membrane permeability by passive diffusion and thereby in enhanced intracellular accumulation, then this substance property may not only explain the observed enhanced antiproliferative activity of simvastatin and cerivastatin compared to pravastatin, but also the lack of cell specificity observed for those substances.

Another mechanism governing transmembrane transport is the expression and function of drug transporters. Especially uptake transporters, facilitating cellular entry and thereby intracellular activity, may alter cell specificity of their substrate drug, when differentially expressed in target and non-target cells. One of the uptake transporters known to be involved in the pharmacokinetics of statins is OATP2B1, which apart from its expression in hepatocytes and enterocytes has been detected in human platelets and cardiac endothelial cells [24,50]. The expression function of this transporter in myocytes is assumed to influence drug effects such as the statin-induced myotoxicity — a side effect associated with accumulation of statins in skeletal muscle [25].

The observed higher accumulation of atorvastatin in HCASMC can be explained by our finding that smooth muscle cells exhibit higher endogenous OATP2B1 expression compared to endothelial cells. From a

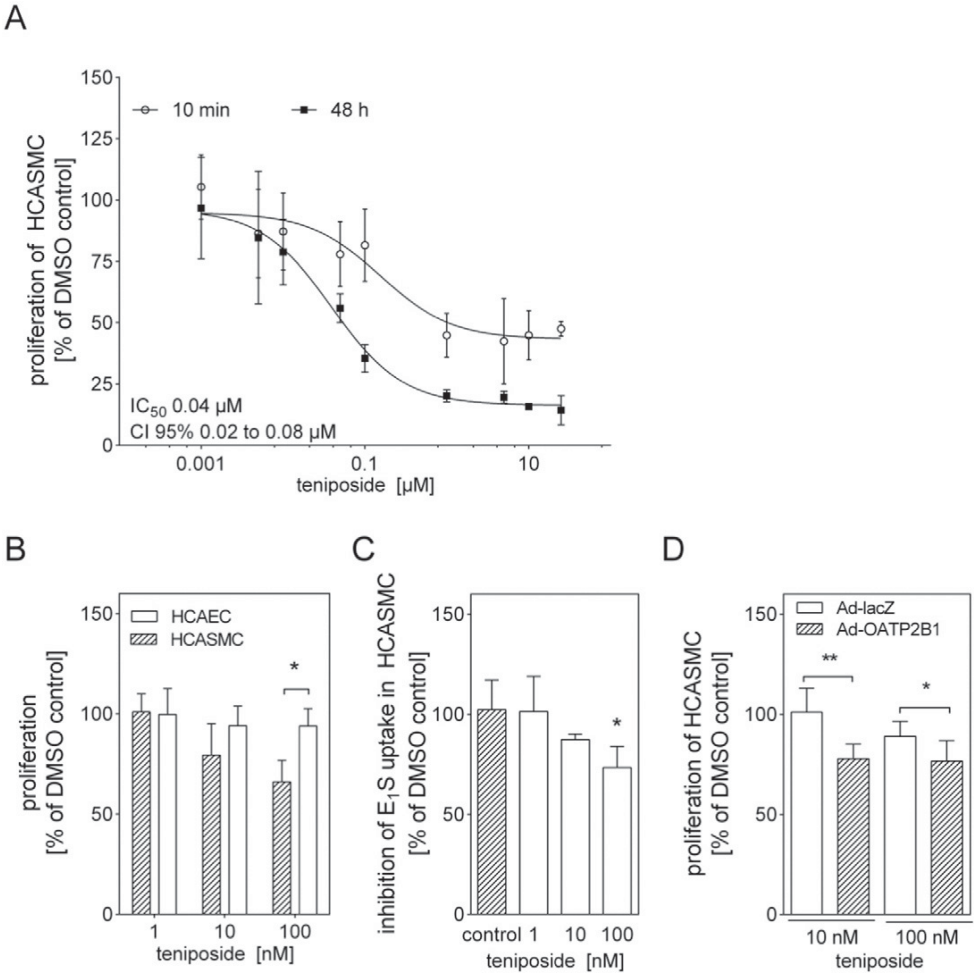

Fig. 6. Influence of teniposide on cell proliferation in naïve and OATP2B1 infected cells. (A) Naïve HCASMC were incubated 10 min or 48 h with increasing concentration of teniposide (1 nM–100 µM) and proliferation was determined by BrdU ELISA. IC_{50} value (48 h) was calculated by non-linear regression. (B) Proliferation was determined by BrdU ELISA 48 h after incubating naïve HCASMC and HCAEC with 1, 10, or 100 nM for 10 min (*$p < 0.05$, Student's *t*-test). (C) Uptake of [^{3}H]-E_1S in presence of teniposide (1, 10, 100 nM) was determined in HCASMC 48 h after infection with 50 pfu/cell Ad-OATP2B1 (*$p < 0.05$ One-way ANOVA). (D) Cell proliferation was determined by BrdU ELISA 48 h after treatment. HCASMC were incubated for 10 min with 10, and 100 nM teniposide (*$p < 0.05$, Student's *t*-test Ad-lacZ vs Ad-OATP2B1). Data are presented as mean ± SD of at least n = 3 experiments conducted in triplicates.

clinical perspective atorvastatin acid is administered as the calcium salt. This pharmacological active derivative is spontaneously and enzymatically catalyzed converted to its inactive lactone exhibiting higher lipophilicity compared to the educt [51,52]. Even if taken up in a higher concentration, no difference was observed comparing accumulation of the lactone in HCAEC and HCASMC. Other known statin uptake transporters including OATP1B1 and OATP1B3 [53] were not detected in coronary artery cells (data not shown). Our assumption is further supported by enhanced cytotoxicity and transport of atorvastatin in cells overexpressing OATP2B1. We did not test the influence of inhibition or knock-down of endogenous OATP2B1 on atorvastatin efficacy which is a limitation of our study. With respect to all herein reported statins we also observed inhibition of OATP2B1 activity by cerivastatin and simvastatin. However, the effective concentration of both statins was higher than 50 µM, indicating that the observed antiproliferative effect of those statins is more likely a result of passive diffusion than of active transport by OATP2B1. Interestingly, mevastatin stimulated the E_1S uptake of OATP2B1, which may be explained by different affinity sites for E_1S [54]. Nevertheless the moderate lipophilicity of mevastatin may contribute to the detected unspecific cellular effects.

Taken together the data suggest that endogenous expression of OATP2B1 is a determinant of cellular uptake and may therefore be a preclinical tool to identify substances with enhanced cellular accumulation in muscle cells. An often used in vitro method to identify competitive inhibitors and thereby substrates are transport inhibition studies using overexpressing cell lines and radiolabeled model substrates. Using MDCK II cells overexpressing OATP2B1 and [^{3}H]-estrone-3-sulfate we screened a variety of cytostatics for their interaction with OATP2B1. This approach revealed teniposide as a potent inhibitor of OATP2B1 in vitro. In order provide evidence, that teniposide is not only an inhibitor, but also a substrate of OATP2B1 we performed proliferation assays thereby assessing an indirect measure of enhanced cellular accumulation. From a pharmacokinetic point of view compared to atorvastatin and paclitaxel, teniposide is a moderate lipophilic substance suggesting a higher influence of OATP2B1 on the observed antiproliferative activity. In accordance with the assumption that enhanced intracellular accumulation is linked to a better efficacy of a substrate drug, we detected a significantly higher impact on cell proliferation by teniposide in presence of artificial induced overexpression of OATP2B1 in smooth muscle cells. Importantly, comparing the effect of teniposide on proliferation of naïve

cells revealed higher sensitivity of HCASMC to teniposide compared to HCAEC thereby further supporting our hypothesis that OATP2B1 modulates cellular uptake and pharmacological activity.

5. Conclusion

We identified the HMG-CoA reductase inhibitor atorvastatin as a candidate drug to be used on drug-eluting stents, which exhibits higher activity in smooth muscle cells. Based on the herein reported data the enhanced muscle cell targeting is associated with higher endogenous expression of the atorvastatin uptake transporter OATP2B1 in these cells. As a determinant of pharmacokinetics of compounds used in the microcompartment of the vessel wall this transporter may therefore be a preclinical tool suitable to identify substances for the use on drug delivery devices, as shown for teniposide.

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Author declaration

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. We further confirm that there is no conflict of interest.

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3.4. Pimecrolimus increases the expression of interferon-inducible genes that modulate human coronary artery cells proliferation

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Contribution J. Hussner: author and performance of all experiments

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Pimecrolimus increases the expression of interferon-inducible genes that modulate human coronary artery cells proliferation

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ABSTRACT

The pharmacodynamics of the loaded compounds defines clinical failure or success of a drug-eluting device. Various limus derivatives have entered clinics due to the observed positive outcome after stent implantation, which is explained by their antiproliferative activity resulting from inhibition of the cytosolic immunophilin FK506-binding protein 12. Although pimecrolimus also binds to this protein, pimecrolimus-eluting stents failed in clinics. However, despite its impact on T lymphocytes little is known about the pharmacodynamics of pimecrolimus in cultured human coronary artery cells. We were able to show that pimecrolimus exerts antiproliferative activity in human smooth muscle and endothelial cells. Furthermore in those cells pimecrolimus induced transcription of interferon-inducible genes which in part are known to modulate cell proliferation. Modulation of gene expression may be part of an interaction between calcineurin, the downstream target of the pimecrolimus/FK506-binding protein 12-complex, and the toll-like receptor 4. In accordance are our findings showing that silencing of toll-like receptor 4 by siRNA in A549 a lung carcinoma cell line reduced the activation of interferon-inducible genes upon pimecrolimus treatment in those cells. Based on our findings we hypothesize that calcineurin inhibition may induce the toll-like receptor 4 mediated activation of type I interferon signaling finally inducing the observed effect in endothelial and smooth muscle cells. The crosstalk of interferon and toll-like receptor signaling may be a molecular mechanism that contributed to the failure of pimecrolimus-eluting stents in humans.

1. Introduction

The natural occurring cytotoxic macrolide sirolimus (rapamycin) and the anti-cancer drug paclitaxel were the first compounds used on drug-eluting stents resulting in a significant reduction of in-stent restenosis rates after stent implantation (Garg and Serruys, 2010; Moses et al., 2003; Stone et al., 2007). Supported by experimental studies in animals and humans identifying inflammation as one of the major driving forces for development of in-stent restenosis after bare metal stent implantation (Kornowski et al., 1998; Okamoto et al., 2001), it became evident that the clinical success of sirolimus is not only based on the suppression of smooth muscle cell proliferation, but also on the reduction of inflammatory processes in the vessel wall (Guerra et al., 2014). Sirolimus is assumed to bind to the cytosolic immunophilin FK506-binding protein 12 (FKBP12) thereby inhibiting the mammalian target of rapamycin (mTOR) and preventing the degradation of the cell-cycle regulator $p27^{kip}$ which in turn regulates migration and proliferation of vascular smooth muscle cells (Martin et al., 2004).

On the basis of the immunosuppressive function sirolimus was origin for further pharmacological developments leading to the evaluation of a variety of limus drugs to be loaded on drug-eluting stents. While everolimus and zotarolimus have been shown to be clinical efficient and safe, other limus compounds failed during development (Barbash et al., 2014; Chevalier et al., 2013; Tamburino et al., 2009). One of those is the macrolide derivative pimecrolimus (SDZ ASM981). This molecule has been first described as potent anti-inflammatory and immunomodulatory drug (Mrowietz et al., 1998) and was finally approved for the topical treatment of exfoliative skin diseases (Elidel® cream, Novartis Pharmaceutical Corp., East Hanover, N.J., USA) (Luger et al., 2001).

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0014-2999/

Regarding the use of this molecule in cardiovascular diseases preclinical studies using pimecrolimus-eluting stents in a porcine coronary model revealed the promising reduction of neointima formation accompanied by complete endothelialization of the stent struts (Berg et al., 2007; Waksman et al., 2007). Based on those preclinical findings these stents were assumed to overcome the problem of late stent thrombosis, an adverse event commonly observed with the first generation DES. Subsequently, Ormiston and colleagues performed a clinical study showing excessive neointimal formation in humans, which has been confirmed by a prospective multicenter study (Ormiston et al., 2009; Verheye et al., 2009a). Taken together, pimecrolimus-eluting stents failed to show clinical advantage even if preclinical data were more than promising. Even though the controversy of preclinical and clinical assessments may certainly be explained by species differences in the role of inflammation in neointima formation little is known about pharmacodynamics of pimecrolimus in cells of the human coronary artery, as commented by Vorpahl et al. (2009).

Hence, the discrepancy of previous observations was basis of the herein reported study investigating the cellular effects associated with pimecrolimus treatment. Although pimecrolimus induced the expression of interferon-inducible genes and slightly inhibited the proliferation of smooth muscle and endothelial cells as observed using human coronary cells these effects may not be sufficient to overcome the strong proliferative stimuli of stent implantation.

2. Materials and methods

2.1. Materials

Human recombinant interferon-alpha2a (IFN-α) and interferon-beta 1a (IFN-β) were purchased from Biomol GmbH (Hamburg, Germany) and Lucerna-Chem AG (Luzern, Switzerland), respectively. Pimecrolimus was purchased from BioVision Inc. (Milpitas, CA, USA), cyclosporin A and ruxolitinib were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. Cell culture

Human coronary artery endothelial (HCAEC) and human coronary artery smooth muscle cells (HCASMC) derived from different donors were obtained from PromoCell GmbH (Heidelberg, Germany) and cultured in their optimized media namely Smooth Muscle Cell Growth Medium 2 and Endothelial Cell Growth Medium MV (PromoCell). To compare the impact of pimecrolimus on cell proliferation and gene expression we used cells of from 1 up to 4 individuals as indicated in the result section. For the mechanistic studies we used cells isolated from one individual. The human lung carcinoma cell line A549 (ATCC®, CCL-185™, Washington, USA) was cultured in DMEM containing 10% FCS and 1% GlutaMAX™ (Gibco, Life Technologies, Carlsbad, CA, USA). All experiments were conducted in technical triplicates and repeated in three independent experiments. Cells were cultivated in a humidified atmosphere at 37 °C supplemented with 5% CO_2.

2.3. Cell viability assay and bromodeoxyuridine (BrdU) cell proliferation assay

Cell viability and cell proliferation was determined in one *in vitro* assay. At first, cells were seeded in 96-well plates at a density of 5×10^3 cells/ well. After 24 h cultivation with the respective growth media cells were starved for 24 h with basal media containing only 0.5% FCS or 0.05% FCS for HCAEC or HCASMC, respectively. After treatment with the compounds for 24 h BrdU labeling solution was added to the medium and cells were cultured for additional 24 h. Prior to the detection of the proliferation status, cell viability was determined using the Fluorometric Cell Viability Kit I obtained from Vitaris AG (Baar, Switzerland). The reduction of the cell-permeable compound resazurin to the fluorescent red dye resorufin is an indicator for the metabolic activity of cells. After determination of viability, cells were washed with PBS and proliferation was determined using the commercially available colorimetric BrdU Cell Proliferation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Fluorescence or luminescence were detected using the microplate reader Infinite® 200 Pro (Tecan, Männedorf, Switzerland). Cells treated with the solvent served as control. Data are presented as percent of control.

2.4. Interferon beta ELISA

The secreted amount of biological active human interferon beta (IFN-β) in supernatants of HCAECs treated for 48 h with 10 μM pimecrolimus or solvent control (DMSO) was determined using the Human Interferon-β ELISA Kit obtained from Life Technologies (Carlsbad, CA, USA). In brief, after treatment, the culture supernatant was collected and concentrated using Pierce Concentrator 10 K obtained from Life Technologies followed by quantification using the above mentioned ELISA according to the manufacturer's protocol. The reaction with the supplied color developer was stopped after 30 min and the absorbance at 450 nm was detected with the microplate reader Infinite® 200 Pro. The amount of human IFN-β in the supernatants was interpolated by linear curve fitting using the INF-β calibration curve (IU/ml). Secretion of IFN-β is given as fold of DMSO-treated cells.

2.5. Small interfering RNA (siRNA) transfection

Knockdown of toll-like receptor 4 (TLR4) with Silencer® Select siRNA (s14195, Ambion®, Life Technologies) was performed by reverse transfection using DharmaFect transfection reagent 1 (Thermo Fisher Scientific, Reinach, Switzerland). First, the mixture consisting of DharmaFECT 1 and TLR4-siRNA (final concentration of 25 pmol/ml) was prepared and added to each well of a 12-well plate. After incubation for 30 min at room temperature 1.5×10^5 cells/ well were seeded on top. In addition to TLR4-siRNA, cells were transfected with Silencer® Select GAPDH positive control siRNA or negative Control No.1 siRNA (Life Technologies). Forty-eight hours after transfection cells were treated with 10 μM pimecrolimus for further 48 h and RNA was isolated. Cells transfected with negative control siRNA served as reference and data are presented in % of DMSO-treated cells.

2.6. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from cells was isolated with the NucleoSpin® RNA (Macherey-Nagel, Düren, Germany) in accordance to the manufacturer's instructions. After reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) gene expression was carried out with the Viia7™ (Life Technologies). Therefore, TaqMan® gene expression assays from Life Technologies were used detecting interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*, Hs01675197_m1), interferon-induced protein with tetratricopeptide repeats 3 (*IFIT3*, Hs00155468_m1), 2′-5′-oligoadenylate synthetase 1 (*OAS1*, *IFI4*, Hs00973637_m1), interferon regulatory factor 9 (*IRF9*, Hs00196051_m1), toll-like receptor 4 (*TLR4*, Hs00152939_m1). The expression of 18 S rRNA

(4310893E) was used as endogenous controls. The relative expression ratio was determined by the ΔΔCt comparative method (Livak and Schmittgen, 2001).

2.7. Western blot analysis

Protein expression was determined in cells harvested in commercially available RIPA buffer supplemented with 1% protease inhibitor cocktail both obtained from Sigma-Aldrich. After quantification of protein content using the bicinchoninic acid assay (Pierce, Thermo Fisher Scientific) 10 μg of each sample were separated by SDS-PAGE and electro-transferred to nitrocellulose membranes using a Tank blotting system (Bio-Rad Laboratories AG, Cressier, Switzerland). Prior to overnight incubation with the primary antibody membranes were placed in 5% FCS in TBS-T (0.25 M Tris-base, 0.026 M KCl, 0.04% Tween 20) for 1 h. The antibodies used were anti-pSTAT1 (5167, Cell Signaling, Cambridge, UK), anti-STAT1 (sc-592) and anti-β-Actin (sc-1616) both from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA) diluted in an appropriate dilution in TBS-T supplemented with 0.5% BSA. After several washing steps with TBS-T membranes were incubated with the respective HRP-labeled secondary antibody for 1 h at room temperature and luminescence was visualized using the ECL Western blotting substrate (Pierce, Thermo Fisher Scientific Inc.). Signals were digitalized with the ChemiDoc XRS system (Bio-Rad Laboratories) and densitometry was conducted using Image Lab 4.1 software from Bio-Rad Laboratories.

2.8. Statistical analysis

Data are represented as mean ± standard deviations (S.D.) from at least three independent experiments conducted in technical replicates. Graphs and calculations were performed using GraphPad Prism© software (GraphPad Software, Inc., La Jolla, USA). Data were statistically analyzed using one-way ANOVA followed by Dunnetts post-hoc test to compare the mean values of more than two groups. Data from interferon beta ELISA were analyzed by Student's *t*-test to compare treated and non-treated cells. The results of qRT-PCR were analyzed a nonparametric test for not normally distributed samples. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Impact of pimecrolimus on cellular proliferation and viability

At first, four individuals of each, human coronary artery smooth muscle (HCASMC) and endothelial cells (HCAEC) were incubated with increasing concentrations of pimecrolimus to determine cellular behavior of human cardiovascular cells. As shown in Fig. 1A 48 h of pimecrolimus treatment inhibited the proliferation

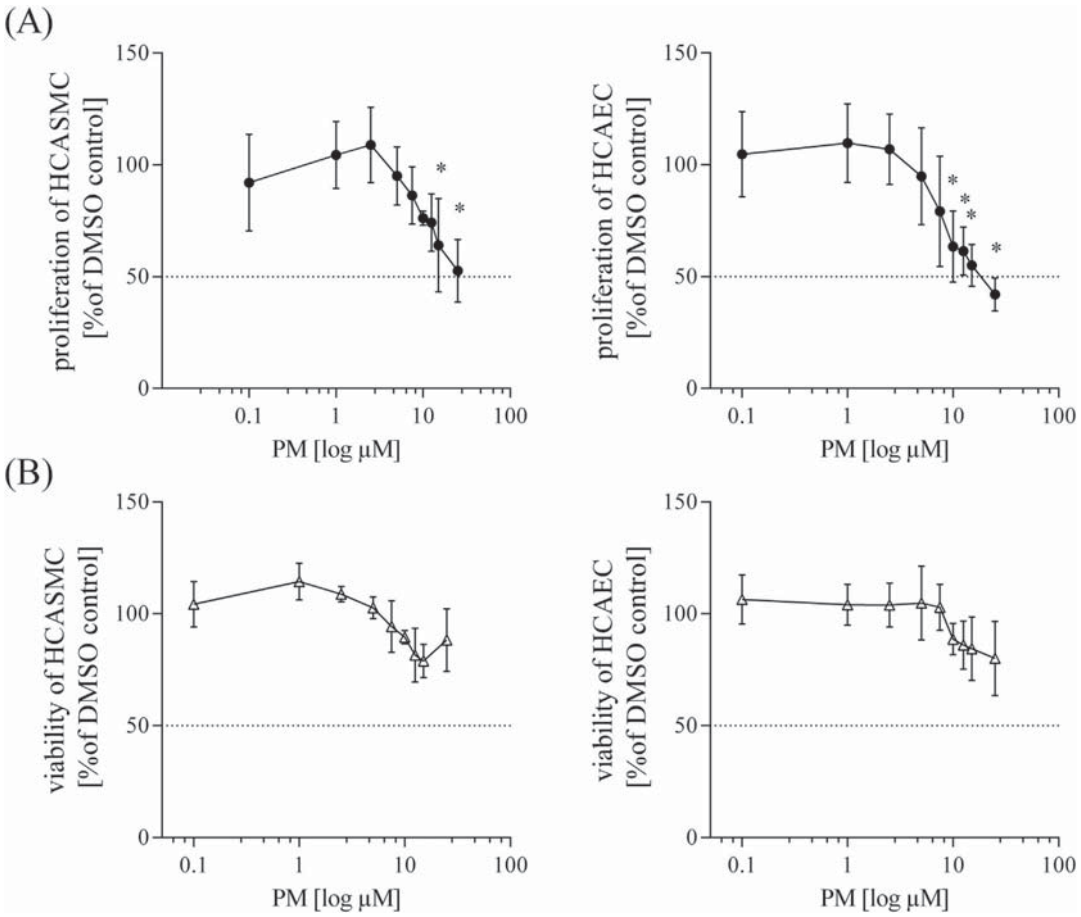

Fig. 1. Impact of pimecrolimus on cell behavior. Human coronary artery smooth muscle (HCASMC) and endothelial cells (HCAEC) of different individuals were incubated with pimecrolimus (PM, 0.1 μM–25 μM) for 48 h. **(A)** Cell proliferation or **(B)** viability was determined by BrdU ELISA or fluorometric resazurin, respectively. Data are presented as mean ± S.D. (log μM) in % of DMSO treated cells. n = 4 in triplicates, $^*P < 0.05$ One-way ANOVA PM *vs.* DMSO treated cells.

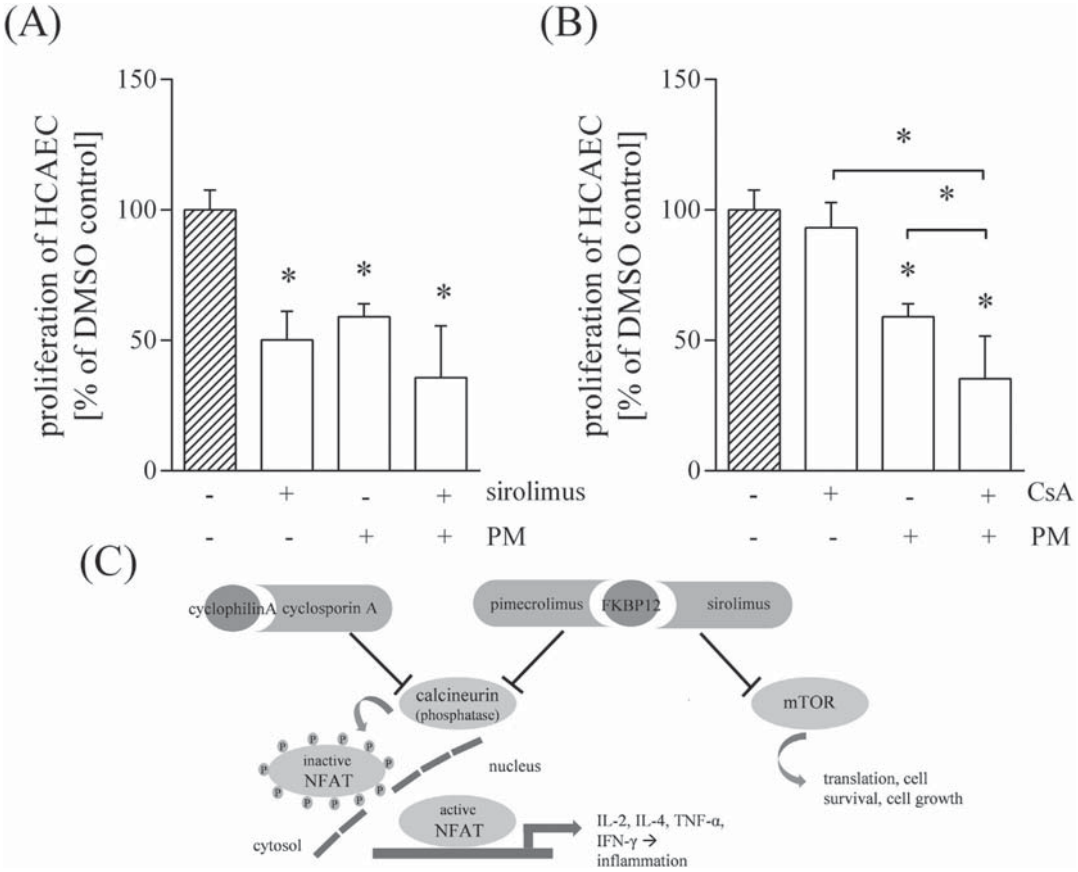

Fig. 2. Impact of immunophilin inhibitors. Human coronary endothelial cells (HCAEC) of one individual were incubated with 10 μM pimecrolimus (PM) in presence or absence of **(A)** 1 μM sirolimus or **(B)** 1 μM cyclosporin A (CsA) for 48 h. Proliferation status was assessed by BrdU ELISA. Mean ± S.D., n=3 in triplicates, $^*P < 0.05$ One-Way ANOVA. **(C)** Interaction of immunophilins cyclophilin A and FK506 binding protein 12 (FKBP12) with cyclosporin A, pimecrolimus or sirolimus and their subsequent inhibition of calcineurin or mamalian target of rapamycin (mTOR). NFAT (nuclear factor of transcription); interleukin (IL)-2 and 4; tumor necrose factor (TNF)-α; interferon (IFN)-γ.

of HCAEC or HCASMC at concentrations higher than 10 μM or 15 μM, respectively (mean in % of control ± S.D., HCASMC 64.0 ± 20.7%, HCAEC 63.4 ± 15.9%, $^* < 0.05$ one-way ANOVA). Due to solubility problems of the compound, an IC_{50} value was not determined. However, pimecrolimus did not influence cell viability (Fig. 1B). Taken together these observations suggested that pimecrolimus has a cytostatic effect on the cells occurring at relatively high concentrations.

3.2. Impact of pimecrolimus on calcineurin activation

Pimecrolimus and sirolimus bind to the immunophilin FKBP12 thereby modulating different downstream pathways. In order to determine which pathway contributes most likely to the pharmacodynamics of pimecrolimus in HCAEC we tested whether sirolimus or cyclosporine A, which binds the immunophilin cyclophilin A, are able to modulate the pimecrolimus effect. Therefore, one individual of HCAEC was incubated with pimecrolimus in presence of sirolimus or cyclosporine A. As shown in Fig. 2A sirolimus did not influence the antiproliferative activity of pimecrolimus (mean proliferation in % of control ± S.D., 59.1 ± 5.0%). However, concomitant treatment with the specific calcineurin inhibitor cyclosporine A, which itself did not influence proliferation of the cells (Fig. 2B; 93.2 ± 9.6%) significantly enhanced the reduction of cell proliferation observed for pimecrolimus (35.3 ± 16.5%; $^*P < 0.05$ one-way ANOVA). At this point we hypothesized due to enhanced antiproliferative activity in presence of cyclosporine A that inhibition of the calcineurin-calmodulin complex is at least in part playing a role in the cellular signaling of pimecrolimus.

3.3. Expression of genes from the interferon pathway upon pimecrolimus or sirolimus treatment

One of the signaling pathways modulated by the phosphatase activity of calcineurin is that of interferons (IFN) (Kang et al., 2007; Wang et al., 2012). Based on preliminary data of an RNA microarray (data not shown) comparing the gene expression pattern in pimecrolimus treated and non-treated HCAEC and HCASMC suggesting a modulation of type I interferon pathway, we determined the expression of target genes by real-time PCR (involved genes are depicted in Fig. 3). HCASMC and HCAEC, each cell type isolated from four individuals, were treated with 10 μM pimecrolimus for 48 h and the expression of known target genes namely *IFI4*, *IRF9*, *IFIT3*, and *IFIT4* was investigated in both cell types (Fig. 3A and B). Pimecrolimus enhanced the expression of the interferon-induced protein with tetratricopeptide repeats 1 and 3 (*IFIT1* and *IFIT3*) in HCAEC (mean of mRNA expression ± S.D.; 9.42 ± 4.13 and 3.68 ± 1.07, $^*P < 0.05$ Mann-Whitney test). Moreover, pimecrolimus induced the expression of interferon regulatory factor 9 (*IRF9*), and of 2′-5′-oligoadenylate synthetase (*IFI4*) (3.43 ± 2.21 and 3.55 ± 1.26, $^*P < 0.05$ Mann-Whitney test). Similar results

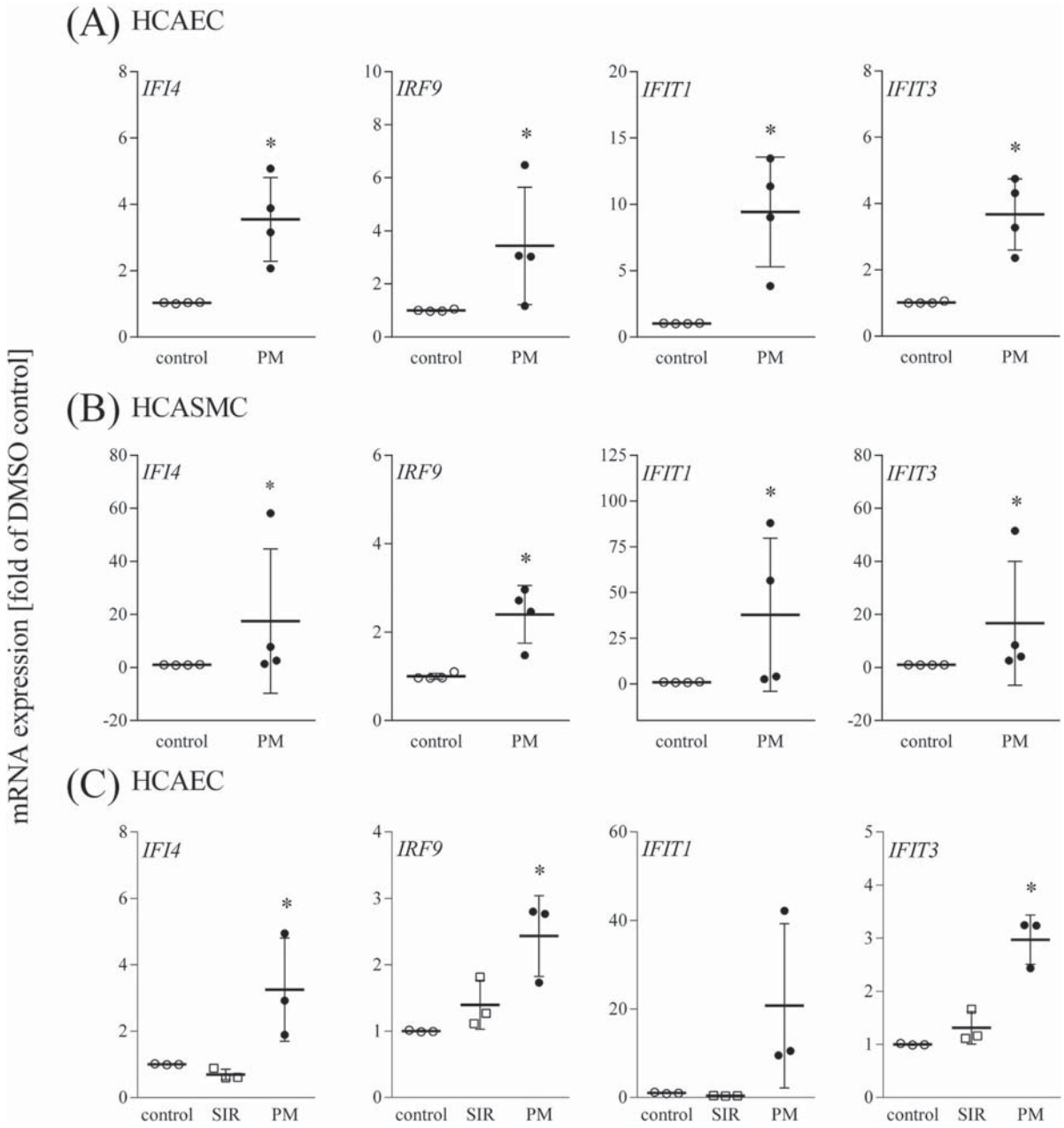

Fig. 3. Gene expression analysis upon compound treatment. **(A)** Human coronary artery endothelial cells (HCAEC) and **(B)** smooth muscle cells (HCASMC) isolated from four individuals each were incubated with DMSO or 10 μM pimecrolimus. Gene expression of *IFIT1* (interferon-induced protein with tetratricopeptide repeats 1), *IFIT3* (interferon-induced protein with tetratricopeptide repeats 3, *IRF9* (interferon regulatory factor 9), *IFI4* (2′-5′-oligoadenylate synthetase), and 18S rRNA was determined by qRT-PCR. Fold of mRNA expression is presented as single data points each representing the mean of one individual normalized to 18 S rRNA and related to the mean of the DMSO control using the comparative ΔΔCt-method. Mean ± S.D., n=4 in technical triplicates, $^{*}P < 0.05$ Mann-Whitney test pimecrolimus vs. DMSO. **(C)** Endothelial cells of one individual were incubated for 48 h with 1 μM sirolimus (SIR) or 10 μM pimecrolimus (PM) and gene expression of *IFIT1, IFIT3, IRF9, IFI4*, and 18S rRNA was determined and analyzed using the ΔΔCt-method. Data are presented as mean ± S.D., n=3 in triplicates, $^{*}P < 0.05$ Kruskal-Wallis test compound *vs*. DMSO.

were observed for HCASMC. The pronounced effect on gene expression by pimecrolimus even increased with higher concentration (data not shown). No such influence on expression was detected for sirolimus as determined in HCAEC of one individual (compare Fig. 3C).

3.4. Pimecrolimus induced secretion of IFN-β

Our data revealed induction of genes known to be associated with an activation of type I interferon receptors. In order to test whether the observed transcriptional regulation is associated with an increase in type I interferon secretion and thereby direct activation of the type I interferon receptor the amount of secreted IFN-β was quantified in the supernatant of cardiovascular cells exposed to pimecrolimus. As illustrated in Fig. 4A the immunosuppressant slightly increased the levels of INF-β released into the supernatant of HCAEC (INF-β fold increase as mean ± S.D., pimecrolimus vs. DMSO, 1.22 ± 0.05 *vs.* 1.00 ± 0.03, $^{*}P < 0.05$ Student's *t*-test). No such effect was observed for HCASMC.

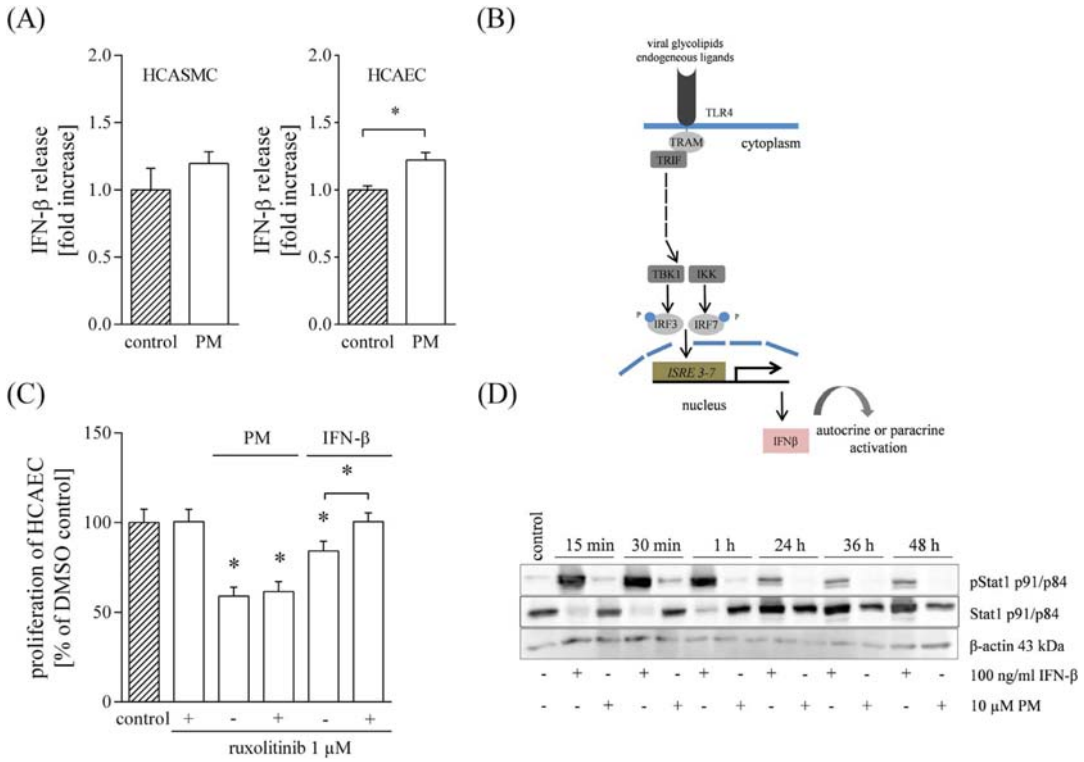

Fig. 4. Activation of type I IFN signaling pathway. (A) Human coronary artery endothelial cells (HCAEC) of one individual were incubated with 10 μM pimecrolimus (PM) for 48 h and the release of IFN-β in cell supernatant was quantified using a specific ELISA. Data are presented as fold increase of DMSO treated cells, mean ± S.D., n=3 in triplicates, $^{*}P < 0.05$ Student's t-test. **(B)** Activation of TLR4 cascade with subsequent IFN-β activation. **(C)** Endothelial cells of one individual were incubated with pimecrolimus (PM) or IFN-β in presence or absence of 1 μM ruxolitinib. Proliferation was determined after 48 h by BrdU ELISA. Mean ± S.D., n=3 in triplicates, $^{*}P < 0.05$ One-way ANOVA. **(D)** Endothelial cells of one individual were incubated with IFN-β or pimecrolimus (PM) for 15 min, washed with PBS and incubated for 48 h in growth medium. Protein expression of phospho signal transducers of activation (pSTAT1), STAT1, and β-actin was determined in whole protein lysates using specific antibodies.

3.5. Influence of type I interferons on cell proliferation

Treatment of HCAEC or HCASMC with IFN-β significantly inhibited the proliferation of those cells (Fig. 5B and C). In detail, HCASMC or HCAEC treated with 100 ng/ml exhibited a reduction of the proliferation to 32.9 ± 13.9% or 65.2 ± 18.8%, respectively, when compared to control treated cells ($^{*}P < 0.05$ One-way ANOVA). No such effect was detected using IFN-α. Importantly as observed for pimecrolimus, INF-β treatment did not influence cell viability still suggesting that the IFN-β pathway is part of the pimecrolimus-induced inhibition of HCASMC and HCAEC.

3.6. Activation of Jak/STAT signaling upon pimecrolimus or IFN-β treatment

Hitherto our data suggest that pimecrolimus induces target genes of type I interferons. Although this may be in part explained by the slight increase of INF-β secretion in presence of the immunosuppressant, the levels of INF-β detected in the supernatant were far below those we used to test the influence on the cellular behavior of cardiovascular cells. In order to test whether pimecrolimus directly activates the type I interferon receptor complex activation of intracellular signaling molecules was assessed.

Normally, binding of type I IFNs to the receptor activates an intracellular signaling cascade that finally activates the transcription of IFN target genes (compare Fig. 5A). For detection of STAT1 phosphorylation (pSTAT1) endothelial cells were incubated with IFN-β or pimecrolimus for 15 min, washed and cultivated until detection of pSTAT1. As shown in Fig. 4D IFN-β significantly induced phosphorylation of STAT1 as previously reported (Kalvakolanu, 2003), while pimecrolimus did not influence the phosphorylation status of this signaling molecule. In accordance are findings showing that presence of the Jak1/2 inhibitor ruxolitinib did not influence the antiproliferative activity of pimecrolimus as observed in HCAEC of the same individual (proliferation in % of control ± S.D., PM *vs.* PM & ruxolitinib 59.1 ± 5.0% *vs.* 61.5 ± 5.6%). Contrary, ruxolitinib abolished the effect of INF-β (84.1 + 5.5% vs. 100.6 + 4.8%,$^{*}P < 0.05$ One-way ANOVA, Fig. 4C). However, the incubation with both compounds led to an increase of STAT1 expression with a maximal peak at 24 h (Fig. 5D).

3.7. Impact of TLR4 on interferon signaling cascade

It has previously been reported that the IFN-signaling pathway is modulated by toll-like receptors (compare Fig. 5B). One receptor known for its cross-talking is TLR4, and quantification of mRNA levels showed high expression of this receptor in HCAEC and HCASMC (Supplemental S1). In the following experiments aiming to test the influence of cellular knock-down of the TLR4 on the pimecrolimus effect the immortalized cell line A549 was used, since primary cells are difficult to transfect (compare Fig. 6). Importantly, A549 cells also exhibit the reduction of proliferation by pimecrolimus and significant levels of the *TLR4* (Supplemental S2). Even if the effect of pimecrolimus on expression of INF target genes was less pronounced in those cells compared to that observed in primary cells, the expression of *IFI4* and *IRF9* was on the one hand significantly induced by pimecrolimus and on the other hand markedly reduced in presence of TLR4 siRNA ($P = 0.05$ Mann-

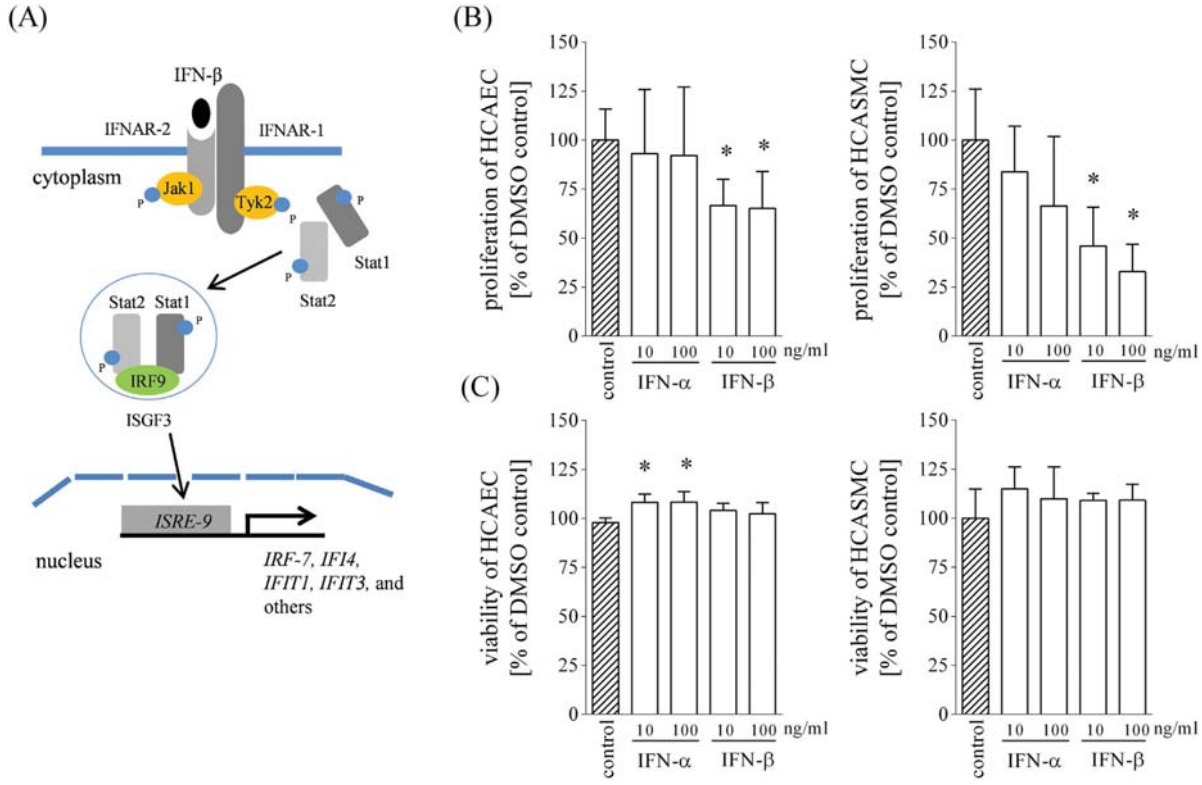

Fig. 5. Impact of type I interferons on cell proliferation and viability. (A) Type I IFN-signaling pathway. **(B,C)** Human coronary artery smooth muscle cells (HCASMC) and endothelial cells (HCAEC) of at least three individuals each were incubated with 10 or 100 ng/ml interferon (IFN-β) for 48 h and **(B)** proliferation or **(C)** viability was determined by BrdU Elisa or Resazurin viability kit, respectively. Mean ± S.D., n=7, $^{*}P < 0.05$ One-way ANOVA.

Whitney test). Taken together those findings supported the notion that pharmacodynamics of pimecrolimus is based on TLR4 activation cross talking to the IFN-signaling cascade.

4. Discussion

Understanding the molecular mechanisms involved in clinical failure and/or success of a compound is a prerequisite for future pharmacological developments. Pimecrolimus, which is an immunosuppressant in clinical use for topical application is one of those compounds that failed during stent development. As mentioned before, pimecrolimus-eluting stents were primarily demonstrated to exert the highly preferred combination of inhibition of neointima formation and promotion of endothelialization in the preclinical setting (Waksman et al., 2007). However, those stents finally failed assessing clinical data in humans (Verheye et al., 2009a, 2009b). It was aim of this study to identify potential cellular mechanisms that may have contributed to the observed performance of pimecrolimus-eluting stents in humans.

First, we determined its impact on cell proliferation and observed a concentration-dependent cytostatic effect. This finding is in accordance with previous findings in HUVECs and human

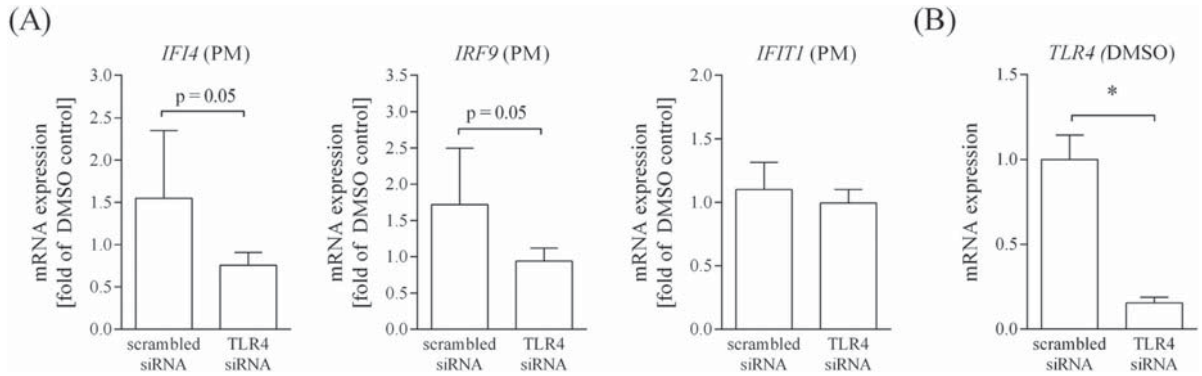

Fig. 6. Impact of TLR4 on pimecrolimus-induced effects. (A) A549 were transfected with siRNA for 48 h and gene expression of *IFI4* (2′-5′-oligoadenylate synthetase), *IRF9* (interferon regulatory factor 9), *IFIT1* (interferon-induced protein with tetratricopeptide repeats 1) was quantified after 48 h incubation with 10 μM pimecrolimus (PM). Data are presented as mean ± S. D. normalized to 18S rRNA, n=4 in triplicates, pimecrolimus vs. DMSO ($2^{-\Delta\Delta Ct}$). **(B)** Expression of *TLR4* (toll-like receptor 4) is presented as mean ± S. D. normalized to 18 S rRNA, n=4 in triplicates, scrambled vs. *TLR4* siRNA ($2^{-\Delta\Delta Ct}$), $^{*}P < 0.05$ Mann-Whitney test.

coronary smooth muscle cells (Grassberger et al., 1999; Konig et al., 2013). The lack of efficacy in clinical studies with an unexpected neointimal response may be due to the low antiproliferative potency of pimecrolimus in HCASMC accompanied by issues of solubility at concentrations higher than 30 μM as observed in our study. However, the successful first generation drug-eluting stent compound sirolimus also functions as a cytostatic in those cells, suggesting that there are differences in molecular mechanisms contributing to the observed clinical outcome. One mechanism assumed to contribute to the high efficacy of sirolimus-eluting stents is the modulation of calcium signaling finally influencing cell proliferation (Konig et al., 2013). Therefore, the failure of pimecrolimus-eluting stents may be a result of ineffective substrate concentrations or is based on a distinct cellular mechanism.

From a pharmacological point of view, the class of limus derivatives exerts its effects by binding to immunophilins such as FKBP12 or cyclophilin A thereby inhibiting distinct pathways. While the pimecrolimus-FKBP12 complex is assumed to inhibit the phosphatase activity of calmodulin-dependent calcineurin (Grassberger et al., 1999), the sirolimus-FKBP12 complex is assumed to reduce serine kinase activity of the mTOR complex (Vilella-Bach et al., 1999). And even though sirolimus does not inhibit calcineurin, it has been shown to suppress the activation of nuclear factor of activated T cells (Konig et al., 2013). Cyclosporine A, another immunomodulatory drug commonly used in clinics, binds to the immunophilin cyclophilin A thereby specifically inhibiting the calcineurin-calmodulin pathway (Liu et al., 1991). Based on our observation of enhanced antiproliferative activity in presence of cyclosporine A, we hypothesized that the calcineurin dependent pathway may be part of the cellular effect of pimecrolimus. Importantly, pimecrolimus exhibits much lower affinity to FKBP12 ($K_i \sim 100$ nM) compared to sirolimus ($K_i \sim 0.2$ nM) thereby potentially explaining the lack of an additive effect in cells treated with both compounds (DeCenzo et al., 1996; Grassberger et al., 1999).

Preliminary mRNA microarray data obtained in our laboratory suggested an upregulation of genes involved in the IFN-signaling pathway in pimecrolimus treated cells. This has been confirmed by real-time PCR showing a significantly increased expression of the interferon-inducible proteins IFIT1, IFIT3, and the 2′-5′-oligoadenylate synthetase IFI4, known to regulate the immune host defense and cell proliferation (de Veer et al., 2001; Der et al., 1998). In terms of interferon signaling, interferon-regulatory factor 9 (IRF9) plays a key role in this pathway. This regulatory protein forms with pSTAT1/2 the functional complex IFN-stimulated gene factor 3. This transcriptionally active complex binds response elements located in promotor regions of many IFN-stimulated genes, and initiates their transcription (Darnell et al., 1994). In addition, IRF9 is assumed to exhibit divergent regulatory activity in terms of proliferation or apoptosis strictly depending on the cell type and/ or the stimulus. Although it has been reported that INF-β stimulated cancer cells are more susceptible to p53-dependent apoptosis (Takaoka et al., 2003), after vascular injury IRF9 and therefore the ISGF3 has been reported to be involved in neointima formation (Zhang et al., 2014). Findings from Zhang and colleagues showed that growth factor stimulated proliferation and migration depends on IRF9.

Based on the herein reported data, it may be assumed that the observed antiproliferative activity of pimecrolimus is associated with secretion of type I interferons. Human type I interferons namely INF-α and INF-β are primarily known for their function in the innate immune response to viral infections, but are also regulators of different cellular processes including proliferation. Indeed, type I interferons and particularly IFN-β are known to modulate proliferation of smooth muscle and endothelial cells (da Silva et al., 2002; Erdmann et al., 2011; Sano et al., 2015). The observed difference in cellular response to INF-α (increased proliferation) compared to INF-β (reduced proliferation) in the herein reported study may be explained by differences in receptor affinities. As it has been shown that cellular signaling at least for IFN-α subtypes are associated correlated to the binding affinity to the interferon receptor (Aguet et al., 1984). No difference in response was observed treating the cardiovascular cells with IFN-β, which is in accordance with findings from previous studies (Palmer and Libby, 1992; Sano et al., 2015).

In order to determine whether pimecrolimus influences interferon signaling by enhancing secretion of INF-β we quantified this type I-interferon in the supernatant of treated cells. Even if secretion of the interferon by HCAEC was slightly increased no such effect was observed in HCASMC. Therefore, we assumed that the activation of type I IFN inducible gene expression in pimecrolimus treated cells is not attributable to a release of IFN-β with a subsequent binding to its receptor. This is supported by the findings on the activation of the Jak/STAT signaling cascade showing that pimecrolimus did not induce a phosphorylation of STAT1. And even though Rani et al. demonstrated that IFN-β can induce the transcription of IRF9 without STAT activation (Rani et al., 2010), there is evidence that calcineurin which is assumed to be inhibited by pimecrolimus negatively regulates IFN response by modulation of toll-like receptor mediated signaling pathways (Kang et al., 2007).

In general, toll-like receptors are transmembrane proteins regulating immune function and inflammation (Takeda and Akira, 2001; Uematsu and Akira, 2006). Especially TLR4 acts through pathways leading to the production of pro-inflammatory cytokines including TNFα and IFN-β (Akira, 2006; Bustamante et al., 2011). In addition, inhibition of the calcineurin-calmodulin complex by FK506 has previously been shown to interact with TLR4 signaling at least in murine macrophages (Kang et al., 2007). In resting macrophages, calcineurin interacts with TLR4, myeloid differentiation protein 88 and TIR-domain-containing adapter-inducing interferon-β (TRIF). In contrast if activated, TLR ligands or FK506 induce the dissociation of calcineurin from these proteins in macrophages. Finally the release of TRIF is responsible for activation of the transcription factor interferon-regulatory factor 3 which results in enhanced IFN-β production and secretion in activated macrophages (Doyle et al., 2002; Kawai et al., 2001). In order to determine whether TLR4 is involved in the antiproliferative effect of pimecrolimus we studied whether knockdown of TLR4 in the cancer cell line A549 diminishes the effect on INF-target gene expression. Indeed, the expression of IFI4 and IRF9 was markedly reduced, suggesting that activation of TLR4 by pimecrolimus may be involved. However, this slight reduction had no impact on cell proliferation (data not shown). Therefore, the impact of TLR4 on cell proliferation has to be further investigated potentially employing alternative approaches. These findings might lead to the assumption that pimecrolimus induced effect is due to calcineurin inhibition by TLR4 mediated activation of type I IFN-signaling. Importantly, the activation of pimecrolimus reduced the TLR4 mRNA expression in HCASMC (Supplemental S2).

It seems noteworthy, that FKBP12 expression in resting smooth muscle cells shows a much low abundance compared to plaque-derived cells or those occurring in neointima (Bauriedel et al., 2008), therefore the antiproliferative effect of pimecrolimus may be significantly higher *in vivo*, assuming that drug-eluting stents are inserted in vascular plaques.

5. Conclusion

Clinical studies showed that drug-eluting stents coated with limus compounds specifically inhibiting mTOR are superior

compared to inhibitors of calcineurin (Qi-Hua et al., 2015; Tamburino et al., 2009). Even though preclinical studies suggested a high impact of pimecrolimus on neointima formation clinical assessments revealed a failure of pimecrolimus-eluting stents. We provide evidence that pimecrolimus functions as an antiproliferative agent *in vitro* using vascular endothelial and smooth muscle cells. These effects are assumed to be based on an interaction with its target protein calcineurin. However, the concentrations needed for the antiproliferative effect are rather high and may be limited by solubility of the compound. In addition, we observed an activation of interferon type I inducible genes, which are part of a network regulating the immune host defense, apoptosis, and cell proliferation. Especially the interferon regulatory factor 9 is controversy described in its efficiency to inhibit cell proliferation. Nevertheless, siRNA knockdown of TLR4 suggested an interaction of pimecrolimus with this cellular receptor by calcineurin inhibition.

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Author declaration

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. We further confirm that there is no conflict of interest.

Conflict of interest

None of the authors have a conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2016.05.020.

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Supplementary data

Pimecrolimus increases the expression of interferon-inducible genes that modulate human coronary artery cells proliferation

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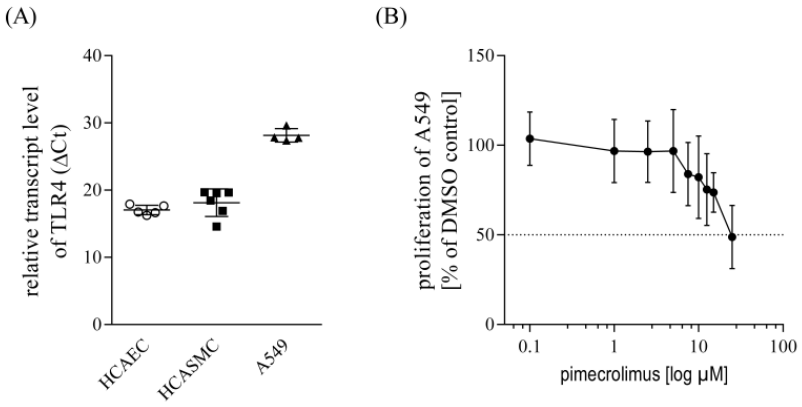

Figure S1 (A) mRNA expression of toll-like receptor 4 (*TLR4*) in human coronary endothelial cells (HCAEC) and smooth muscle cells (HCASMC) compared to A549 cells. Gene expression was determined in different passages and normalized to expression of 18S rRNA. Data are means ± S.D., n = at least 3 different passages. **(B)** Proliferation of A549 (lung carcinoma) was quantified after 48 h incubation with increasing concentration of pimecrolimus (0.1 – 25 log µM). Mean ± S.D. in % of DMSO treated cells.

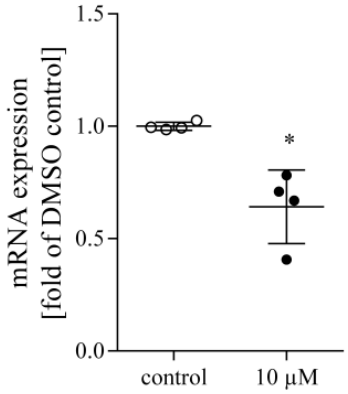

Figure S2 Gene expression of TLR4 after pimecrolimus treatment. Human coronary smooth muscle cells (HCASMC) isolated from four individuals were treated with DMSO or 10 µM pimecrolimus for 48 h. Gene expression of toll-like receptor 4 (*TLR4*) and 18S rRNA was determined by qRT-PCR. Fold of mRNA expression is presented as single data points each representing the mean of one individual normalized to 18S rRNA and related to the mean of the DMSO control using the comparative ΔΔCt method. Mean ± S.D., n = 4 in technical triplicates. *P<0.05 Mann-Whitney test

Chapter 4

Conclusions and outlook

One of the most concerning issues with implantation of drug-eluting coronary stents is the high incidence of late thrombosis associated with high mortality. Therefore, extensive research focused on the development of novel strategies to improve the long-term outcome of DES is necessary. One approach was to adapt stent material or the drug-eluting polymer to reduce the local hypersensitivity reaction that often occurs after stenting with drug release. Additionally, special emphasis was placed on identifying compounds or cellular mechanisms used for a more specific and/or efficient inhibition of SMC proliferation. However, as is often observed, although compounds demonstrated a high impact on neointima reduction, clinical assessments showed an unfavorable outcome. Hence, subsequent studies are necessary to address the underlying mechanisms.

Stent material and drug polymer reduce the local hypersensitivity reaction

It is a rational step to develop DES, where the selected stent material or polymer reduces the hypersensitivity reaction occurring with stent implantation. Stent strut thickness and a fast drug release often result in a pro-thrombotic surface. Current stent materials using platinum or chromium allow thinner stent struts and thus decrease the relative risk of clinical restenosis [50]. Similar results are registered using degradable polymers.

The release kinetics from stent polymer is an important characteristic often associated with the outcome. In our first study using a dual-loading drug technology based on a degradable PLLA polymer we detected a two-phase release profile with an initial burst release phase and slower release phase for both compounds. The initial burst release is characteristic for DES as shown particularly for the Cypher® sirolimus-eluting stent. The polymer elutes around 80% of the drug within the first 30 days. A longer drug release was associated with a better outcome as compared to a DES, which released its dose within 7 days [165]. Furthermore, a high release of around 90% in the first 2 days is unfavorable since the high dose results in toxic drug concentrations or is inefficient due to fast metabolism and/or excretion [166, 167].

Dual-drug loading for a higher efficacy and safety of DES

The regeneration of endothelial cells would decrease the incidence of stent thrombosis and thus avoid the long-term dual antiplatelet therapy that confers a substantial risk of bleeding [139]. As most cytotoxic drugs do not exhibit a cell-specific effect, a pro-healing compound concomitantly used would provide an alternative strategy.

Our developed dual-drug-eluting stent anticipates the inhibition of vascular SMCs without an impact on re-endothelialization. We therefore used an abluminal location of sirolimus and a luminal location of atorvastatin. Our goal was to combine the antiproliferative effect of sirolimus with the general observed effects of atorvastatin whereby minimizing the systemic exposure of sirolimus on ECs. Proliferation assays revealed that cellular effects were less pronounced in ECs than in SMCs. Thus, we suggest that luminal coating with atorvastatin is suitable to enhance re-endothelialization alongside a more targeted effect of sirolimus on SMCs with reduction of systemic toxicity. However, *in vivo* studies are warranted to prove the efficacy and safety of this dual-drug loading stent.

Pharmacokinetics define pharmacodynamics of compounds

Passive and active transport mechanisms play a crucial role in pharmacokinetics determining drug distribution to the target tissues and thus the biological activity of a compound. In the case of the vascular system where non-target (ECs) and target cells (SMCs) are in close vicinity, a coordinated distribution is necessary to obtain optimal drug concentrations in SMCs.

When comparing the lipophilicity of statins one might predict the tissue retention time in the coronary artery after local application. Cerivastatin especially is a highly lipophilic compound compared to pravastatin whose hydrophilic properties result in rapid uptake by diffusion and high transport out of the cell. In accordance with this are findings that pravastatin failed to induce an effect on cell proliferation [156]. Our studies provide evidence that endogenous expression of the drug transporter OATP2B1 is a determinant of statin effects in vascular cells. Using the transport activity of OATP2B1 as a screening approach may serve as a preclinical tool to identify compounds for an SMC-specific activity.

One approach to obtain a cell-specific effect of candidate drugs is the modulation of their pharmacokinetics. It was the aim of our second study to prove the concept that changing the pharmacokinetics by adenoviral-induced gene expression can be used to enhance the cell-specificity. We were able to show that the human drug transporter OCT1 increased the antiproliferative effect in SMCs using the promotor of transgelin (SM22α). Adenovirus-

mediated overexpression of a drug transporter may be a potential strategy in combination with different delivery devices including bioresorbable stents or nanoparticles based on biodegradable polymers.

In vitro studies are necessary to understand molecular mechanisms

Although limus agents including sirolimus and everolimus exhibited a high impact on restenosis reduction, other compounds including tacrolimus and pimecrolimus failed to enter clinics. Since they share the same binding partner FKBP12 another downstream pathway may account for the contrary efficacy on SMC proliferation. As a part of this thesis we studied the effects of pimecrolimus in order to determine its pharmacodynamics in coronary cells. Importantly, we provide evidence that pimecrolimus functions as an antiproliferative agent in smooth muscle and endothelial cells. We believe that the inhibition of calcineurin with modulation of the type I interferon signaling pathway may explain the failure of this limus derivative. Thus, it is necessary to assess the pharmacodynamics of compounds especially in cells affected by vascular modeling processes after stenting.

Our studies demonstrate different therapeutic approaches to improve the outcome of DES. Future developments are warranted which may combine different approaches involving novel drug candidates, polymers and stent surfaces. However, pharmacokinetics is still likely to be underestimated in stent technology but should be considered in future stent design since it often determines the biological activity of drugs in coronary cells.

Chapter 5

Appendix

5.1. Bibliography

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