Protease activated Receptor-1 antagonist, F 16618 reduces arterial restenosis by down-regulation of TNFα and MMP7 expression, and migration and proliferation of vascular smooth muscle cells

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Egr-1: early growth response-1
HB-EGF: heparin binding-epidermal growth factor
MCP-1: monocyte chemoattractant protein-1
MMP: matrix metalloproteinase
PAR: protease activated receptor
PDGF: platelet-derived growth factor
TIMP: tissue inhibitor of metalloproteinase
TNFα: tumor necrosis factor α
vWF: van Willebrand factor

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Abstract:

Wound healing after angioplasty or stenting is associated with increased production of thrombin and the activation of protease activated receptor 1 (PAR1). The aim of the present study was to examine the effects of a new selective PAR1 antagonist, 2-[5-oxo-5-(4-pyridin-2-yl)piperazin-1-yl)-penta-1,3-dienyl]-benzonitrile (F 16618), in restenosis and vascular smooth muscle cell proliferation and migration using both in vivo and in vitro approaches. Daily oral administration of F 16618 inhibited the restenosis induced by balloon angioplasty on rat carotid artery in a dose dependent manner. Furthermore, single intravenous administration of F 16618 during the angioplasty procedure was sufficient to protect the carotid artery against restenosis. In vitro, F 16618 inhibited the growth of human aortic smooth muscle cells (SMCs) in a concentration-dependent manner with maximal effects at 10 µM. At this concentration, F 16618 also prevented thrombin-mediated SMC migration. In vivo, oral and intravenous F 16618 treatments reduced by 30 and 50% the expression of the inflammatory cytokine TNFα 24 h after angioplasty. However, only acute intravenous administration prevented the induction of matrix metalloproteinase 7 expression. In contrast, F16118 treatments had no effect on early SMC de-differentiation and transcription of monocyte chemoattractant protein-1 and IL-6 and late re-endothelialization of injured arteries. Furthermore, F 16618 compensated for the carotid endothelium loss by inhibiting PAR1-mediated contraction. Altogether, these data demonstrate that PAR1 antagonists such as F 16618 is a highly effective treatment of restenosis following vascular injury, by inhibition of TNFα, matrix metalloproteinase 7, and SMC migration and proliferation in addition to an anti-thrombotic effect.
Introduction

Wound healing following vascular injury, and in particular restenosis after balloon angioplasty and stenting, results from different processes such as inflammation, smooth muscle cell (SMC) de-differentiation leading to SMC migration and proliferation, and constrictive remodelling (Muto et al., 2007; Zargham, 2008). In fact, localized loss of endothelium and sub-endothelial structures allows the direct contact of blood with SMC. This leads to generation of large amounts of the serine protease thrombin through activation of the coagulation cascade by the tissue factor pathway (Marmur et al., 1994). By binding the sub-endothelial extracellular matrix, thrombin remains functionally active, localized and protected from inactivation by circulating inhibitors (Schror et al., 2010). Thus thrombin cleaves circulating fibrinogen to fibrin, but also exhibits a wide range of functions by interacting with the surface receptors of platelets, leukocytes, endothelial cells and SMC (Coughlin, 2000; Minami et al., 2004; Steinberg, 2005; Hirano, 2007). Specifically, thrombin modulates endothelial permeability, vasomotor tone, leukocyte trafficking, migration and proliferation of vascular SMC (Minami et al., 2004).

Thrombin cleaves the N-terminal segment of protease activated receptors (PARs). This unmasks a new amino-terminal tethered ligand that binds to the extracellular domain to directly activate these G protein-coupled receptors (Vu et al., 1991; Dery et al., 1998; Coughlin, 2000; Hollenberg and Compton, 2002; Hirano, 2007). In normal arteries, PAR1 expression is detected in platelets, leukocytes and endothelial cells (Macfarlane et al., 2001), but it is limited in SMCs. However, after vascular injury such as balloon angioplasty, PAR1 transcription is up-regulated in SMC (Hirano, 2007). Up-regulation of PAR1 has been hypothesized as a key event in the development of vascular lesions and the hypercontractile response to thrombin (Fukunaga et al., 2006). This leads to neointimal formation and constrictive remodelling.
Thus, PAR1 antagonists may represent a powerful strategy to inhibit the development of vascular lesions after arterial reconstruction procedures. Orally active PAR1 antagonists were first developed as inhibitors of platelet aggregation, with a low impact on bleeding. SCH-530348, which has potent anti-thrombotic properties, is currently in phase 3 clinical trials for the treatment of acute coronary syndrome (Siller-Matula et al., 2010). E5555 moderately inhibits human platelet activity, has anti-inflammatory properties and prevents arterial PAR1 up-regulation and hyper-responsiveness to thrombin (Kai et al., 2007; Siller-Matula et al., 2010). We recently discovered a new PAR1 antagonist, F 16618 that displays potent antithrombotic activity (Perez et al., 2009; Letienne et al., 2010b). In the present study, we aimed to characterize the effects of F 16618 on balloon angioplasty-induced restenosis of the rat carotid artery. To further identify the mechanism of action of F 16618, we also performed proliferation and migration experiments with human aortic SMCs.
Methods

Drugs

F 16618, 2-[5-oxo-5-(4-pyridin-2-ylpiperazin-1-yl)-penta-1,3-dienyl]-benzonitrile hydrochloride was synthesized in the Centre de Recherche Pierre Fabre (Castres, France) by the Division of Medicinal Chemistry, as previously described (Perez et al., 2009). The PAR1 agonist SFLLR (Ser-Phe-Leu-Leu-Arg-Asp) was synthesized by the laboratory of aminoacids, peptides and proteins (Faculté de Pharmacie, Montpellier, France). Mitomycin C (6-Amino-1,1a,2,8,8a,8b-hexahydro-8-(hydroxymethyl)-8a-methoxy-5-methyl azirino[2′,3′:3,4]pyrrolo[1,2-a]indole-4,7-dione carbamate (ester) was purchased by Sigma-Aldrich (St Louis, MO). Human thrombin was from Calbiochem (Merck Biosciences, Darmstadt, Germany).

Carotid artery injury and F 16618 treatment

Rats were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in strict compliance with all applicable regulations and the protocol was carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research. This conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Balloon denudation of the left carotid artery endothelium was performed in male adult Sprague–Dawley rats weighing 250-270 g under isoflurane anesthesia. After exposure of the left carotid artery, a 2F Fogarty balloon catheter was inserted into the external carotid branch of the aortic arch, inflated to produce slight resistance, and moved back and forth three times. F 16618 was administered in the rats by two different routes. F 16618 or vehicle (1% methylcellulose) was administered orally once daily 3 days prior to and 14 days after balloon angioplasty. In another set of experiments, F 16618 or vehicle (40% PEG / 60% NaCl) was
administered by intravenous route 5 minutes before angioplasty and during the chirurgical procedure. For each mode of treatment, the correspondence between the total administered doses and the plasma concentrations of F 16618 was given in table 1. The control group of Sham-operated rats included 11 animals, the vehicle one was constituted of 19 rats. The groups receiving oral or intravenous treatment included 9-13 and 8-10 rats, respectively.

After treatment for 24 h, the animals were sacrificed and segments of non-injured and injured carotid arteries with endothelium at edges of the lesion were collected for mRNA extraction and RT-PCR quantification (Supplemental data). After treatment for 14 days, animals were sacrificed and carotid arteries collected for morphometric and imunofluorescence analysis.

**Carotid artery histomorphometric analysis**

Cross-sections of arteries were fixed with 4 % paraformaldehyde and stained with Hematoxylin / Eosin solution. The internal and external medial areas were measured to determine media and neointima surface. A neointima / media ratio (N/M) was calculated using a video image analysis system (LEICA QWIN, LEICA Imaging Systems, Cambridge, England) and served as an index of restenosis measurement. The analysis was conducted by an investigator blinded to treatment.

**Immunofluorescence**

Segments of carotid artery were mounted in embedding medium (Miles), frozen in isopentane precooled in liquid nitrogen, and stored at –80°C. Immunostaining of PAR1 and vWF was performed on 7-µm-thick cross-sections. Tissue sections were permeabilized and saturated with 0.5% Triton X-100, 1% BSA and 10% goat serum in phosphate buffered saline (PBS) for 60 minutes. Slides were then incubated with PAR-1 (ATAP2, 1:50, Santa Cruz, CA) or vWF (1:50, Acris, Herford, Germany) antibodies or corresponding isotype (mouse IgG, 1:50,
Vector Laboratories, Servion, Switzerland) with 1% BSA, 0.5% Triton X-100 and 3% goat serum in PBS overnight at room temperature. After washing in PBS, Alexa 594-conjugated goat anti-mouse IgG (1:400, Invitrogen, Cergy Pontoise, France) was added with 1% BSA and 3% goat serum in PBS for 1 hour. In some experiments, DAPI (1:500) was incubated with secondary antibody. After washing in PBS, tissues were mounted with Dako fluorescent mounting medium and visualized with an Olympus IX 50 microscope and a Roper Scientific camera. For PAR-1 staining, fluorescence images were automatically collected and deconvoluted using a piezoelectric translator (PIFOC, Karlsruhe/Palmbach, Germany) and a Metamorph software (Universal Imaging Corp, Downingtown, PA). The immunolabelling of endothelium was quantified with NIH Image software program as ratio of mean vWF fluorescence to internal vessel perimeter.

Cell proliferation assays

The human aortic SMCs were obtained from Lonza and cultured following the manufacturer’s instructions. For serum-induced cell proliferation, SMCs were cultured with complete Smooth muscle Basal medium (SmBm) 5 % fetal calf serum and treated with 1-100 µM (0.34-34 mg/L) F16618 for 48 hours. For PAR1 agonist-induced cell proliferation, cells were starved for 24 hours with SmBm containing 0.1 % serum and 0.1 % supplement (insulin, epidermal growth factor and basic fibroblast growth factor) and then incubated for 48 hours with DMSO 1 ‰ (vehicle) or F16618 (1-100 µM) with or without 10 µM SFLLR or 10 UI/ml thrombin (Sigma Chemicals, St Louis, MO). For proliferation tests without PAR1 expression, cells at 60 - 80 % confluence were transfected with scrambled or PAR1 siRNA using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Silencing of PAR1 mRNA was checked by real time PCR and immunofluorescence (Supplemental data, methods and Figure 1). Cell proliferation was assessed using a WST-1 based proliferation assay (Clontech,
Mountain View, CA) according to the manufacturer’s instructions. Each experiment included 12 wells for each condition and was repeated at least three times. For these experiments, cells were used up to 6 passages maximum.

**Cell migration assays**

Cell migration was studied by performing wound healing assays. Human aortic SMCs (20,000 cells) were plated in low 35mm μ-dishes with culture inserts (Ibidi, Martinsried, Germany). In some experiments, confluent cells were incubated in the presence of 20 μM mitomycin C for 2 h to inhibit cell proliferation. Inserts were then removed with sterile forceps to create a wound field of about 500 μm. To start migration, 1 % DMSO without (vehicle) or with stimulating proteins was added to medium either with or without 10 μM F 16618. Cells were then allowed to migrate in a cell culture incubator. At 0 and 8 h, 10 fields of the injury area were photographed with a light microscope at x100 magnification. For each coverslip, the area uncovered by cells at time 0 and 8h was determined by analysis with NIH Image software program. The migration distance was calculated from the difference between the two areas and the known width at time 0.

**Isometric tension recording**

Male Sprague Dawley rats were euthanized by intraperitoneal injection of sodium pentobarbital (160 mg/kg). The left carotid arteries were removed, prepared without endothelium and mounted in organ baths (Emka Technology, Paris, France) as previously described (Bocquet et al., 2009). Cumulative concentration-response curves were obtained with PAR1 agonist peptide SFLLR (0.1-100 μM with half log dose increment). Thrombin induced carotid contraction was assessed using a single dose (10 UI/ml) to avoid receptor desensitization. PAR1 antagonist F16618 or corresponding vehicle (1 % DMSO) was added...
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30 minutes before the SFLLR concentration-response curve. The amplitude of the tension was measured irrespective of the time. A single concentration of antagonist was tested per tissue sample.

Statistical analysis

For tension measurement experiments, results are expressed as % mean (E_{max}) ± SEM where E_{max} was obtained with the higher dose of SFLLR (100 µM). Concentration-response curves were fitted using Origin 7.5 software to calculate EC_{50} and pA_{2} values. For in vitro and in vivo experiments, one-way analysis of variance (ANOVA) was performed followed by a Dunnet’s test, to compare each group. Differences were considered significant when p<0.05.
Results

**F16618 prevents balloon-injury induced restenosis of the rat carotid artery**

The effects of the PAR1 antagonist F 16618 were tested in a rat model of restenosis induced by balloon angioplasty of the rat carotid artery. As seen in Figure 1A (left panel), PAR1 is mainly expressed in endothelial cells and SMCs of uninjured carotids. Fourteen days after balloon angioplasty, PAR1 expression in injured carotid appeared in both neointima and media (Fig. 1A, right panel). The carotid artery developed a neointima layer characteristic of restenosis compared to sham operated rats (Fig. 1B). Daily oral administration of F 16618, 3 days prior to and 14 days after angioplasty, produced a dose-dependent reduction of restenosis with a maximal effect at 10 mg/kg (25 µM) (Fig. 1C). However, F 16618 has a bell shaped response curve with less anti-restenotic activity for higher doses (Fig. 1C). In another set of experiments, the efficacy of F 16618 was evaluated by a single intravenous injection during the surgical procedure. This protocol also inhibited restenosis, but only for the highest dose of 1.25 mg/kg (76 µM) (n=8, P<0.05) (Fig. 1D). These data suggest anti-proliferative and/or anti-migratory effects of F 16618 on SMCs.

**F16618 inhibits human aortic smooth muscle cell proliferation**

To determine whether F 16618 inhibits SMC proliferation, we used human aortic SMCs. As shown by western blot analysis (Supplemental Data), these SMCs expressed PAR1 (Fig. 2A). F 16618 inhibited serum-induced cell proliferation in a concentration-dependent manner with a maximal inhibitory effect of 64.5 ± 0.5% at 100 µM (Fig. 2B). To ascertain the direct effect of F 16618 on PAR1, human aortic SMCs were treated with siRNA directed against PAR1. Transfection of SMCs with PAR1 siRNA significantly lowered the basal cell proliferation and prevented the anti-proliferative effect of F 16618 (Fig. 2B). This shows that PAR1 participates in part in serum-mediated SMC proliferation. We then investigated the F 16618
efficiency on SMC proliferation stimulated by specific PAR1 agonists. As seen in figure 2C-D, F 16618 inhibits the proliferative action of both SFLLR and thrombin in a concentration dependent manner, with a maximal inhibitory effect at 10 µM. At this concentration, F 16618 totally prevented the PAR1 agonist-activated proliferation. In contrast, when human aortic SMCs were stimulated by angiotensin II, F 16618 did not have any effect (data not shown). In addition, transfection of SMCs with PAR1- siRNA abolished the proliferative effect of thrombin and the inhibitory action of F 16618 (Fig. 2C). These data demonstrate that F 16618 inhibits thrombin/PAR1-mediated SMC proliferation.

**F16618 inhibits human aortic smooth muscle cell migration**

To examine whether F 16618 was also able to inhibit cell migration, human aortic SMCs were stimulated by heparin binding-epidermal growth factor (HB-EGF) and monocyte chemoattractant protein-1 (MCP-1), which both contribute to the pro-migratory effects of thrombin (Brandes et al., 2001; Kalmes et al., 2001). At 10 µM, the PAR1 antagonist had no significant effect on basal migration in control medium, but it suppressed the pro-migratory action of MCP-1 and HB-EGF (Fig. 3A). Similar results were observed in the presence of 20 µM mitomycin (not shown). The PAR1 antagonist also suppressed the pro-migratory effect of thrombin, which increased human aortic SMC migration by 27 % (Fig. 3B).

**F 16618 has no effect on expression of smoothelin, Egr-1 and PDGF in injured arteries**

*In vivo*, de-differentiation of vascular SMCs evidenced by loss of their contractile phenotype stimulates their migration and proliferation (Zargham, 2008). To explore whether the F 16618 treatments prevented restenosis by inhibition of SMC de-differentiation, we quantified the mRNA expression of the contractile protein smoothelin 24 h after angioplasty. The injury decreased by 75% the expression of smoothelin mRNA (Fig. 4A). Neither single intravenous
administration of F 16618 nor oral treatment had an effect on smoothelin expression. This indicates that the PAR1 antagonist is unable to block the SMC de-differentiation.

Platelet-derived growth factor (PDGF) is a mediator of SMC de-differentiation, migration and proliferation (Zargham, 2008). Its expression is up-regulated by the transcription factor, early growth response-1 (Egr-1), which is rapidly induced at the endothelial wound edge following balloon-angioplasty (Khachigian, 2006). To better define the in vivo mechanism of action of F 16618, we investigated its effect on the expression of Egr-1 and PDGF 24 h after angioplasty. In injured carotid arteries, the global expression of Egr-1 and PDGF m RNAs decreased by 40% (Fig. 4B). Whatever the treatment, F 16618 had no effect on the expression of Egr-1 and PDGF. Together, the results suggest that the acute phase after injury is characterized by change in SMC phenotype but not yet associated with proliferative events.

**F 16618 reduces TNFα and MMP7 expression without affecting mRNAs of MCP-1, IL-6, TIMP-1 and TIMP-2**

The response to arterial injury is the sequence of inflammatory events and extracellular matrix remodeling, which stimulate SMC migration and proliferation (Inoue and Node, 2009). To characterize how a single i.v. administration prevents restenosis, we next examined the influence of F 16618 on expression of inflammatory cytokines and matrix metalloproteinase (MMP). Tumor necrosis factor α (TNFα) is a key regulator of inflammatory responses (Monraats et al., 2005). Twenty four hours following angioplasty, its mRNA was expressed 4-fold more in injured carotid arteries than in non-injured ones (Fig. 5A). Both oral and intravenous F 16618 treatment significantly reduced the injury-mediated expression of TNFα. Interleukin 6 (IL-6) controls monocyte activation and MCP-1 is involved in monocyte recruitment into injured vascular walls (Welt and Rogers, 2002; Brasier, 2010). Balloon injury caused 24 hours after 9-fold and 13-fold increase in expression of IL-6 and MCP-1
mRNAs, respectively (Fig. 5A). Neither oral nor intravenous F 16618 treatment affected the injury-mediated expression of IL-6 and MCP-1. These results suggest that F 16618 interferes with the primary inflammatory response without preventing monocyte activation and recruitment, which are required for the healing process.

The MMP7 is known to be present in media and stenotic regions of vessels from patients with supravalvular aortic stenosis and Williams Beuren syndrome but it is not expressed in normal artery (Dridi et al., 2005). In rat, the MMP7 mRNA was also poorly expressed in non-injured carotid arteries, but its expression increased 4-fold 24 h after balloon-angioplasty (Fig. 5B). Interestingly, only the acute intravenous administration of F 16618 prevents the injury-mediated expression of MMP7. The oral treatment had no effect. Following angioplasty, the expression of tissue inhibitors of metalloproteinase (TIMP-1, TIMP-2) appeared to be oppositely controlled (Fig. 5B). The mRNA expression of TIMP-1 was upregulated whereas that of TIMP-2 was repressed. Neither oral nor intravenous F 16618 treatment affected TIMP-1 and TIMP-2 expression. Altogether, these results show that single administration of F 16618 during the surgical procedure prevents early activation of MMP7 without affecting the endogenous inhibitors TIMP-1 and TIMP-2.

**Effect of F 16618 on re-endothelialization of injured carotid surface**

We next studied whether F 16618 was able to modulate re-endothelialization, which is an essential step in the normal wound healing process of injured vessels. In all uninjured carotids, the labeling of endothelial marker, von Willebrand factor, was visible on the entire endothelial layer (Fig. 6A, upper panel). At magnification x400, proximity of the internal elastin lamina from the endothelial monolayer lets appear a yellow labelling. Under our conditions of balloon angioplasty, the re-endothelialization remained incomplete 14 days after the carotid artery injury (Fig. 5A, lower and left panel). Re-endothelialization was also partial in injured carotids of rats treated with oral or intravenous F 16618 (Fig. 6A, lower and
medium-right panels). Analysis of the vWF-labeled vessel perimeter showed that the decrease in fluorescence was similar in vehicle- and F 16618-treated rats (Fig. 6B). This suggests that the PAR1 antagonist reduces the formation of neointima without significant effect on the re-endothelialization process in our model of vessel injury.

**F16618 abolishes the SFLLR- and thrombin-induced rat denuded carotid artery contraction**

The loss of endothelium is associated with enhanced contractile response induced by both SMC exposure to the vessel lumen and up-regulation of PAR1 (Fukunaga et al., 2006; Kai et al., 2007). Thus, we investigated the effect of F 16618 on vasoconstriction of rat denuded carotid arteries. Thrombin produced a contraction of the carotid, which was prevented with F 16618 treatment in a concentration dependent manner (Fig. 7A). Half maximal inhibitory concentrations of F 16618 were ~ 10 µM. The PAR1 agonist SFLLR induced a concentration-dependent constriction of denuded carotid rings with an EC50 value of 3.83 µM. Addition of F 16618 blocked the SFLLR-induced contraction of the denuded carotid in a concentration dependent manner with pA2 value of 7.7 (Fig. 7B). These results clearly demonstrate that the PAR1 antagonist F 16618 protects vessels from vasoconstriction induced by endothelium loss.
Discussion

Recently, we have characterized a new potent and selective PAR1 antagonist F 16618, which prevents thrombosis (Perez et al., 2009; Letienne et al., 2010b). Herein, we show that F 16618 inhibits late neointimal formation in rat injured carotid artery through anti-inflammatory activities and exhibits anti-proliferative, anti-migratory and anti-vasoconstrictor properties \textit{in vitro} and \textit{ex vivo}.

Chronic oral and acute intravenous administration of F 16618 similarly inhibited neointimal formation. This shows that the blockade of PAR1 by a single administration of F 16618 during balloon angioplasty is sufficient to prevent neointimal hyperplasia. Anti-restenotic effect of an acute perivascular administration of PAR1 antagonist has been previously reported in the rat carotid model (Andrade-Gordon et al., 2001). Single administration of 25 mg/kg RWJ-58259 effectively reduced the neointimal formation in injured arteries. In our study, a single administration of 1.25 mg/kg F 16618 by intravenous route was adequate to reduce neointimal hyperplasia. The higher efficiency of F 16618 could result from direct contact with circulating cells, and also from its anti-thrombotic effect, a property not shared by RWJ-58259 (Andrade-Gordon et al., 2001).

In our experimental restenosis model, thrombosis precedes neointima formation. We recently demonstrated that a single i.v. administration of selective PAR1 antagonists including F 16618 exerts anti-thrombotic activity in an arterio-venous shunt model in rats (Letienne et al., 2010a; Letienne et al., 2010b). Previous experiments with PAR1 agonist peptides suggested that PAR1 is not functional in rat platelets (Kinlough-Rathbone et al., 1993). PAR1 antagonists may exert their anti-thrombotic effect by inhibiting activation of endothelial cells at wound edge. Early induction of Egr-1 and PDGF has been detected at endothelial wound edge after catheter scrape injury to rat aorta (Khachigian, 2006). Here, we observed a reduction of Egr-1 and PDGF expression in the acute phase following angioplasty. These
reductions indeed paralleled those of the endothelial markers thrombomodulin and intercellular adhesion molecule-1 (Supplemental data, Fig. 2). Such decreases likely result from removal of endothelium. Thus, it is hard to conclude with our global mRNA analysis whether activation of endothelial cells at wound edge is inhibited by F 16618.

By denuding the vessel of its protective endothelium, balloon angioplasty creates a local environment that favors vasospasm and contributes to constrictive remodeling. Thrombin is a potent vasoconstrictor that participates in initiation of vasospasm at the site of vascular injury. In addition, upregulation of PAR1 expression contributes to the enhanced contractile response to thrombin in injured arteries (Fukunaga et al., 2006). In the present study, F 16618 shows no effect on re-endothelialization 14 days after injury but inhibits the contractile response to PAR1 agonists in endothelium-denuded carotid arteries. Similar results have been obtained with other arteries (Bocquet et al., 2009). In vivo, the acute treatment with F 16618 may compensate for the vasoconstriction induced by endothelial loss and artery enlargement thereby preventing vasospasm-induced remodeling.

During the acute phase following de-endothelialization, neointimal formation is initiated by vasospasm and also by local inflammatory reaction (Zargham, 2008). Consequently, activated platelets and leukocytes release cytokines, growth factors and MMPs, which initiate SMC proliferation and migration (Welt and Rogers, 2002; Muto et al., 2007; Zargham, 2008; Inoue and Node, 2009). Increased plasma levels of MCP-1, MMPs, and TIMP-1 have been observed in human post-angioplasty restenosis (Cipollone et al., 2001; Jones et al., 2009). In addition, a haplotype of the human gene coding for the inflammatory cytokine TNFα is associated with increased risk of restenosis (Monraats et al., 2005). In our experimental model, vascular injury induces marked expression of TNFα, IL-6, MCP-1, MMP7 and TIMP-1. Both acute and chronic F 16618 treatments reduce the expression of TNFα, which is produced by endothelial cells and leukocytes. In contrast, only acute delivery of F 16618 inhibits the
expression of MMP7. This suggests requirement of high F 16618 concentration to penetrate into the vascular tissue to target macrophages and fibroblasts, which synthesize MMP7 (Woessner, 1995; Galis and Khatri, 2002). It should be noted that F 16618 partly affects the inflammatory response required for vascular healing since it has no effect on IL-6 and MCP-1 expressions.

We also demonstrate that F 16618 suppresses in vitro migration and proliferation of human aortic SMCs at a concentration of 10 µM which is below those efficient in vivo. Indeed, in the case of the daily oral administration, maximal inhibitory effect was observed at a plasma concentration of 25 µM. Since, at this concentration, F 16618 does not prevent the loss of the contractile protein smoothelin, chronic administration of F 16618 could inhibit restenosis by reducing TNFα expression and migration/proliferation of de-differentiated SMC. For the i.v. administration during angioplasty, a concentration of 75 µM is needed for efficient inhibition of TNFα and MMP7 expression, and neointima formation. In vivo, F 16618 interacts with SMC, but also with endothelial cells, fibroblasts and circulating leukocytes. In humans, F 16618 will also interact directly with platelets. Therefore, the effective concentration is necessary higher in vivo than in vitro.

Although our animal model of arterial injury provides evidence for a role of PAR1 signaling in the restenosis process, it cannot predict the efficacy of PAR1 antagonist in the human pathology. In fact, immunosuppressors, anti-inflammatory drugs, anti-platelets agents, anticoagulants, calcium-channel blockers, and angiotensin-converting enzyme inhibitors reduced the late neointimal thickening in experimental models but have failed to show any benefit for the prevention of restenosis in humans (Welt and Rogers, 2002; Inoue and Node, 2009). By systemic administration, only drugs with pleiotropic actions show efficiency in humans (Douglas, 2007; Wessely, 2010). With its potent anti-oxidant and anti-proliferative properties, the lipid lowering agent probucol is effective in preventing restenosis in both rats and humans.
(Douglas, 2007). The phosphodiesterase type III blocker cilostazol, which is used as antiplatelet agent, exerts anti-proliferative and lipid-lowering effect and also promotes SMC relaxation (Douglas, 2007). It is effective in humans too. Since the PAR1 antagonist F 16618 negatively modulates thrombosis, inflammation, cell proliferation and migration, and regulation of vascular tone, it could be suitable to prevent post-angioplasty restenosis in humans.

However, the direct thrombin inhibitors heparin and bivalirudin, which also have pleiotropic actions, have demonstrated beneficial effect on restenosis in rabbit models but not in humans (Burchenal et al., 1998). This paradox may be explained by the fact that inhibition of thrombin alone is not sufficient to prevent restenosis in humans and that drugs have been administered for only 24 hours after angioplasty in the clinical trial. Since F 16618 blocks PAR1 auto-activation, this compound inhibits signaling events induced not only by thrombin but also by coagulation factors VII and X and some MMPs (Camerer et al., 2000; Borensztajn et al., 2008). Moreover, F 16618 is an orally active drug, with fewer side effects than direct thrombin inhibitors (Letienne et al., 2010b). This allows to consider chronic treatment until complete healing. All these characteristics suggest that chronic treatment with F 16618 could be effective in the prevention of human restenosis.

Whether systemic administration of F 16618 fails to prevent post-angioplasty restenosis in humans, it remains an attractive strategy to locally inhibit in stent-restenosis. Indeed, the immunosuppressive, anti-proliferative and anti-migratory drug sirolimus prevented intimal hyperplasia in rat injured artery but has failed to show any benefit in humans when it was systemically administered (Inoue and Node, 2009). However, sirolimus and its derivates have beneficial effect when they are locally and chronically released by drug-eluting stents (Inoue and Node, 2009; Wessely, 2010). Stented arteries are subjected to sustained pro-inflammatory and pro-thrombotic status (Welt and Rogers, 2002). Hence, these processes may be prevented
by the anti-thrombotic and anti-inflammatory properties of F 16618, in addition to its effect on neointimal hyperplasia. Moreover, at the high concentration of 75 µM, intravenous administration of F 16618 significantly delayed the time of thrombotic occlusion without affecting the bleeding time and without hemodynamic effect in rat (Letienne et al., 2010b). Indeed, the potent antithrombotic activity of F 16618 is potentiated when combined with aspirin or clopidogrel without further increasing the bleeding time. The broad therapeutic range of F 16618 may facilitate its use on coated stent in the context of standard of care in percutaneous coronary intervention (PCI) and coronary artery bypass graft.

In conclusion, our results suggest that F 16618 prevents restenosis by limiting early inflammatory events, MMPs release, SMC migration/proliferation, and vascular contraction. These properties could be of particular interest for acute treatment during PCI or chronic release by eluting stents.
**Authorship Contributions**

*Participated in research design:* Chieng-Yane, Bocquet, Letienne, Le Grand and David-Dufilho

*Conducted experiments:* Chieng-Yane, Bourbon, Sablayrolles and Perez

*Performed data analysis:* Chieng-Yane and Bocquet

*Wrote or contributed to the writing of the manuscript:* Chieng-Yane, Bocquet, Lompre and David-Dufilho

*Others:* Hatem contributed to discussion.
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References


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b) The Groupe d’Etude sur l’Hémostase et la Thrombose; the Société Française d’Hématologie; and the Paris Descartes University provided a grant to P.C.Y to achieve her PhD.
Figure legends

**Fig. 1.** Anti-restenotic properties of F 16618 in a rat model of carotid injury by balloon angioplasty. A, PAR1 immunostaining of carotid artery cross-sections (7-µm-thick) was examined by fluorescence microscopy with monoclonal PAR1 antibody and secondary Alexa 594-coupled antibody (red). Nuclei were labelled with DAPI (405 nm) and elastin autofluorescence was revealed by fluorescence at 488 nm (green). Fluorescence images were collected with magnification 600x and automatically deconvoluted using a piezoelectric translator and Metamorph software. B, Hematoxylin/Eosin staining of carotid artery cross-sections from sham-operated (n=11) and balloon angioplasty-operated rats after treatment with vehicle (n=19) or F 16618, administered either orally (n=9-13) or intravenously (n=8-10). Magnification 100x. C, Effect of oral F 16618 treatment on Neointimal (N) and medial (M) ratios of carotid cross-sectional areas. D, Effect of intravenous F 16618 treatment on N/M ratios. The N/M cross-sectional area ratios were determined with a Leica Qwin software. Bar graphs represent mean ± s.e.m. * p<0.05, ** p<0.01 and *** p<0.001 vs vehicle.

**Fig. 2.** Effects of F 16618 on PAR1-dependent growth of human aortic smooth muscle cells. A, PAR1 expression was assessed by Western blot analysis. Blot is representative of 3 experiments. B, Proliferation of human aortic SMCs that are cultured in 5% serum for 48 h with 1‰ DMSO (Vehicle) or F 16618 (n=3-6). Proliferation of human aortic SMCs that are transfected with scrambled siRNA (-) (n=3) or PAR1-directed siRNA (n=3) and cultured for 48h in 5% serum with either 1‰ DMSO (Vehicle) or 100 µM F 16618. Serum-starved SMCs were treated with 1‰ DMSO (Vehicle, n=5) or F 16618 ranging from 1 µM to 10 µM (n=3). C, Serum-starved SMCs transfected or not with scrambled siRNA (-) or PAR1-directed siRNA were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 µM to 10 µM. Cells were then stimulated or not by thrombin (n=3-5). D, Serum-starved SMCs were treated...
with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 µM to 10 µM. Cells were then
stimulated or not by SFLLR (n=5) for 48 h. Cell proliferation for 48 h was quantified by
colorimetric assay with WST-1 reagent. Results are means ± s.e.m of n experiments. *
P<0.05, *** P<0.001 vs vehicle; # P<0.05, ### P<0.001 vs SFLLR or thrombin.

Fig. 3. Migration of human aortic smooth muscle cells is prevented by F 16618. When SMC
monolayers reached confluence, the insert was removed to create a wound field of about 500
µm width and cells were stimulated in medium containing 10 % serum. Cell migration to the
wound surface was monitored for 0 and 8 hours. Ten fields of the wound area were
photographed with a light microscope at x100 magnification. A, Human aortic SMCs were
treated with 1‰ DMSO (Control) or with 10 µM F 16618 and stimulated or not with 25
ng/ml MCP-1 and 25 ng/ml HB-EGF. The migration distance of the wound edge was
quantified using a NIH Image software program. Histograms are mean ± s.e.m (n=4/7).
*P<0.05 and ** P<0.01. B, Cells were incubated with 20 µM mitomycin C for 2 h to inhibit
cell proliferation. After removing the insert, cells were treated with 1‰ DMSO (vehicle) or
10 µM F 16618 and stimulated or not with 10 UI/ml thrombin. Histograms are mean ± s.e.m
of 3-5 different cultures, *P<0.05.

Fig. 4. F 16618 does not modify the expression of smoothelin, transcription factor Egr-1 and
growth factor PDGF. Operated rats were treated with vehicle or F16618 administered either
orally 3 days prior to angioplasty at the dose of 10 mg/kg/day or intravenously during the
surgical procedure at the dose of 1.25 mg/kg. 24 h after balloon angioplasty, non-injured and
injured carotids were harvested and total mRNA was extracted. The cDNA expression was
evaluated by real time PCR analysis. The expression level of targeted mRNA was expressed
as ratio to HPRT. Data are mean ± s.e.m of 4-6 rats/group. **P<0.01 and ***P<0.001 vs. non injured carotid.

**Fig. 5.** F 16618 inhibits the expression of TNFα and MMP7 without affecting MCP-1, IL-6 and TIMPs. A, Effect of F 16618 on injury-induced expression of inflammatory mediators. B, Effect of F 16618 on expression of MMP7 and metalloproteinase inhibitors. A-B, Operated rats were treated with vehicle or F16618 administered either orally 3 days prior to angioplasty at the dose of 10 mg/kg/day or intravenously during the surgical procedure at the dose of 1.25 mg/kg. 24 h after balloon angioplasty, non-injured (■) and injured carotids (□) were harvested and total mRNA was extracted. The cDNA expression was evaluated by real time PCR analysis. The expression level of targeted mRNAs was expressed as ratio to HPRT. Data are mean ± s.e.m of 4-6 rats/group. *P<0.05 and ***P<0.001 vs. non injured carotid. #P<0.05, ##P<0.01 and ###P<0.001 vs. injured carotid.

**Fig. 6.** Re-endothelialization of injured carotid artery is not prevented by F 16618. Operated rats were treated with vehicle or F16618 administered either orally 3 days prior to angioplasty at the dose of 10 mg/kg/day or intravenously during the surgical procedure at the dose of 1.25 mg/kg. At 14 days following balloon angioplasty, non-injured and injured carotids were harvested, mounted in embedding medium and frozen. A, vWF immunostaining of carotid artery cross-sections (7 µm-thick) was examined by fluorescence microscopy with a monoclonal antibody revealed with a Alexa 594-coupled goat anti-mouse antibody (red). The auto-fluorescence of elastin is revealed at 488 nm (green). For each rat, three cross-sections were analyzed with magnification 100x. B, vWF labelling in non-injured (■) and injured carotids (□) was quantified on 100x magnification images as the ratio of mean vWF
fluorescence to internal vessel perimeter with NIH Image software program. Results are mean ± s.e.m of 4-5 rats for each condition. *P<0.05, **P<0.01 vs. non-injured carotid.

**Fig. 7.** Thrombin- and SFLLR-induced contraction of denuded rat carotid is suppressed by F16618. Endothelium-denuded vessel rings were stabilized for 30 minutes, tested using a potassium rich solution, then pre-incubated with 1% DMSO (Vehicle, n=13-33) or 0.01-100 µM F 16618 for 30 minutes (n=7-18). A, Contraction of carotid rings in response to 10 UI/ml thrombin. Results are mean ± s.e.m * p<0.05, *** p<0.001 vs vehicle. B, Contraction of carotid rings in response to various concentrations of SFLLR. Concentration-response curves were fitted using Origin 7.5 software to calculate EC$_{50}$ and pA$_2$ values. C, Expression of log (EC$_{50}$ drug – EC$_{50}$ vehicle) as function of a range of F 16618 concentrations.
Table 1: Correspondence between oral and intravenous doses of F 16618 and maximal plasma concentrations

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<td>(mg/L)</td>
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<td>(µM)</td>
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<td>(µM)</td>
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For oral treatment, maximal plasma concentrations of F 16618 were detected one hour after force-feeding. For intravenous treatment, the plasma concentrations were calculated from the mean rat weight of 260 g and the corresponding plasma volume (Lee and Blaufox, 1985).
Fig. 1

A Non Injured Carotid  Injured Carotid

B Angioplasty

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Oral

Intravenous

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C

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D

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<tr>
<td>F 16618 (mg/kg)</td>
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**Fig. 3**

(A) 0 8 h

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Migration distance (µm)

- **CTRL**: 80-90 µm
- **H B-EGF+MCP-1**: 110-120 µm
- **F 16618**: 80-90 µm
- **H B-EGF+MCP-1 + F 16618**: 90-100 µm

(B) 0 8 h

<table>
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Migration distance (µm)

- **Thrombin**: 80-90 µm
- **Thrombin + F 16618**: 70-80 µm
Fig. 4

![Bar charts showing gene expression levels](chart.png)

- **Smoothenin/HPRT**
  - Non injured carotid
  - Injured carotid

- **Egr-1/HPRT**
  - Oral
  - Intravenous

- **PDGFB/HPRT**
  - Vehicle
  - F 16618

*Significance levels indicated with:
  - ***p < 0.001
  - **p < 0.01
  - *p < 0.05
Fig. 5

A

TNFα/HPRT

B

MMP7/HPRT

IL-6/HPRT

TIMP1/HPRT

MCP-1/HPRT

Vehicle  
F 16618

Vehicle  
F 16618

Oral  
I. V.