Weekly Dosing With the Platelet-Derived Growth Factor Receptor Tyrosine Kinase Inhibitor SU9518 Significantly Inhibits Arterial Stenosis

Yasundo Yamasaki, Kazuhisa Miyoshi, Nobuyuki Oda, Motomu Watanabe, Hidekazu, Miyake, Julie Chan, Xueyan Wang, Li Sun, Cho Tang, Gerald McMahon, Kenneth E. Lipson

Abstract—The platelet-derived growth factor (PDGF) ligands and their receptors have been implicated as critical regulators of the formation of arterial lesions after tissue injury. SU9518 (3[5-{5-bromo-2-oxo-1,2-dihydroindol-3-ylidenemethyl]-2,4-dimethyl-1H-pyrrol-3-yl}propionic acid) is a novel synthetic indolinone that potently and selectively inhibits the cellular PDGF receptor kinase and PDGF receptor–induced cell proliferation. Inhibition of PDGF receptor phosphorylation in cell-based assays occurs within 5 minutes after drug exposure and persists for >6 hours after drug removal. The pharmacokinetics indicate plasma levels that exceeded the effective concentration required to inhibit the PDGF receptor in cells for up to 8 hours or 7 days after a single oral or subcutaneous administration, respectively. In the rat balloon arterial injury–induced stenosis model, once-daily oral or once-weekly subcutaneous administration of SU9518 reduced intimal thickening of the carotid artery (ratio of neointimal to medial area, 1.94±0.38 versus 1.03±0.29 [P<0.01] 2.21±0.32 versus 1.34±0.45 [P<0.01], respectively). These studies provide the rationale to evaluate PDGF receptor tyrosine kinase inhibitors, including inhibitors related to the indolinone, SU9518, for the treatment of arterial restenosis. (Circ Res. 2001;88:630-636.)

Key Words: angioplasty • platelet-derived growth factor • restenosis • tyrosine kinase • indolinone

It is currently estimated that >350 000 coronary angioplasty procedures will be performed in the United States in the next few years.1,2 The growth of angioplasty has been suggested to be the result of improvements in surgical procedures, increased success rates, and a reduction in acute complications. Percutaneous transluminal coronary angioplasty (PTCA) for primary treatment of arterial occlusion after acute myocardial infarction has been demonstrated to be superior to thrombolytic therapy with regard to the restoration of normal coronary blood flow and may be associated with lower rates of recurrent infarction.3,4 However, restenosis at the site of the primary occlusion can occur within the first 6 months after the PTCA procedure leading to additional procedures or complications. Of patients who undergo PTCA, 35% to 50% will develop angiographic renarrowing, and as many as 25% to 35% are at risk for clinical recurrence.5–7

The underlying mechanisms of restenosis comprise a combination of effects ranging from vessel recoil, negative vascular remodeling, and thrombus formation to neointimal hyperplasia.8,9 The neointimal hyperplasia process has been suggested to be associated with the expression of growth factors and their cognate receptors including tyrosine kinases. The growth factors themselves are released by local thrombi and the angioplasty-injured arterial segment itself and may serve to enhance the expression of other growth-regulatory events. These processes result in an inflammatory reaction and a myofibroblast proliferative response, which worsen vessel narrowing caused by negative remodeling and result in the formation of a clinically significant restenotic lesion.10,11

Platelet-derived growth factor (PDGF) is one of several growth factors implicated in the restenosis process. In the animal balloon-injury model, PDGF acts primarily to induce smooth muscle migration and secondarily to promote intimal proliferation.12,13 PDGF receptor (PDGFr) autophosphorylation increases within a few days after injury and persists for several weeks.14 Furthermore, in human restenotic lesions in comparison with nonlesioned sites, the presence of PDGF-A and -B as ligands and PDGFrβ is detected after PTCA.15,16

The finding of the expression of these factors and receptor in vascular damaged and repaired regions strongly suggests that autocrine or paracrine stimulation of these receptor systems are important in the biological regulation of this tissue injury process.

Triazolopyrimidine (trapidil), a mild competitive inhibitor of the PDGFr, has been evaluated in 3 clinical trials to date.17–19 In one of these studies, Maresta et al19 found a...
Figure 1. SU9518 is a potent inhibitor of PDGF tyrosine kinase. A, Chemical structure of SU9518. B, Quiescent 3T3 cells expressing human PDGFrα or PDGFrβ were treated with SU9518 at indicated concentrations for 1 hour followed by stimulation of cells with PDGF for 5 minutes. Lysates of treated cells were fractionated by SDS-PAGE and transferred to nitrocellulose. Amount of PDGFr and phosphorylated PDGFr was visualized with anti-PDGFr and anti-phosphotyrosine (Anti-pY) antibodies, respectively. C, Ability of SU9518 to inhibit rat PDGFr kinase in A7r5 aorta cells was tested as described above.

Figure 2. Kinetics of SU9518 inhibition of PDGF tyrosine kinase. A, Rapidity of inhibition of PDGF receptor kinase was shown by exposing 3T3/PDGFrβ cells to SU9518 (2 μmol/L) for indicated times before stimulation with PDGF. B, To test the duration of PDGF kinase inhibition, quiescent 3T3/PDGFrβ cells were exposed to SU9518 (2 μmol/L) for 1 hour. The compound was then removed from cells for indicated times before stimulation with PDGF for 5 minutes. None indicates that the compound was not removed, whereas 0 minutes of wash out indicates that the compound was removed and cells were immediately stimulated with PDGF. For both panels, lysates of treated cells were fractionated by SDS-PAGE and transferred to nitrocellulose. Amount of PDGFr and phosphorylated PDGFr was visualized with anti-PDGFr and anti-phosphotyrosine (Anti-pY) antibodies, respectively.

Cellular Kinase Assay

Confluent cells were deprived of serum overnight, treated with the test compound for 1 hour, and then stimulated with 3.3 nmol/L (100 ng/mL) PDGF-AA or -BB for 5 minutes at 37°C. Cells were then lysed with SDS sample buffer and fractionated by SDS-PAGE. Phosphorylated PDGFr were detected by Western blot analysis using anti-phosphotyrosine antibodies (4G10) with enhanced chemiluminescence (ECL) detection. The amount of PDGFr in each lane was determined by using anti-PDGFrα, anti-PDGFrα, or anti-PDGFrβ antibodies and ECL detection.

Ligand-Induced Bromodeoxyuridine (BrdU) Incorporation Assay

3T3/EGF cells (3T3 cells overexpressing EGFr) in a 96-well plate were made quiescent by serum deprivation for 24 hours. The serum-deprived cells were then stimulated with (in nmol/L) fibroblast growth factor (FGF) 2/basic FGF 1.5, EGF 4, or PDGF 3.8 in the absence or presence of the indicated concentrations of SU9518 for 20 hours. BrdU was added for a 2-hour labeling period, and the cells were fixed. The amount of BrdU incorporation was determined with an ELISA kit (Roche Molecular) using anti-BrdU/POD (peroxidase). Cytotoxicity was assessed under identical conditions by using a Tox-8 kit (Sigma).

Pharmacokinetic Analysis

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Taiho Pharmaceutical Co. Male Wistar rats (7 weeks old, Charles River Japan, Inc [Tsukuba]) were housed in constant temperature facilities and given standard laboratory chow and water ad libitum. Blood samples (1 mL) were obtained at 2, 4, and 8 hours after oral administration of SU9518 or 1, 2, 4, and 7 days after subcutaneous administration. Blood was sampled from a cervical vein, and the plasma was stored at −20°C until analysis was performed. SU9518 was extracted from the plasma with acetonitrile, acidified, and extracted with ethyl acetate. HPLC analysis was performed on a Mightysil RP-18 GR column, 5-μm particle size, 4.6 × 150 mm using a Waters HPLC with a gradient of...
90% 5 mmol/L phosphate buffer (pH 2.8) and 10% acetonitrile to 30% 5 mmol/L phosphate buffer (pH 2.8) and 70% acetonitrile.

Rat Arterial Injury Model
Male Sprague-Dawley rats (12 to 13 weeks old, Clea Japan Inc, Tokyo, Japan) were housed in constant-temperature facilities and given standard laboratory chow and water ad libitum. Balloon catheter denudation of the carotid artery endothelium was performed according to the method described by Clowes et al.22 Briefly, the rats were anesthetized with a gas mixture of N₂O/O₂ (30:70) containing 2% halothane. After a median incision of the abdominal skin had been made, the right iliac artery was dissected and cannulated with a 2F balloon catheter (embolectomy catheter arterial balloon, Medical Technology Transfer). The catheter was inflated with saline and passed through the left common carotid artery 4 times to produce a distending, de-endothelializing injury.

Histopathologic Evaluation
On the 14th day after balloon injury, the rats were anesthetized with ether and perfused transcardially with saline, followed by 10% buffered formalin. The left carotid artery (from the aortic arch to bifurcation) was removed, postfixed, and embedded in paraffin. Sections 3 μm thick (5 per artery) were cut and stained with hematoxylin and eosin. The cross-sectional areas of the intima and the media on photographs were measured with a digital analyzer (Digitizer, Wacom).

PDGFr Protein Expression and Phosphorylation
Detection of PDGFr phosphorylation in injured arteries was performed according to the method of Panek et al.23 The injured artery was excised and cut into strips 7 days after balloon catheter injury. The strips were immediately frozen in liquid nitrogen, and protein was extracted, subjected to PDGFrβ immunoprecipitation, and separated by SDS-PAGE. Phosphorylated PDGFr was visualized by anti-phosphotyrosine immunoblotting (4G10) with ECL detection. The amount of PDGFr in each lane was determined using anti-PDGFrβ antibody. The signal intensity of PDGFr and phosphorylated PDGFr in vehicle- or SU9518-treated rats (subcutaneous injection of 100 mg/kg) was compared with that of uninjured rat artery PDGFr (n=3 in each group) by densitometry.

In Vivo Migration Assay
Quantification of smooth muscle cell migration into the intima was performed by the method of Bendeck et al.24 Four days after balloon injury, the artery was fixed with 4% buffered paraformaldehyde. The middle of the artery was cut lengthwise and pinned intimal side up onto a Teflon plate. The arteries were incubated with monoclonal antibody against human nuclei and chromosomes (MAB 1276, 1:100) overnight at 4°C, and immunoreactive cells were visualized with a commercially available detection system (Vector Laboratories). The number of cell nuclei per mm² was counted by light microscopy.

In Vivo Proliferation Assay
Quantification of medial and intimal smooth muscle cell proliferation was performed by the method of Muranaka et al.25 Four or 7 days after injury, the artery was fixed with 4% buffered paraformaldehyde and embedded in paraffin for preparation of 4 sections 3 μm thick. The sections were incubated with biotinylated anti–proliferating cell nuclear antigen (PCNA) monoclonal antibody (PCNA15, 1:250) overnight at 4°C, and counterstained with hematoxylin to visualize all nuclei.

In Vivo Proliferation Assay
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Drug Administration and Dosage
SU9518 (50 mg/kg in 0.5% hydroxypropyl methylcellulose solution [10 mL/kg]) was administered orally by gavage once daily from 1 hour before denudation to the day before removal of the artery for evaluation. Alternatively, SU9518 (100 mg/kg in carboxymethylcellulose sodium USP [0.5%], sodium chloride [0.9%], polysorbate 80 [0.4%], benzyl alcohol [0.9%], and deionized water [4 mL/kg]) was administered subcutaneously 1 day before denudation and again 6 days after the operation.

Reagents
SU9518 (Figure 1A) was synthesized by SUGEN using methods previously described.26 ECL reagents were purchased from Amer sham. PDGF-AA, PDGF-BB, and anti-BrdU/POD conjugate were purchased from Roche Molecular. Anti-phosphotyrosine monoclonal (4G10) and anti-PDGFrαβ polyclonal antibodies were from Upstate Biotechnology Inc, whereas anti-PDGFrα and anti-PDGFrβ antibodies were purchased from Santa Cruz Biotechnology. Biotinylated anti-PCNA monoclonal antibody was purchased from Caltag Laboratories. Anti-nuclei and chromosomes were purchased from Chemicon International. All other reagents were obtained from Sigma.

Statistical Analysis
All data are expressed as mean ± SD. Statistically significant differences between 2 groups were calculated by (2-tailed) Aspin-Welch t test. A P value <0.05 was considered to indicate statistical significance.

Results
SU9518 Inhibits PDGFr Kinase Tyrosine Phosphorylation
SU9518 was evaluated for its ability to inhibit PDGFr kinase using mouse fibroblasts expressing human PDGFrα or PDGFrβ. Cells were made quiescent by serum deprivation and contact inhibition and were then stimulated with PDGF and various concentrations of SU9518 (Figure 1B). Within 5 minutes of exposure to PDGF, significantly increased tyrosine phosphorylation was observed. SU9518 was found to inhibit the autophosphorylation of both receptors in a dose-dependent manner leading to complete inhibition of receptor activity at a concentration of 80 nmol/L.

To demonstrate that SU9518 would also inhibit rat PDGFr kinase, a similar experiment was performed with A7r5 cells (Figure 1C), which only express PDGFrβ (not shown). PDGFrβ in A7r5 cells were inhibited with a dose response similar to that observed in mouse fibroblasts overexpressing human PDGFrαs.

Kinetics of SU9518 Inhibition of PDGFr Kinase in Fibroblasts
The temporal inactivation of the PDGFr kinase by SU9518 was studied to determine the pharmacological requirements to maintain the inhibitory activity. In the first experiment, receptor autophosphorylation was measured using 3T3/PDGFrβ fibroblast cells after exposure of cells to SU9518 for different periods of time (Figure 2A). Complete inhibition of PDGF-induced receptor autophosphorylation was observed after incubation of the cells with SU9518 at all time points tested, indicating that <5 minutes of exposure was required to block the activation of PDGFr kinase. The rapid penetration and inactivation of the receptor may be due in part to the high relative lipophilicity of the compound and its ability to penetrate cell membranes.

Cells were incubated with SU9518 for 1 hour, followed by the removal of the media, to determine the persistence of the inhibition. They were then incubated for various periods of time before stimulation with PDGF to induce receptor autophosphorylation. Figure 2B illustrates that PDGFr kinase was completely inhibited for up to 2 hours after compound removal. This durable inhibitory effect was further substan-

Table 1. Effect of Subcutaneously Administered SU9518 on Intimal Thickness of Injured Rat Carotid Arteries

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Intima/Media</th>
<th>Medial Area, mm²</th>
<th>Intimal Area, mm²</th>
<th>Lumen Area, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated</td>
<td>6</td>
<td>2.21±0.32</td>
<td>0.110±0.026</td>
<td>0.229±0.029</td>
<td>0.210±0.017</td>
</tr>
<tr>
<td>SU9518-treated (100 mg/kg)</td>
<td>6</td>
<td>1.34±0.45*</td>
<td>0.121±0.020</td>
<td>0.154±0.052*</td>
<td>0.243±0.034</td>
</tr>
<tr>
<td>Percentage inhibition</td>
<td>39.4%</td>
<td>...</td>
<td>...</td>
<td>32.8%</td>
<td>...</td>
</tr>
</tbody>
</table>

I/M ratio was measured as described in Materials and Methods. I/M, medial area, intimal area, and lumen area values are given as mean ± SD. SU9518 was administered to rats by subcutaneous injection once weekly for 14 days (2 injections).

Table 2. Effect of Subcutaneously Administered SU9518 on Migration and Proliferation of Smooth Muscle Cells in Injured Rat Carotid Arteries

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Intima (4 days)</th>
<th>Media (4 days)</th>
<th>Intima (7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated</td>
<td>5</td>
<td>72±9</td>
<td>24.1±5.3</td>
<td>59.6±14.7</td>
</tr>
<tr>
<td>SU9518-treated, 100 mg/kg</td>
<td>5</td>
<td>33±7*</td>
<td>16.9±4.2†</td>
<td>36.8±14.2†</td>
</tr>
<tr>
<td>Percentage inhibition</td>
<td>54.2%</td>
<td>29.9%</td>
<td>38.3%</td>
<td></td>
</tr>
</tbody>
</table>

Number of migrating smooth muscle cells and labeling index (%) in medial and intimal layers were measured as described in Materials and Methods and are presented as mean ± SD. SU9518 was administered to rats by subcutaneous injection once weekly for 14 days (2 injections).

*P<0.01, †P<0.05 (vs vehicle, Aspin-Welch’s t test).
SU9518 Inhibits PDGF-Induced Cell Proliferation

The observation that SU9518 potently inhibited PDGF-induced receptor autophosphorylation suggested that the compound should also inhibit cellular responses mediated by PDGFrs. Mouse fibroblasts containing expressed human PDGFrb were stimulated with PDGF in the absence or presence of SU9518 (2 μmol/L) for 18 hours, after which they were exposed to BrdU for an additional 1.5 hours to label cells in the S phase of the cell cycle. The cells were subsequently fixed and stained for total DNA or BrdU incorporation (Figure 3). Very few of the serum-deprived cells were in S phase at the time of BrdU labeling. In contrast, the majority of PDGF-simulated cells incorporated BrdU at 18 hours, indicating that most had entered the cell cycle and progressed to the S phase. SU9518 was a very effective inhibitor of this process, which resulted in the blockade of PDGF-induced cell-cycle progression. The relative IC_{50} values for SU9518 inhibition of PDGF-, FGF- and EGF-induced BrdU incorporation using mouse fibroblasts were 0.053 ± 0.004 (n=5), 4.40 ± 1.13 (n=13), and 9.63 ± 2.98 (n=7), respectively. SU9518 demonstrated excellent potency for inhibition of PDGF-induced proliferation and good selectivity relative to that for FGF- and EGF-induced proliferation. This latter finding was expected, because SU9518 exhibited no inhibition of isolated EGFr kinase using a biochemical assay (not shown). SU9518 demonstrated no cytotoxicity at concentrations of up to 50 μmol/L (the highest concentration tested; n=3). Together, these data demonstrate that SU9518 is a potent and selective inhibitor of PDGFrb kinase activity in cells.

Plasma Pharmacokinetic Analysis of SU9518 in Rats

Pharmacokinetic studies (Figure 4, left panel) conducted with SU9518 at a single oral dose of 50 mg/kg in rats indicated plasma levels of 0.96 ± 0.23, 1.76 ± 0.64, and 1.06 ± 0.09 μg/mL at 2, 4, and 8 hours, respectively. This analysis indicated that plasma levels above 1 μmol/L were maintained for >8 hours after a single oral administration of this compound. These data along with the durable inactivation of the receptor provided a rationale to administer the compound on a daily basis by the oral route at a dose of 50 mg/kg. A similar analysis for subcutaneous delivery (Figure 4, right panel) showed plasma levels of SU9518 after a single administration at 100 mg/kg to be 0.87 ± 0.10, 0.63 ± 0.14, 0.55 ± 0.09, and 0.46 ± 0.09 μg/mL at 1, 2, 4, and 7 days, respectively. In this latter case, plasma levels exceeded 1 μmol/L for >7 days after the single subcutaneous injection.

SU9518 Inhibits Intimal Thickening of Rat Carotid Artery

Because PDGFrb has been strongly implicated as a contributor to the thickening of arterial walls after balloon injury in the rat, SU9518 was evaluated in a neointimal thickening model. For these experiments, rat carotid arteries were denuded of endothelium with a balloon catheter, and then SU9518 was administered orally at 50 mg/kg per day after the procedure. After 14 days, the arteries were removed and examined for the degree of neointimal thickening. Figure 5 shows a cross section of arteries from vehicle-treated control (panel A) or SU9518-treated (panel B) rats. Without SU9518 treatment, substantial thickening was observed by 14 days after vascular injury in vehicle-treated control rats. In this case, the ratio of neointimal area to medial area (I/M ratio) was 1.94 ± 0.38. Oral administration of SU9518 at a dose of 50 mg/kg significantly reduced the I/M ratio by 46.7% to 1.03 ± 0.29 (P<0.01). Moreover, this reduction of neointimal thickening was accomplished with no apparent changes in clinical symptoms, body weight, or organ morphology or color (not shown).

For evaluation of efficacy after subcutaneous administration of SU9518 at 100 mg/kg, the first dose was administered 1 day before balloon catheter denudation of the carotid artery endothelium, and the second dose was administered 6 days after arterial injury. Control untreated animals experienced substantial intimal thickening 14 days after vascular injury (I/M ratio, 2.21 ± 0.32) (Table 1). Weekly administration of SU9518 led to a significant reduction (39.4%) in arterial thickening, which was mainly due to inhibition of intimal thickening without statistically significant changes of the medial and luminal areas. As in the case of the oral administration, no obvious changes in symptoms, body weight, or organs were observed (not shown).

SU9518 Inhibits Migration and Proliferation of Smooth Muscle Cells in Rats

Smooth muscle cell migration and proliferation are thought to be the main processes leading to arterial thickening in the rat balloon injury model. To examine the early steps in arterial thickening, the number of migrating cells was examined 4 days after the initial trauma. After endothelial denudation, the intima is usually devoid of cells. However, 4 days after balloon injury, cells were observed in the intima, as previously reported.27 Treatment with SU9518 significantly reduced the number of intimal cells per mm² compared with vehicle-treated control rats (Table 2).

To assess the extent of cell proliferation induced by balloon injury, the medial and intimal layers were examined for PCNA-positive cells after 4 and 7 days, respectively.
Discussion

Synthetic inhibitors of tyrosine kinases have emerged as important new therapies for the potential treatment of human cancers and metastases. In addition, there have been several published reports of kinase inhibitors as potential therapeutic agents for the treatment of disease associated with blood vessel injury. In this regard, the PDGFr kinases have been published reports of kinase inhibitors as potential therapeutic agents for the treatment of disease associated with blood vessel injury. To determine the effect of administration of SU9518 on PDGFr expression and phosphorylation, immunoblots were examined 7 days after carotid artery injury (Figure 6). Balloon injury increased PDGFrβ expression 2-fold in vehicle-treated (1.81 ± 0.10-fold) and SU9518-treated (1.75 ± 0.18-fold) rats, compared with normal rats. In vehicle-treated rats, PDGFr phosphorylation increased (3.27 ± 0.20-fold) in parallel with the increase of PDGFrβ expression. Administration of SU9518 decreased the level of PDGFr phosphorylation without significantly affecting the receptor expression (1.11 ± 0.26, 95.2% inhibition, *P* < 0.01).

SU9518 Inhibits Arterial PDGFr Phosphorylation

PDGFr autophosphorylation has been reported to increase within a few days after balloon injury and to persist for several weeks. To determine the effect of administration of SU9518 on PDGFr expression and phosphorylation, immunoblots were examined 7 days after carotid artery injury (Figure 6). Balloon injury increased PDGFrβ expression 2-fold in vehicle-treated (1.81 ± 0.10-fold) and SU9518-treated (1.75 ± 0.18-fold) rats, compared with normal rats. In vehicle-treated rats, PDGFr phosphorylation increased (3.27 ± 0.20-fold) in parallel with the increase of PDGFrβ expression. Administration of SU9518 decreased the level of PDGFr phosphorylation without significantly affecting the receptor expression (1.11 ± 0.26, 95.2% inhibition, *P* < 0.01).

Acknowledgments

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