SCH 602539, a Protease-Activated Receptor-1 Antagonist, Inhibits Thrombosis Alone and in Combination With Cangrelor in a Folts Model of Arterial Thrombosis in Cynomolgus Monkeys

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Objective—To determine the antithrombotic effects of SCH 602539, an analog of the selective protease-activated receptor (PAR)-1 antagonist vorapaxar (formerly SCH 530348) currently in advanced clinical development, and the P2Y_12 ADP receptor antagonist cangrelor, alone and in combination.

Methods and Results—Multiple platelet activation pathways contribute to thrombosis. The effects of SCH 602539 and cangrelor alone and in combination on cyclic flow reductions were evaluated in a Folts model of thrombosis in cynomolgus monkeys. The effects of these treatments on ex vivo platelet aggregation and coagulation parameters were also monitored. Dose-dependent inhibition of cyclic flow reductions was observed after treatment with SCH 602539 alone and cangrelor alone (P<0.05 versus vehicle for the 2 highest concentrations of each agent). The combination of SCH 602539 and cangrelor was associated with synergistic antithrombotic effects (P<0.05 versus vehicle for all combinations tested). The 2 highest doses of SCH 602539 inhibited platelet aggregation in response to PAR-1–selective high-affinity thrombin receptor agonist peptide by greater than 80% but did not affect platelet aggregation induced by other agonists; also, they did not affect any coagulation parameters.

Conclusion—The combined inhibition of the PAR-1 and the P2Y_12 ADP platelet activation pathways had synergistic antithrombotic and antiplatelet effects. The addition of a PAR-1 antagonist to a P2Y_12 ADP receptor antagonist may provide incremental clinical benefits in patients with atherothrombotic disease, both in short- and long-term settings. These hypotheses need to be tested clinically. (Arterioscler Thromb Vasc Biol. 2010;30:2143-2149.)

Key Words: thrombin ■ thrombosis ■ PAR-1 antagonist ■ antiplatelet ■ platelet activation pathways

Atherothrombotic disease is associated with considerable morbidity and mortality. Although the benefits of dual antiplatelet therapy with aspirin and a P2Y_12 adenosine diphosphate (ADP) receptor antagonist have been demonstrated in a broad range of patients with atherothrombotic disease, many of these patients continue to have recurrent ischemic events. This high residual risk can be attributed to the fact that aspirin and P2Y_12 ADP receptor antagonists (eg, clopidogrel and prasugrel) only have a partial inhibitory effect on platelet-mediated thrombosis because they each target only 1 of many platelet activation pathways. As a result, thrombosis mediated by other platelet activation pathways, including stimulation of protease-activated receptor (PAR)-1 by thrombin, continues to occur even in the presence of aspirin and a P2Y_12 ADP receptor antagonist, leading to ischemic events.

Thrombin is the most potent platelet agonist because it stimulates platelet activation at low subnanomolar concentrations. PAR-1 is the principal receptor for thrombin on human platelets, whereas the secondary PAR-4 receptor may contribute to platelet activation at high concentrations of thrombin. Because the PAR-1 pathway is a key contributor to platelet-mediated thrombosis, PAR-1 is a valid therapeutic target for the development of novel antiplatelet agents. Previous preclinical studies with PAR-1 antagonists have demonstrated antithrombotic activity without an effect on bleeding time or coagulation parameters, supporting the clinical potential of this therapeutic approach.

The present study was designed to evaluate the antithrombotic efficacy of SCH 602539, a thrombin receptor antagonist selective for PAR-1 (SCH 602539 is an analog of vorapaxar [previously known as SCH 530348], a PAR-1 antagonist currently in phase 3 clinical development), alone and in combination with a P2Y_12 ADP receptor antagonist cangrelor in a Folts model of thrombosis. SCH 602539 and cangrelor were chosen as the prototypes for the antagonism of the PAR-1 and P2Y_12 ADP receptors because both agents can be administered parenterally and titrated.
Methods

Animals

Given the existence of species differences in the platelet thrombin receptor, we used cynomolgus monkeys because they have the same distribution of thrombin receptors (PAR-1 and PAR-4) on their platelets as do humans. Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act, in a program accredited by the American Association for Accreditation of Laboratory Animal Care.

Foils Model of Thrombosis in Anesthetized Monkeys

The procedures used in this study are similar to the acute artery occlusion model developed by Foils and others. Cynomolgus monkeys were sedated with ketamine hydrochloride, 10 mg/kg IM, followed by anesthetization with sodium pentobarbital, 20 mg/kg IV bolus and 5 mg/kg per hour IV infusion for the duration of the experiment. The body temperature was maintained at 37°C to 39°C, and fluids infused were maintained at body temperature. The right carotid artery was exposed and dissected free of surrounding tissue, and an appropriately sized transonic flow probe was placed around the vessel. A constrictor (Lexan; 5-mm length) was placed onto the carotid artery. The constrictor was sized to abolish reactive hyperemia and to reduce mean carotid blood flow by no more than 50% to 60%. Mechanical damage to the endothelium was induced in the constricted segment of the artery. Cyclic flow reductions would typically return in 3 to 4 minutes. The CFR frequency was calculated as the number of such cycles (CFRs) over a 30-minute period. Based on the pilot studies, CFRe were evaluated in a maximum of 4 consecutive 30-minute collection periods, and mechanically mediated endothelial injury was performed at 30-minute intervals to ensure exposure of the thrombogenic endothelium.

Animals instrumented to produce CFRe were administered SCH 602539, cangrelor, or a combination of SCH 602539 and cangrelor. The dosing regimens of the PAR-1 antagonist SCH 602539 were selected to provide complete inhibition of PAR-1-selective high-affinity thrombin receptor agonist peptide (haTRAP)-induced ex vivo platelet aggregation observed with the PAR-1 antagonist vorapaxar in a phase 2 trial. The doses of cangrelor were chosen to achieve 40% to 60% inhibition of ex vivo platelet aggregation induced by 10 μmol/L ADP, the magnitude of inhibition typically achieved with the standard 75-mg maintenance dose of clopidogrel, an oral P2Y12 ADP receptor antagonist. Clopidogrel was not used in the present study because its features (ie, it has to be administered orally, requires hepatic conversion to the active metabolite, and binds to the P2Y12 ADP receptor irreversibly) make it unsuitable for the protocol used in this study. The design of the study is shown in Figure 1. Group 1 received drug vehicle only (20% hydroxypropyl betacyclodextrin or saline, 2 mL IV). Group 2 received SCH 602539 dissolved in 20% hydroxypropyl betacyclodextrin administered as sequential IV boluses of 0.1, 0.3, or 1 mg/kg, at 30-minute intervals. Group 3 received cangrelor dissolved in saline and administered as an intravenous infusion of 0.1, 0.2, or 0.3 μg/kg per minute for 30 minutes each. Group 4 received a combination of SCH 602539 bolus plus cangrelor infusion, at respective doses as follows: (1) 0.05 mg/kg plus 0.05 μg/kg per minute; (2) 0.1 mg/kg plus 0.1 μg/kg per minute; and (3) 0.15 mg/kg plus 0.1 μg/kg per minute. The 3-dose regimens within each group were administered sequentially over the consecutive 30-minute periods coinciding with the reinduction of endothelial injury. Study drug or vehicle was administered after stable CFRe were achieved. On the completion of a 30-minute baseline CFR collection period, drug(s) or vehicle was administered incrementally (every 30 minutes) either as a bolus (SCH 602539) or as an infusion (cangrelor), and their effects on CFRe were monitored in the next three 30-minute observation periods. Blood samples, 3 mL, were collected from the femoral arterial catheter at the end of each 30-minute observation period for assessment of ex vivo platelet aggregation and coagulation parameters.

Ex Vivo Platelet Aggregation

Platelet aggregation studies were performed ex vivo on blood samples obtained from the monkeys subjected to the Folts model, using a whole blood aggregometer (model 540VS; ChronoLog, Havertown, Pa). Briefly, 0.5 mL of blood was incubated with 0.5 mL of normal saline at 37°C in a cuvette containing a stir bar for 2 minutes. Platelet agonists used in this study included TRAP, 3 μmol/L; ADP, 10 μmol/L; the thromboxane A2 mimetic U46619, 10 μmol/L; and collagen, 3 μg/mL. Platelet aggregation was monitored for 5 minutes after the addition of the agonist. The peak aggregation response was recorded in ohms. In addition, standard coagulation parameters, including prothrombin time (PT), activated partial thromboplastin time (aPTT), and activated clotting time (ACT), were assessed.

PT and aPTT Evaluation

These assays were performed in plasma obtained from the animals at the indicated time points. The PT assay is used for the detection of deficiencies in the extrinsic coagulation system, especially for factors VII and X. Briefly, a 100-μL plasma sample was warmed to 37°C for 3 minutes. Then, 200 μL of thromboplastin reagent was added to the plasma sample and inserted into the well of the automated instrument (Coag-A-Mate) to evaluate and record the time to the formation of a clot. The aPTT test is used to detect deficiencies in the intrinsic coagulation system, especially for factors VIII, IX, XI, and XII. Briefly, a 100-μL plasma sample was incubated with the aPTT reagent at 37°C for 5 minutes. Then, 100 μL of calcium chloride was added to the plasma sample to initiate clot formation, the speed of which was detected and measured with the automated instrument (Coag-A-Mate).

ACT Evaluation

The ACT test measures the clotting time of fresh whole blood and was performed in an automated coagulation timer (ACT II; Medtronic). Briefly, 0.2 mL of citrated whole blood was added to each well of the cartridge and the test was initiated by inserting the cartridge into the machine (ACT II). The end point, clot formation, was measured by the rate of fall of the plunger-9 flag mechanism contained in each cartridge. The plunger assembly falls rapidly through an unclotted sample, but the fibrin web formed during clotting impedes the rate of decent, which can be detected by a photo-optical system and displayed. These measurements were performed in duplicate.

Evaluation of Synergy

The in vivo effect of coadministration of PAR-1 and P2Y12 ADP receptor antagonists on the inhibition of CFRe (synergy, additive, or antagonism) was tested using the RACT cartridges (Medtronic). Briefly, 0.2 mL of citrated whole blood was added to each well of the cartridge and the test was initiated by inserting the cartridge into the machine (ACT II). The end point, clot formation, was measured by the rate of fall of the plunger-9 flag mechanism contained in each cartridge. The plunger assembly falls rapidly through an unclotted sample, but the fibrin web formed during clotting impedes the rate of decent, which can be detected by a photo-optical system and displayed. These measurements were performed in duplicate.

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Evaluation of Synergy Resulting From Dual P2Y₁₂ and PAR-1 Inhibition

The Folts model of CFRs, resulting from fixed stenosis of an arterial bed, is the result of the in vivo interplay of platelet-sensitive vasoactive molecules, which closely mimics the clinical scenario of unstable angina. The mean ± SEM EC₅₀ values calculated from dose-response curves are 0.100 ± 0.042 mg/kg and 0.099 ± 0.039 µg/kg per minute for SCH 602539 and cangrelor, respectively.

Five experiments were conducted for each of the 3 combinations partnering SCH 602539 and cangrelor. The corresponding equipotent doses for each compound compared with the different dose combinations were calculated from the respective dose-response curve (Figure 2D and Figure 3). The in vivo CFR inhibition exerted by SCH 602539 or cangrelor, when combined with its partner, was greater than that of the individual components administered alone.

The presence of a synergistic interaction is suggested by the 50% reduction in CFR frequency with SCH 602539, 0.05 mg/kg, in combination with cangrelor, 0.05 µg/kg per minute; and the complete extinction of CFR production in 4 of 5 animals receiving SCH 602539, 0.1 mg/kg, plus cangrelor, 0.1 µg/kg per minute. Similar antithrombotic effects were also observed with the combination of SCH 602539, 0.15 mg/kg, plus cangrelor, 0.1 µg/kg per minute, administered in the subsequent 30-minute period. The CFR frequency for baseline and each of the 3 doses administered, along with the calculated doses, are shown for SCH 602539 and cangrelor in Figure 2D and Figure 3, respectively.

Synergism was confirmed because the synergistic factor was less than 1 in 12 of 15 combination experiments (data not shown) and was seen in the lowest tested dosage combination of 0.05 plus 0.05. By using the model of Berenbaum, the calculated mean ± SEM synergistic factor for all 15 combination experiments was 0.41 ± 0.17.

Effects on Ex Vivo Platelet Aggregation

The effects of SCH 602539 alone, cangrelor alone, and the combination of SCH 602539 and cangrelor on ex vivo platelet aggregation mediated by various agonists (eg, haTRAP, ADP, thromboxane A₂ mimetic U46619, and collagen) in the Folts model of thrombosis in anesthetized cynomolgus monkeys were also evaluated. Table 1 outlines the inhibition of platelet aggregation noted at each of the tested dosing regimens of study drug. The 2 highest doses of SCH 602539, 0.3 and 1 mg/kg, were associated with potent (>80%) and dose-related inhibition of ex vivo platelet aggregation induced by 3-µmol/L haTRAP but did not affect the aggregation induced by 10-µmol/L ADP, 10-µmol/L thromboxane A₂ mimetic U46619, and 3-µg/mL collagen, demonstrating selectivity of SCH 602539 for the PAR-1 receptor pathway. The 2 highest doses of cangrelor, 0.2 and 0.3 µg/kg per minute, inhibited ADP-mediated platelet aggregation by approximately 50% to 60% (as targeted) but did not interfere with the aggregation stimulated by 3-µmol/L haTRAP, 10-µmol/L thromboxane A₂ mimetic U46619, or 3-µg/mL collagen. Finally, the combination of SCH 602539 and cangrelor inhibited the aggregation induced by 3-µmol/L haTRAP and 10-µmol/L ADP in a dose-related manner; and

Values of less than 1 indicate the presence of synergy, whereas values equal to 1 are indicative of an additive effect.

\[
\frac{\text{Dose of } A_{\text{in combination with } B}}{\text{Dose of } A_{\text{alone}}} + \frac{\text{Dose of } B_{\text{in combination with } A}}{\text{Dose of } B_{\text{alone}}}
\]

Results

Of the 24 monkeys studied, 22 exhibited stable CFRs after instrumentation. There was minimal reduction in blood flow in the context of abolishing hyperemic blood flow. The heart rate, blood pressure, and body temperature were unchanged for the duration of the study.

Antithrombotic Effects in the Folts Model of Thrombosis

The results of 22 experiments with the vehicle are shown in Figure 2A. The stability and utility of the surgical model were demonstrated by the consistent and reproducible CFRs achieved in animals treated with vehicle for the 2-hour study period. In the 22 experiments, the control frequency of the CFRs was 5.6 ± 1.8 per 30 minutes (range, 7–13) (Figure 2A).

Intravenous bolus doses of SCH 602539 reduced the number of CFRs in a dose-dependent manner (Figure 2B). The CFR frequency was reduced from baseline by approximately 50% with the 0.1-mg/kg dose and by greater than 80% with the 0.3- and 1.0-mg/kg doses (P < 0.05 versus vehicle for both). The CFRs were completely abolished in 4 of 6 animals with both the 0.3- and 1.0-mg/kg doses.

Continuous infusions of cangrelor reduced the CFR frequency from baseline by approximately 40%, 70%, and 90% with the 0.1-, 0.2-, and 0.3-µg/kg per minute 30-minute infusion doses, respectively (Figure 2C). The reductions achieved with the 2 highest doses were statistically significant versus vehicle. The intermediate and highest doses of cangrelor completely prevented CFRs in 3 and 5 of 6 animals, respectively. No complete suppression was evident in any of the animals treated with the lowest dose of cangrelor.

To assess the antithrombotic effects of SCH 602539 in combination with cangrelor, doses of each agent that provided only modest inhibition of CFRs when used alone were chosen (SCH 602539, 0.1 mg/kg; and cangrelor, 0.1 µg/kg per minute). Doses of SCH 602539 and cangrelor that were estimated not to affect CFR frequency when administered alone (SCH 602539, 0.05 mg/kg; and cangrelor, 0.05 µg/kg per minute), and a slightly higher dose of SCH 602539, 0.15 mg/kg, were used to explore the possibility that the antithrombotic effects of a PAR-1 antagonist and a P2Y₁₂ ADP receptor antagonist were synergistic. Initial treatment with SCH 602539, 0.05 mg/kg, plus cangrelor, 0.05 µg/kg per minute, significantly reduced the mean CFR frequency to 2.4 and completely abolished CFRs in 2 of 5 animals (Figure 2D). Treatment with a combination of SCH 602539, 0.1 mg/kg, plus cangrelor, 0.1 µg/kg per minute, completely abolished the CFRs in 4 of 5 animals and reduced the number of CFRs by approximately 50% in the remaining animal (Figure 2D). Similar antithrombotic effects were observed with a combination of SCH 602539, 0.15 mg/kg, plus cangrelor, 0.1 µg/kg per minute (Figure 2D).
the level of inhibition was the same as for each agent used alone. No inhibition of platelet aggregation induced by 10-μmol/L thromboxane A₂ mimetic U46619 or 3-μg/mL collagen was observed. These findings suggest that the ex vivo inhibition of haTRAP- and ADP-mediated platelet aggregation pathways is not predictive of the synergistic effect of combined therapy with SCH 602539 and cangrelor on the reduction of CFRs.

### Effects on Coagulation Parameters

The administration of SCH 602539 alone, cangrelor alone, and the combination of SCH 602539 and cangrelor had no effect on the coagulation parameters, including PT, aPTT, and ACT (Table 2). These findings are consistent with the fact that both SCH 602539 and cangrelor are antiplatelet agents that interact with specific platelet receptors and do not interfere with the activity of the coagulation cascade.

### Discussion

In the present study, we have demonstrated the antithrombotic effects of PAR-1 antagonism with SCH 602539 in a Folts model of thrombosis. More important, we have demonstrated that the in vivo antithrombotic effects of PAR-1...
antagonism, in combination with P2Y12 ADP receptor antagonism, are synergistic. The inhibitory activity of SCH 602539 was specific for platelet aggregation induced by haTRAP. The aggregation induced by other agonists, such as ADP, thromboxane A2 mimetic U46619, and collagen, was not affected, demonstrating the specificity and selectivity for the PAR-1 receptor. Cangrelor specifically inhibited aggregation induced by ADP and demonstrated a modest numerical inhibition of aggregation induced by collagen, but not by haTRAP or U46619. These findings parallel those reported in the literature.24,25

As expected, neither SCH 602539 nor cangrelor (nor the combination of the 2) had any notable effect on coagulation parameters, a finding that is consistent with the fact that these agents interact with specific platelet receptors and do not interfere with the coagulation cascade. SCH 602539 is structurally related to vorapaxar, a PAR-1 antagonist being investigated in clinical trials.26,27 These findings parallel those reported in the literature.28,29

Synergistic antithrombotic effects similar to those observed with the combination of SCH 602539 and cangrelor in this study have been reported with other combinations of antiplatelet agents18,19 that target distinct platelet activation pathways contributing to thrombosis. In the Folts model of thrombosis in pigs, the combination of low oral doses of clopidogrel, 0.1 mg/kg, and aspirin, 1 mg/kg, completely eliminated CFRs at 90 minutes, whereas the higher doses of each agent alone (clopidogrel, 5 mg/kg, and aspirin, 7 mg/kg) reduced, but did not completely abolish, the CFRs.18 A separate study19 in rabbits that used several different models of thrombosis demonstrated that the addition of oral aspirin to oral clopidogrel was associated with potent antithrombotic effects, and also additive bleeding effects, possibly related to the combined inhibitory activity of 2 antiplatelet agents on collagen-induced platelet aggregation. The additive antithrombotic effects of combined inhibition of the thromboxane A2 and P2Y12 ADP receptor platelet activation pathways with aspirin and clopidogrel observed in these studies are consistent with significant reductions in ischemic events with dual antiplatelet therapy over aspirin alone reported in large clinical trials.28,29,30

There are 2 possible explanations for this synergism. The first is via the concurrent inhibition of the G protein–coupled
reacting to either thrombin or P2Y1-mediated ADP stimulation and are localized to the P2Y1 transmembrane receptors. Blockade of PAR-1 may directly activate platelets by phospholipase C–mediated calcium secretion. Thrombin, the most potent platelet agonist, activates platelets directly by phospholipase C–mediated calcium release and Rho-mediated platelet shape change. However, thrombin also generates activation amplification through the secondary release of ADP from the dense granules. Blocking thrombin-mediated platelet secretion may result in a reduction in the total ADP pool available to the localized site. This would result in less agonist/antagonist competition for P2Y12 receptors and a greater level of inhibition of ADP-mediated platelet aggregation. Such triple inhibition may allow for lower levels of ADP receptor blockade using available agents while increasing efficacy and reducing the risk of bleeding. In addition, triple therapy may obviate the perceived clinical need for potent P2Y12 ADP receptor antagonists.

The challenges clinically are to determine whether the addition of a PAR-1 antagonist to the standard of care of aspirin and a P2Y12 ADP receptor antagonist will provide incremental clinical benefit without incremental bleeding risk and to determine the optimal dose for each agent. The combined inhibition of thromboxane A2 and P2Y12 ADP receptor platelet activation pathways with aspirin and a P2Y12 ADP receptor antagonist provides more potent antithrombotic activity than either agent alone and has been documented to reduce the rate of ischemic outcomes compared with aspirin alone.2,3 Recent studies of a more potent P2Y12 ADP receptor antagonist, prasugrel, resulted in an incremental 19% reduction in clinical events; however, there remains a 10% prevalence of clinical events complicated with a 32% increased bleeding risk, including major hemorrhage during coronary artery bypass grafting and intracranial hemorrhage during stroke.2 A conceptual therapeutic window model of P2Y12 inhibition has been proposed by Gurbel and Tantry25; this model defines the delicate balance between efficacy and bleeding. Such a model would establish a warfarin international normalized ratio–like range that factors the type of ADP inhibition and the presence of PAR-1 inhibition. These data would suggest that in the context of the model of Gurbel and Tantry, PAR-1 inhibition would improve therapeutic outcomes, widen the therapeutic range of ADP inhibition, and reduce ADP-mediated risk of bleeding by allowing lower levels of ADP-mediated inhibition of platelet aggregation.

The clinical potential of PAR-1 antagonists as a novel class of oral direct-acting antiplatelet agents for the management of atherothrombosis is supported by the results of 2 recent phase 2 trials with the PAR-1 antagonist vorapaxar, which showed strong trends toward reduced incidence of ischemic events without an accompanying increase in bleeding.14,26 Two large ongoing trials (Clinicaltrials.gov identifiers: NCT00526747 and NCT00527943) are investigating the clinical efficacy and safety of vorapaxar in combination with standard antiplatelet therapy among patients presenting with an acute coronary syndrome and those with a history of a coronary artery, cerebrovascular, or peripheral artery disease.

In conclusion, this study demonstrates that combined P2Y12 and PAR-1 antagonism results in the synergistic inhibition of CFRs in the Folts model of fixed arterial stenosis, suggesting the possible clinical benefit of blocking multiple platelet pathways. Such a concept is being tested in the ongoing phase 3 megatrials of vorapaxar.

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