Combined Administration of Aspirin and a Specific Thrombin Inhibitor in Man

Robert J. Clarke, MD; Gail Mayo, RN; Garret A. FitzGerald, MD; and Desmond J. Fitzgerald, MD

Background. Heparin is of limited value as an antithrombotic drug in the presence of platelet activation and residual thrombus. Greater anticoagulant activity can be achieved in vivo with more specific thrombin inhibitors. Heparin may also increase the risk of bleeding by an effect on platelets that is independent of its thrombin inhibitory activity.

Methods and Results. The pharmacodynamic and pharmacokinetic effects of a novel thrombin inhibitor, argatroban, were examined alone and in combination with aspirin in normal male volunteers. Argatroban induced a dose-dependent prolongation of the thrombin time and the activated partial thromboplastin time (aPTT). aPTT had returned to its pretreatment value 1 hour after stopping the infusion of argatroban. Six male subjects received an infusion of 1 μg/kg/min argatroban after the administration of two doses of 162.5 mg aspirin or a matching placebo. At this dose, aspirin decreased serum thromboxane B_2 by a mean of 99% and prolonged the bleeding time (230±52 versus 320±113 seconds, p<0.01). Argatroban given alone increased thrombin time by 454±18% and aPTT by 160±3%. Steady-state plasma concentrations were achieved at 1 hour and declined exponentially with an elimination half-life of 24±4 minutes. Neither the anticoagulant effects nor the plasma concentrations of argatroban were altered by aspirin. Furthermore, argatroban did not increase the bleeding time when given alone and did not further prolong the bleeding time when combined with aspirin.

Conclusion. The combination of aspirin and argatroban may prove to be an effective therapeutic strategy in the prevention of coronary thrombosis. (Circulation 1991;83:1510-1518)

Coronary thrombosis is the precipitating event in unstable angina and acute myocardial infarction and complicates many therapeutic interventions in patients with cardiovascular disease. Heparin, the only available anticoagulant suitable for acute administration, is not entirely effective in preventing coronary thrombosis. Although heparin accelerates the inactivation by antithrombin III of a number of coagulation factors, including prothrombinase and thrombin, it is not the ideal anticoagulant in the presence of platelet activation and an existing thrombus. Heparin is neutralized by platelet products, including platelet factor 4 and thrombospordin, and the heparin-antithrombin III complex is a weak inhibitor of prothrombinase on the platelet surface. Furthermore, thrombin bound to the clot surface or to cells is weakly inhibited by heparin. Heparin administration is also associated with an increased risk of bleeding. Although this may be a result of its inhibition of thrombin, heparin exerts an antplatelet effect in vivo that is unrelated to its anticoagulant activity. In addition, Heiden and coworkers reported an interaction between aspirin and heparin in which there is a marked prolongation in the bleeding time when these two agents are combined in some individuals.

More effective anticoagulation may be achieved by specific inhibitors of thrombin. Several compounds have been described that bind to the active site of thrombin and are not dependent on antithrombin III for their activity. These agents are not neutralized by platelet products and are effective against clot-bound thrombin. In addition, they may avoid the nonspecific effects of heparin on platelets. Argatroban is an arginine derivative that binds to the
hydrophobic pocket close to the active site of thrombin and is a competitive inhibitor of thrombin-induced platelet activation and clotting (Figure 1).16,17 In experimental models, argatroban has proved to be more effective than heparin in preventing coronary thrombosis.18-21 In open-label clinical studies, argatroban has been shown to produce an anticoagulant effect, and it was used in place of heparin in a patient with heparin-induced thrombocytopenia.22 In the present study, we examined the effect of argatroban on bleeding time, an index of platelet and vascular function in vivo, and on ex vivo indexes of coagulation in normal male volunteers. Many patients with coronary artery disease take aspirin regularly. Furthermore, a potent interaction has been demonstrated between argatroban and thromboxane A2 inhibition in preventing reocclusion in experimental models of coronary thrombolysis.18,21 Because it is likely that argatroban would be combined with aspirin in the clinical setting, we also explored the potential interaction between these two drugs regarding hemostatic function.

Methods

Nine healthy male volunteers (age range, 20–35 years; mean ±SD age, 20±6 years) participated in the study and gave informed, written consent to a protocol that had been approved by the Committee for the Protection of Human Subjects, Vanderbilt University Medical Center. All subjects were admitted overnight to the Elliot Newman Clinical Research Center, where the infusions were carried out. None of the subjects had taken aspirin or any other medication for 2 weeks before the study.

Dose-Finding Study

Three male volunteers were administered an intravenous infusion of argatroban in incremental doses ranging from 0.1 to 2 μg/kg/min during a 3-hour period. Activated partial thromboplastin time (aPTT) and thrombin time (TT) were monitored hourly during the infusion and at 1 and 2 hours during recovery. The 3-hour infusions were separated by a washout period of at least 3 days.

Interaction Between Argatroban and Aspirin

In the second part of the study, six volunteers were infused with argatroban in combination with either aspirin or a matching placebo in a double-blind, crossover study. Argatroban was administered in a dosage of 1 μg/kg/min, which was determined from the dose-finding study to prolong the aPTT by at least 50%. The treatments were separated by a washout period of at least 10 days. Argatroban was administered intravenously as a constant infusion during a 4-hour period. Aspirin in a dose of 162.5 mg or placebo was administered on two occasions, at 26 and 2 hours before the infusion. Bleeding time was measured before the administration of aspirin or placebo and at 30 minutes before and 210 minutes after the start of the argatroban infusion. Coagulation function was measured before aspirin and argatroban and at hourly intervals during the infusion and the recovery period, until these had returned to normal. Serum for thromboxane B2 estimations was taken before aspirin or placebo therapy and 5 minutes before and 4 hours after the start of argatroban infusion. Urine was collected at intervals before and during drug administration for determination of 2,3-dinor-6-keto-prostaglandin F1α (PGF1α) and 2,3-dinor-thromboxane B2, the enzymatic metabolites of prostacyclin and thromboxane A2, respectively.23,24 Blood was taken at 60 and 5 minutes before the infusion for estimation of plasma concentrations of aspirin and salicylate. Plasma argatroban levels were determined before and at intervals during the infusion and after the withdrawal of the drug.

Bleeding Time

Bleeding time was determined using the Ivy method, with a Simplate bleeding time device (Organon, Teknika, Parsippany, N.J.) as previously described.25 A blood pressure cuff was placed around the upper arm and inflated to 40 mm Hg. A site on the volar surface of the forearm perpendicular to the antecubital crease was used, taking care to avoid blood vessels. The spring-loaded lancet produced a uniform incision 5 mm long and 1 mm deep. This single incision wound was blotted every 20 seconds with filter paper, taking care that the filter paper did
not contact the wound. Blotting was continued at the same interval until bleeding stopped; the time until bleeding stopped was recorded. All bleeding time determinations were conducted by the one operator who was blinded to the subjects’ treatment categories throughout the study.

Coagulation Assays

Plasma for these measurements was obtained from venous blood mixed with 3.8% sodium citrate (9:1, vol/vol). For determination of aPTT, 100 µl of plasma was added to 100 µl Actin FS (American Dade, Aguada, P.R.) and incubated at 37°C. One hundred microliters of 0.025% CaCl₂ was added after 5 minutes, and the time to clot formation was recorded by a fibrometer (BBL, Becton Dickinson and Co., Cockeysville, Md.). The normal range for aPTT was 25–40 seconds. For determination of TT, 100 µl thrombin (1 µm/µl) (Armour Pharmaceutical Co., Kanakee, Ill.) was added simultaneously with 100 µl of 0.025% CaCl₂ to 100 µl of plasma at 37°C, and the time to clot formation was recorded (normal range for TT, 14–20 seconds). For determination of prothrombin time (PT), 100 µl of plasma was added to 200 µl thromboplastin C (American Dade) after incubation at 37°C, and the time to clot formation was recorded. The normal range for PT was 10–13 seconds.

Biochemical Analyses

Urinary excretion of 2,3-dinor-thromboxane B₂ and 2,3-dinor-6-keto-PGF₁α was determined by a stable isotope dilution assay with quantitation by gas chromatography–negative ion, chemical ionization–mass spectrometry (GC-NICI-MS), as previously described.23 Briefly, 5 ng of a deuterated internal standard was added to a 5-ml aliquot of urine. After extraction and back-extraction under alkaline and acidic conditions, the sample was derivatized as the pentafluorobenzyl ester. After further purification by thin-layer chromatography, derivatization was completed by the formation of the trimethylsilyl ether derivative. Final separation and quantitation were achieved by a gas chromatograph in series with a Nermag R10-10 mass spectrometer (Houston, Tex.) operated in the negative ion mode. Determinations were made while monitoring mass/charge (m/z) 586 and 590 for the endogenous 2,3-dinor-6-keto-PGF₁α and the tetradeuterated internal standard, respectively.23 Similarly quantitation of 2,3-dinor-thromboxane B₂ and its internal standard were made while monitoring m/z 614 and m/z 618 for the endogenous compounds and internal standard, respectively.24

Serum thromboxane B₂. Venous blood was collected into a plain glass tube, allowed to clot, and incubated for 45–60 minutes at 37°C. The sample was centrifuged at 3,000g for 10 minutes, and the serum was separated and stored at −20°C for later analysis. Quantitation of thromboxane B₂ was carried out by GC-NICI-MS, as described above. Briefly, 4 µl of 0.5-ng/µl solution of tetradeuterated thromboxane B₂ standard was added to 25 µl of plasma. The sample was acidified, extracted into ethyl acetate, derivatized to the methoxime, pentafluorobenzyl ester, and purified by thin-layer chromatography. A final derivatization, to the trimethylsilyl ether, allowed quantitation by selected ion monitoring at m/z 618 for the internal standard and m/z 614 for thromboxane B₂.

Aspirin and salicylate. Aspirin and salicylate levels in plasma were determined using a method that has been previously described.26 Venous blood was collected into ice-cooled tubes containing heparin (30 µl of 1:1,000) with 10% KF·2H₂O (30 µl) to prevent deacetylation and immediately centrifuged at 3,000g at 4°C for 10 minutes. The plasma was removed and stored at −20°C for later analysis. Aliquots (2 µl) of standard solutions containing aspirin and salicylate heptadeuterobenzyl esters were added to aliquots (100 µl) of plasma. These samples were diluted with 4 ml of a phosphate buffer (pH 6.5) containing 0.05 M tetrahexylammonium hydroxide and 20 µl benzylbromide; 4 ml dichloromethane was added, and the samples were shaken for 15 minutes. After separation, drying, and evaporation of organic solvent, the compounds were partitioned between water and hexane. After final separation by the thin-layer chromatography, the samples were eluted with ethyl acetate, evaporated and reconstituted in 15 µl dodecane. Quantitation was carried out by GC-NICI-MS monitoring at m/z 228 for endogenous aspirin and salicylic acid and m/z 235 for the heptadeuterated internal standards.

Argatroban determinations. Argatroban levels in plasma were determined by Mr. V. Anicetti at Ge- nentech Inc. by using a high-performance liquid chromatographic (HPLC) method and a coagulation assay. The HPLC method used an ethanol extraction procedure in which 400 µl of plasma was added to 950 µl of reagent alcohol, vortexed, and centrifuged. The supernatant was evaporated and reconstituted into 100 µl of 50% methanol. Chromatography was carried out on a Hewlett-Packard 1090L liquid chromatograph using an internal surface reverse-phase column (Regis Chemical, Morton Grove, Ill.), which was connected in turn to a nonporous column (Glycotech Inc., New Haven, Conn.). Twenty-five microliters was injected at a temperature of 60°C and a run time of 11 minutes. Concentrations were determined from a standard curve that was constructed for each subject with his own plasma. Quantitation of argatroban was also carried out using a coagulation method, which was basically a modification of the TT assay, as described above. This measured the ability of argatroban to inhibit clot formation in plasma with exogenously added thrombin and calcium chloride. The coagulation assay was more sensitive than the HPLC assay (6.25 versus 31.3 ng/ml), but there was a good correlation between both methods (r=0.89).

Pharmacokinetic calculations. The area under the plasma concentration–time curves (AUCs) of argatroban was calculated by the trapezoid method, extrapolated to infinity. The clearance of argatroban was calculated from the equation clearance equals
dose administered divided by AUC. The data were fitted to a one-compartment model; this permitted calculation of the elimination rate constant, terminal half-life, and volume of distribution at steady state.

Statistical Analysis

All values are reported as mean±SEM or percent of baseline (mean±SEM). The data were analyzed by nonparametric analysis of variance or a paired Student’s t test when appropriate.

Results

Dose-Ranging Study

Argatroban produced a dose-related prolongation of all coagulation indexes (n=3) (Figure 2). Argatroban in a dosage of 1 μg/kg/min prolonged aPTT to 181±7%, TT to 511±27%, and PT to 116.5±5.5% of their respective baseline values (Figure 2). aPTT and TT were 107±2.3% and 123±11%, respectively, of baseline values at 1 hour and 104±4% and 105±0%, respectively, at 2 hours after discontinuation of the drug.

Combination With Aspirin

Plasma concentrations of aspirin were 998±172 and 152±33 ng/ml at 1 hour and 5 minutes, respectively, before argatroban infusion in those receiving active drug (n=6). The corresponding salicylate levels were 2,581±250 and 2,491±172 ng/ml. Both compounds were below the detection limit of the assay in those receiving placebo. The bioavailability of aspirin was confirmed by its effect on serum thromboxane B₂. Serum thromboxane B₂ levels at 4 hours of argatroban were unchanged (284±44 versus 271±30 ng/ml at baseline) in the placebo phase but markedly depressed (5.8±3.5 ng/ml) after aspirin administration.

Argatroban (1 μg/kg/min) infused over 4 hours prolonged aPTT and TT to maximums of 160±3% and 454±18% of baseline values, respectively. Only a minor increase in PT was observed, the peak reaching 116±1% of baseline. These effects were unaltered by the prior administration of aspirin (Figure 3). Steady-state plasma concentrations were achieved after 1 hour of infusion, which is consistent with the effect on TT and aPTT (Figure 4). The elimination of the drug followed first-order kinetics and was best fitted by a single-compartment model in most individuals, although two phases were apparent in some (Table 1). Similar results were achieved with both the functional and HPLC assays. Aspirin did not alter the prolongation in aPTT (160±4%), TT (467±10%), or PT (115±1%) induced by argatroban (Figure 3) and had no effect on its plasma disposition (Figure 4 and Table 1).

The bleeding times were similar at baseline during each phase of the study (243±17 versus 230±24 seconds). As expected, this index of hemostasis was
prolonged by aspirin (320±54 versus 230±25 seconds) (p<0.01) but unchanged by placebo. Argatroban had no effect when given alone and did not further prolong bleeding time after aspirin administration (p>0.05) (Figure 5).

Excretion of the urinary thromboxane metabolite 2,3-dinor thromboxane B2 was unaltered by treatment with argatroban but decreased significantly after treatment with aspirin (306±118 versus 80±10 pg/mg creatinine) (p<0.05) (Figure 6). Similarly, the prostacyclin metabolite 2,3-dinor-6-keto-PGF1α was not significantly altered by argatroban but was reduced after aspirin therapy (159±50 versus 70±28 pg/mg creatinine) (p<0.05) (Figure 7).

Discussion

Coronary thrombosis complicates the course of coronary artery disease despite the use of heparin and is a major cause of morbidity and mortality. In experimental models, more effective anticoagulation can be achieved with agents that specifically inhibit thrombin.18-21 Unlike heparin, these compounds are insensitive to platelet products and inhibit clot bound as well as free thrombin.8 In the present study, we examined the pharmacology of one such compound, argatroban, and its interaction with aspirin in normal male volunteers.

Stereoisomers of 4-methyl-1-[N2-((3-methyl-1,2,3,4-tetrahydro-8-quinolyl)sulfonyl)-l-arginy]-2-piperidine carboxylic acid inhibit thrombin in a competitive fashion by binding to the hydrophobic pocket close to the active site of thrombin. Argatroban, the most potent of these stereoisomers, inhibits the effects of thrombin on fibrinogen and platelets with a Kᵢ of 19 and 40 nM, respectively.16,17 In animal models, argatroban is more effective than heparin in preventing coronary thrombosis and reocclusion after coronary thrombolysis.18,20 The antithrombotic effect is dose dependent and occurs over a range that induces a modest (less than twofold) increase in aPTT.18 Argatroban inhibits platelet aggregation by thrombin but has no effect on the response to other agonists.18 This is consistent with in vitro data demonstrating that argatroban specifically inhibits thrombin-induced platelet activation.17 Although argatroban inhibits other enzymes, including tissue-type plasminogen activator,27 the Kᵢ for this activity (78 μM) is well above concentrations that inhibit thrombosis in vivo, and argatroban has been shown to enhance thrombolysis in a number of experimental models.18,27

Argatroban prolonged aPTT and TT in a dose-dependent fashion in men. At a dosage of 1 μg/kg/min, aPTT increased 1.6–1.8-fold, and TT increased nearly fivefold. This degree of anticoagulation has

Table 1. Clearance, Half-life, and Volume of Distribution at Steady State of Argatroban in Presence or Absence of Aspirin When Measured by Both High-Performance Liquid Chromatography and Coagulation Methods

<table>
<thead>
<tr>
<th></th>
<th>Argatroban alone</th>
<th>Argatroban and aspirin</th>
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<tbody>
<tr>
<td></td>
<td>HPLC (n=6)</td>
<td>Coagulation (n=6)</td>
</tr>
<tr>
<td>Clearance (ml/min/kg)</td>
<td>5.0±0.5</td>
<td>4.4±0.4</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td>24.4±3.5</td>
<td>21.4±2.0</td>
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<tr>
<td>VD₅₀ (l)</td>
<td>11.7±1.3</td>
<td>9.7±1.0</td>
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HPLC, high-performance liquid chromatography; VD₅₀, volume of distribution at steady state.
been shown to exert antithrombotic effects in a canine model of experimental thrombosis. In this model, argatroban (0.5 mg/kg/hr) accelerated reperfusion in response to tissue-type plasminogen activator and, when combined with a thromboxane A2 receptor antagonist, completely abolished reocclusion. At this dosage, aPTT was prolonged 1.8-fold and TT was prolonged sevenfold, which was similar to the anticoagulant effect achieved in healthy volunteers in the present study.

Interestingly, despite its racemic nature, there was little interindividual variation in the anticoagulant responses, although the pharmacodynamic consequences of differences in stereospecific metabolism may become evident in larger studies. Furthermore, there was a close correlation between plasma levels of argatroban as determined by HPLC and by inhibition of thrombin, suggesting that the majority of the activity resulted from the parent compound and not from active metabolites. Pharmacokinetic analysis during a steady-state infusion of 1 μg/kg/min demonstrated a high clearance rate and a single phase of elimination in most individuals, with a half-life of 24±4 minutes. Thus, anticoagulation was achieved rapidly and little thrombin inhibitory activity was detected 1–2 hours after discontinuation of the infusion. This contrasts with heparin, which has a half-life of 60 minutes, and frequently attainment of the steady state may be delayed for as long as 48 hours of continuous heparin infusion.

We examined the pharmacology of argatroban when combined with the antiplatelet agent aspirin.
Aspirin inhibits the formation of thromboxane A$_2$, a potent platelet activator that plays a pathogenic role in a number of conditions associated with coronary thrombosis.$^{30-33}$ Thromboxane A$_2$ has been shown to limit the effects of thrombin inhibitors, including argatroban, in vivo.$^{18}$ Therefore, it is likely that these two drugs would be combined in the clinical setting. The dose of aspirin was selected to induce a maximum reduction in serum thromboxane B$_2$. At this dose, thromboxane A$_2$ biosynthesis, determined by excretion of 2,3-dinor-thromboxane B$_2$, a major enzymatic metabolite, was markedly suppressed, and bleeding time was prolonged. As expected, aspirin also induced a moderate reduction in prostacyclin biosynthesis.

Aspirin did not alter the anticoagulant effects, volume of distribution, or clearance of argatroban. There was also no interaction between aspirin and argatroban regarding bleeding time, an index of platelet and vascular function in vivo. Previous studies have demonstrated the formation of thrombin in bleeding time blood and its suppression by aspirin.$^{34}$ Furthermore, heparin at therapeutic doses prolongs bleeding time.$^{12}$ These findings suggest a role for thrombin in determining this index of hemostasis. However, the lack of response to argatroban, even when combined with a thromboxane A$_2$ inhibitor, suggests that this is not the case and that the prolongation of bleeding time with heparin is a result of direct inhibition of platelets in vivo. Whether this translates into a lower risk of hemorrhagic events with argatroban, however, is unknown because the relation between bleeding time and frequency of bleeding in patients receiving antiplatelet and anticoagulant agents is ill defined.$^{35,36}$

In conclusion, argatroban prolonged TT and aPTT in normal male volunteers to an extent that has been shown to prevent experimental thrombosis with greater efficacy than heparin.$^{18}$ The half-lives of the

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**Figure 6.** Bar graphs of effects of argatroban (Arg) therapy alone and combined with aspirin on thromboxane A$_2$ biosynthesis, determined as excretion of 2,3-dinor-thromboxane B$_2$ (2,3-dinor-TxB$_2$). Each interval represents urine collected over 2 hours. Arg had no effect on 2,3-dinor-TxB$_2$ production ($p>0.05$). Although 2,3-dinor-TxB$_2$ was significantly reduced by aspirin therapy ($p<0.01$), the combination produced no additional reduction ($p>0.05$). cr, Creatinine.

**Figure 7.** Bar graphs of effects of argatroban (Arg) alone and combined with aspirin on prostacyclin biosynthesis, determined as excretion of 2,3-dinor-6-keto-prostaglandin F$_{1\alpha}$ (2,3-dinor-6-keto-PGF$_{1\alpha}$). Arg had no significant effect on 2,3-dinor-6-keto-PGF$_{1\alpha}$. Although metabolite excretion was significantly reduced by aspirin therapy ($p<0.05$), the combination produced no additional effect ($p>0.05$). cr, Creatinine.
anticoagulant effect and of the parent compound were about 24 minutes; this predicts a more rapid onset and offset of activity than is seen with heparin. Argatroban did not alter bleeding time, even after administration of aspirin, which suggests that it has no direct effect on platelets at the dosages used. The combination of argatroban and aspirin may prove to be an effective antithrombotic strategy in the treatment of coronary thrombosis.

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