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Antithrombotic and hemostatic effects of a small molecule factor XIa inhibitor in rats

William A. Schumacher *, Steven E. Seiler, Thomas E Steinbacher, Anne B. Stewart, Jeffrey S. Bostwick, Karen S. Hartl, Eddie C. Liu, Martin L. Ogletree

Discovery Biology, Bristol-Myers Squibb Company, Pennington, New Jersey, USA

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Abstract

The effect of inhibiting activated blood coagulation factor XIa was determined in rat models of thrombosis and hemostasis. BMS-262084 is an irreversible and selective small molecule inhibitor of factor XIa with an IC_{50} of 2.8 nM against human factor XIa. BMS-262084 doubled the activated thromboplastin time in human and rat plasma at 0.14 and 2.2 μM, respectively. Consistent with factor XIa inhibition, the prothrombin time was unaffected at up to 100 μM. BMS-262084 administered as an intravenous loading plus sustaining infusion was effective against $FeCl_2$-induced thrombosis in both the vena cava and carotid artery. Maximum thrombus weight reductions of 97 and 73%, respectively ($P<0.05$), were achieved at a pretreatment dose of 12 mg/kg+12 mg/kg/h which increased the *ex vivo* activated thromboplastin time to 3.0 times control. This dose level also arrested growth of venous and arterial thrombi when administered after partial thrombus formation. BMS-262084 was most potent in $FeCl_2$-induced venous thrombosis, decreasing thrombus weight 38% ($P<0.05$) at a threshold dose of 0.2 mg/kg+0.2 mg/kg/h. In contrast, doses of up to 24 mg/kg+24 mg/kg/h had no effect on either tissue factor-induced venous thrombosis or the *ex vivo* prothrombin time. Doses of up to 24 mg/kg+24 mg/kg/h also did not significantly prolong bleeding time provoked by either puncture of small mesenteric blood vessels, template incision of the renal cortex, or cuticle incision. These results demonstrate that pharmacologic inhibition of factor XIa achieves antithrombotic efficacy with minimal effects on provoked bleeding.

Keywords: Anticoagulant; Bleeding time; Blood coagulation; Factor XIa; Hemostasis; Thrombosis

1. Introduction

Coagulation initiated by tissue factor activation is further amplified by factor XIa (Naito and Fujikawa, 1991; Gailani and Broze, 1991). This leads to maximal activation of the factor VIIIa:factor IXa and factor Va:factor Xa complexes resulting in dramatically increased thrombin production (Butenas et al., 1999). Factor XIa can also be generated by factor XIIa, via contact-activation of the traditional intrinsic cascade (Colman, 2003). The renewed importance of factor XIa gleaned from these *in vitro* experiments has been extended to the whole animal. Factor XI null mice were protected in a variety of thrombosis models (Rosen et al., 2002; Wang et al., 2005), while factor XIa inhibition with neutralizing antibodies was efficacious in rabbit (Minnema et al., 1998; Yamashita et al., 2006) and nonhuman primate (Gruber and Hanson, 2003) models of thrombosis. A peptidomimetic inhibitor of factor XIa was effective in rat venous thrombosis (Lin et al., 2006). Less is known about the *in vivo* activity of small molecule inhibitors of factor XIa.

BMS-262084 was identified from a series of compounds originally synthesized as tryptase inhibitors (Sutton et al., 2002). This compound, whose structure is shown in Fig. 1, is a mechanism-based inhibitor which covalently binds to the active site of serine proteases. When BMS-262084 was fortuitously found to prolong the activated partial thromboplastin time, a more extensive testing of serine proteases involved in coagulation and fibrinolysis was conducted. This revealed the irreversible and time-dependent inhibition of factor XIa by BMS-262084. The aqueous solubility and *in vivo* tolerability of BMS-262084 has made it a useful tool for investigating the effect of factor XIa inhibition on thrombosis and hemostasis in experimental animals.

* Corresponding author. Bristol-Myers Squibb Company, 311 Pennington-Rocky Hill Road., Pennington, New Jersey 08534-2130 USA. Tel.: +1 609 818 4333; fax: +1 609 818 7877.
E-mail address: william.schumacher@bms.com (W.A. Schumacher).

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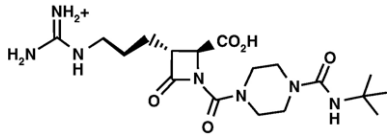

Fig. 1. BMS-262084. Structure of the β-lactam serine protease inhibitor.

The current studies were designed to evaluate BMS-262084 in rat models of venous and arterial thrombosis and provoked bleeding time. We concentrated on oxidative injury as the thrombogenic stimulus, since this method has been used to evaluate the factor XI knock-out mice. A model of venous thrombosis induced by tissue-factor was included as a control in which factor XIa inhibition was not expected to be effective. Recognizing the limited bleeding liability reported for factor XI null mice, several models of provoked bleeding were employed to increase the chance of detecting activity. The effect of BMS-262084 on rat platelets was investigated *in vitro* using whole blood aggregometry. Factor XIa selectivity *in vitro* was demonstrated with a panel of 12 human serine proteases and by selective prolongation of the plasma activated partial thromboplastin time over the prothrombin time (rat-derived serine proteases were not commercially available).

2. Materials and methods

2.1. Chemical compounds and reagents

BMS-262084 was synthesized at Bristol-Myers Squibb. In all *in vitro* enzyme assays BMS-262084 was dissolved in DMSO in a stock solution of up to 5 mM, and typically 2 μl of DMSO stock was added to a total 300 μl assay volume. These assays used a DMSO solvent because they were designed to evaluate large numbers of compounds of varying solubility. For *in vivo* studies and *in vitro* platelet assay, BMS-262084 was prepared in saline at a concentration of up to 12 mg/ml. Saline was also the vehicle for heparin (Baxter Healthcare Corp., USA).

Human enzymes used included: factor XIa and factor VIIa (Enzyme Research Labs, USA), factor Xa and plasmin (Diapharma Group, Inc., USA), factor IXa, factor XIIa and plasma kallikrein (American Diagnostica, USA), urokinase (Abbokinase; Abbot Labs, USA), α-thrombin (Sigma, USA), trypsin (Calbiochem, USA), human lung tryptase (Elastin Products, USA), recombinant tissue plasminogen activator (Genentech, USA) and recombinant tissue factor (Innovin; Dade Behring, USA). Synthetic substrates for these serine proteases included S-2222, S-2238, S-2251, S-2288, S-2366 and S-2444 from Diapharma Group, Inc. (USA), spectrozyme-tissue plasminogen activator, spectrozyme-factor IXa, spectrozyme-factor XIIa, and spectrozyme-plasma kallikrein from American Diagnostica (USA), Z-gly-pro-arg-AMC from Bachem (USA) and Chromozyme-TRY from Roche Molecular Biochemicals (USA).

2.2. Selectivity against human serine proteases

Factor XIa activity and all other enzyme assays were measured at room temperature in 96-well microplates using a 3-min incubation of enzyme (0.5 nM for factor XIa) with BMS-262084 in a buffered solution (for factor XIa this was 145 mM NaCl, 5 mM KCl, 1 mg/ml PEG 8000, 30 mM HEPES at pH 7.4). After incubation the appropriate synthetic substrate was added to start the reaction (100 μM S-2366 for factor XIa having K_m=86 μM). Enzyme velocity was measured at 405 nm in a Spectro Max Plus plate reader (Molecular Devices, USA) operated in kinetic mode and analyzed using the associated SOFTmax Pro® software. The BMS-262084 concentration producing 50% inhibition (IC_{50}) was calculated by XLfit (ID Business Solutions Ltd., Guildford, UK) using data from at least 3 separate experiments.

Applying the method described above, enzymatic activity of human α-thrombin (0.03 U/ml) was measured in 0.145 M NaCl, 0.005 M KCl, 1 mg/ml polyethylene glycol (PEG-8000), 0.030 M HEPES (pH 7.4) using 10 μM S-2238 (K_m=2.54 μM). Factor Xa (0.033 U/ml), tissue plasminogen activator (3703 U/ml), and urokinase (111 U/ml) were assayed in the same buffer as α-thrombin using 100 μM of S-2222 (K_m=87 μM), spectrozyme tissue plasminogen activator (K_m=90 μM), and S-2444 (K_m=31 μM), respectively. Plasmin activity (0.23 nM) was measured in 50 mM Tris (pH 7.8) using 100 μM S-2251 (K_m=98 μM). The factor XIIa (15 nM) assay included 150 mM NaCl, 50 mM Tris (pH 8.2), 50 mM imidazole and 100 μM spectrozyme-FXIIa (K_m=40.2 μM). Factor IXa (20 nM) activity was measured in 100 mM NaCl, 5 mM $CaCl_2$, 33% ethylene glycol, 50 mM Tris (pH 7.5) with 100 μM spectrozyme-factor IXa (K_m>100 μM). Activity of tissue factor complexed to factor VIIa was measured using factor VIIa (1 nM) with equimolar tissue factor in 20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM CHAPS and 1 mg/ml PEG 6000 (pH to 7.4) and using 100 μM S-2288 (K_m>500 μM). The kallikrein (5 nM) assay utilized 50 mM Tris (pH 8.2), 50 mM imidazole and 150 mM NaCl with 50 μM Spectrozyme-plasma kallikrein (K_m=15.2 μM). Trypsin (0.33 μg/mL) activity was measured in 50 mM Tris (pH 8.0) and 2 mM $CaCl_2$ with 100 μM Chromozyme-TRY (K_m=26.6 μM). The tryptase (0.67 nM) assay included 100 mM Tris (pH 8.0), 200 mM NaCl, and 100 μg/ml low molecular weight recombinant heparin with 200 μM Z-gly-pro-arg-AMC (K_m=237 μM).

2.3. In vitro clotting time and platelet function assays

The activated partial thromboplastin time and prothrombin time were measured in plasma using a BBL fibrometer and the procedure described for Dade Actin FSL and Dade Thromboplastin-C reagents, respectively (Baxter Healthcare Corp., Miami, FL). The international sensitivity index of the prothrombin time reagent was 2.0. The aggregation response of rat platelets to 20 μg/ml collagen was determined in whole blood using the impedance procedure and reagents described for a Model 560-CA Chrono-Log aggregometer (Havertown, PA). Plasma and blood samples were from freshly drawn arterial blood collected into 1/10 vol of 3.8% Na-citrate obtained from anesthetized rats as described in Section 2.4, or by venipuncture from consenting human volunteers.

2.4. Animal preparation and dosing for in vivo studies

Male Sprague Dawley rats (310 to 390 g) were obtained from Harlan labs and all experimental procedures conducted on these