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Supplementary Materials for

Blockade of protease-activated receptor-4 (PAR4) provides robust antithrombotic activity with low bleeding

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Materials and Methods

Generation of polyclonal antibodies against thrombin cleavage site of guinea pig PAR4

The first two exons of guinea pig PAR4 were cloned using guinea pig genomic DNA and PCR primers containing the forward primer 5' CTG TGG CCC CTG GTG CTG GGG 3' and the reverse primer 5' GCC AGG TAG CGA TCC AGG CTG AT3'. A peptide spanning the putative thrombin cleavage site (RTPQQVAPRSFPGQC) (Fig. 1) was synthesized, conjugated to keyhole limpet hemocyanin, and used to immunize rabbits (Lampire Biological Laboratories). Serum samples from rabbits were tested in solid-phase binding assays using BSA-conjugated guinea pig PAR4 immunogen peptide. High-titer antiserum was then affinity-enriched over a column prepared by coupling the immunogen peptide to Sulfolink Coupling Gel (Pierce Biotechnology). The antibodies were further purified by size exclusion chromatography using a 16/60 Superdex 200 column (Amersham Bioscience) followed by dialysis. The resulting antibody preparation was homogenous as judged by SDS-PAGE and highly selective based on lack of activity towards platelet activation induced by PAR1 AP, ADP, or a thromboxane A_2 mimetic U-46619.

Anti-PAR4 antibodies and inhibition of platelet aggregation in guinea pigs

Affinity-purified polyclonal antibodies were obtained from rabbits immunized with a peptide spanning the putative thrombin cleavage site of guinea pig PAR4 (Supplemental Methods). An in vitro platelet aggregation assay with the antibodies was conducted in a microplate assay format as described previously (*7*). Guinea pig blood was collected in 0.38% sodium citrate and centrifuged at 150 x g for 10 minutes to collect PRP, which was pre-incubated with anti-PAR4 antibodies or normal rabbit IgG for 30 min, and then stimulated with 50 nM γ-

thrombin. For ex vivo studies, guinea pigs were intravenously dosed with anti-PAR4 antibodies at 0.21, 0.7, and 2.1 mg (0.34, 1.13, or 3.4 mg/kg average body weight) or normal rabbit IgG at 2.1 mg (3.4 mg/kg average body weight), and blood was collected in 0.38% sodium citrate at the end of the guinea pig thrombosis experiment as described below. Platelet aggregation was determined as described for the in vitro experiments in response to PRP stimulation with 50 nM of γ-thrombin, 667 μM of PAR1 AP (SFFLRR) (*7*), or 2.5 mM of guinea pig PAR4 AP (SFPGQA) (*56*).

Radioligand binding of [^{3}H]BMS-986120 to human PAR4

A stable HEK293 cell line expressing human PAR4, in which the thrombin cleavage site of the human PAR4 protein was replaced by a histidine tag, was used for the binding studies. Cell membranes were prepared after centrifugation of homogenized cells at 40,000 x g for 20 min. The binding reaction was carried out in a 96-well plate at room temperature by incubation of 0.5 μg of cell membrane extract and [^{3}H]BMS-986120 in a binding buffer containing 75 mM Tris-HCl pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA, 1% BSA, and protease inhibitors. The reaction was stopped by filtration using GF/C 96-well plates. Saturation binding was measured by testing 0 to 2 nM of [^{3}H]BMS-986120 for binding to the membrane extract during a 2-hour incubation at room temperature. Specific binding was determined by subtracting non-specific binding to a cell membrane extract derived from the parental HEK293 cells. Association kinetics of [^{3}H]BMS-986120 was measured using 0.1 to 0.5 nM of the radioligand. Dissociation kinetics of [^{3}H]BMS-986120 was measured after 2 h incubation of 0.5 nM of [^{3}H]BMS-986120 with isolated membranes at room temperature followed by addition of 125 nM of unlabeled BMS-986120, with the reaction stopped at 0.5 to 210 minutes by filtration.

BRET and AlphaScreen biosensor signaling assays

β-arrestin-2 recruitment and activation of Gq family members were performed using BRET biosensor assays as described previously (*57*, *58*). ERK1/2 activity was detected using AlphaScreen SureFire assay kit (Perkin Elmer). HEK293 cells were transfected with either human *PAR4* cDNA and biosensor constructs or with human *PAR4* cDNA alone using the FuGENE 6 transfection method (Promega Corporation). Forty-eight hours after transfection, the media were gently removed, and the transfected cells were washed twice with 200 µl of PBS, followed by the addition of 80 µl de Tyrode buffer. Subsequently, 10 µl of a concentration range of BMS-986120 were added to the corresponding wells and incubated for 1 hour at 37 ^{0}C and 5% CO_2. Receptor activation was initiated by adding 10 µl of 10X stock solution of PAR4 AP at EC80 for each assay (fig. S2) or Tyrode buffer (as non-stimulated control) and incubated 10 min at 37 ^{0}C, 5% CO_2.

Calcium mobilization assays

Fluorometric Imaging Plate Reader (FLIPR) calcium mobilization assays were performed using HEK293 cells and human platelets. HEK293 Aequorin cells that stably express human PAR4 were used to screen for PAR4 antagonist activity. In addition to PAR4, these cells express endogenous PAR1, and the same cells were also used to determine selectivity against PAR1 and agonist activity for both receptors. Cells were preincubated with a fluorescent calcium indicator (Codex Biosolutions) according to manufacturer's instructions and then treated with test compounds. Changes in fluorescence intensity were measured using a Functional Drug Screening System (FDSS, Hamamatsu) to determine agonist activities. The cells were then

incubated for 30 min at room temperature followed by addition of AP for measuring antagonist activity. A PAR4 AP, Ala-(L-4-F-Phe)-Pro-Gly-Trp-Leu-Val-Lys-Asn-Gly, with improved potency was discovered from a peptide library and used at its EC_{50} of 5 μM. Compound potency was derived from 11-point concentration-response curves. Selectivity against PAR1 was tested in the same cells using a PAR1 AP (SFFLRR) (*7*), and selectivity against PAR2 was tested using CHO cells expressing human PAR2 and a PAR2 AP (2-furoyl-LIGRLO-NH_2) (*6*). The effects of BMS-986120 and BMS-200261 on α-thrombin-induced activation of washed human platelets were tested using Fluro-4AM (Molecular Probes) according to manufacturer's instructions, and changes in fluorescence intensity were measured using FLIPR Tetra Systems (Molecular Devices).

Protease Inhibition Specificity Assay

To rule out potential off-target activities of BMS-986120 against coagulation enzymes including alpha-thrombin and gamma-thrombin, a panel of 14 protease assays was applied, using chromogenic substrates selective for each protease. Microplate kinetic assays were performed to obtain reaction velocities (slope of progress curve) over 20 min at 37°C in the presence of varying concentrations of BMS-986120. Ki values were calculated based on competitive inhibition using the formula: $K_i = IC_{50} / (1+S/K_m)$ for a competitive inhibitor.

γ-Thrombin–induced human platelet-rich plasma (PRP) aggregation assay

Fresh blood was drawn by antecubital venipuncture from consenting normal healthy people. In vitro PRP aggregation was conducted in a 96-well plate format as described above. The test compounds were preincubated with PRP for 5 min at 37 ^oC, and aggregation was

initiated by addition of 50-100 nM of γ-thrombin (Haematologic Technologies), which was titrated daily to achieve 80% platelet aggregation. To prevent PAR1 activation induced by residual α-thrombin contamination, PRP was treated with recombinant hirudin (Refludan, Berlex Laboratories) at a final concentration of 1 U/mL.

α-Thrombin–induced washed human platelet aggregation assay

PRP from citrated human blood was collected as described above and treated with 100 μM of aspirin and 15% acid citrate dextrose (ACD, pH 4.4). Platelets were pelleted by centrifugation at 800 x g for 5 min, washed with 15% ACD in saline, and re-suspended in Tyrode's-HEPES buffer (137 mM NaCl, 2 mM KCl, 1.0 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 0.1% human serum albumin and 50 μM of aspirin. Platelet concentration was adjusted to $2x10^8$/ml, and aggregation was measured by light transmission using Chrono-Log 700 Aggregometer (Chronolog Corporation) or 96-well plate format as described above.

Quantitation of clopidogrel active metabolite H4 and BMS-986120 in monkey plasma

Blood samples for the determination of the clopidogrel active metabolite H4 were processed as described previously (*41*). Briefly, 3 ml of blood was collected by free flow into 3 ml vacutainer tubes (BD Vacutainer with K2EDTA). Immediately, 25 μl of 500 mM 2-bromo-3-methyoxyacetophenone was added and the tube capped and gently inverted 5 to 8 times to mix the anticoagulant and derivatizing agent. The tube was placed on chipped wet ice, and within 15 minutes of blood collection, was centrifuged at 1500 g for 15 minutes at 4°C. The plasma layer

was pipetted into an appropriately labeled screw-capped polypropylene transfer tube, capped, and stored at -80 ^{o}C until shipment for analysis. Plasma samples were sent to the PPD Bioanalytical Lab (Richmond, Virginia) to determine concentrations of the clopidogrel active metabolite H4 by liquid chromatographic mass spectrometry.

Plasma concentrations of BMS-986120 were determined by liquid chromatography-tandem mass spectrometry. BMS-986120 was isolated from monkey plasma by protein precipitation and detected using a Sciex API 4000 Qtrap interfaced with a turbo ion spray ionization source. The limit of quantitation was 1 nM.

In vitro and ex vivo whole blood aggregation assays

Adult male cynomolgus monkeys (5 to 7 kg; Bioculture Ltd) instrumented with arterial vascular access ports were used for ex vivo studies. Blood for in vitro experiments was obtained from male cynomolgus monkeys and adult male or female human volunteers. All blood samples were anticoagulated by collection into hirudin (250 mg/mL final concentration; Refludan, Berlex Laboratories) and allowed to rest for 20 min before assay. All whole blood platelet assays were performed by impedance measurements in a Model 592A aggregometer with Aggro/link WBA software according to manufacturer's instructions (Chronolog Corporation). Maximum impedance aggregation response observed 8 min after addition of platelet activator was recorded.

In the in vitro assays, BMS-986120 was added at varying concentrations, and after 10 min the blood sample was activated by adding ADP (10 µM), collagen (5 µg/ml), U46619 (thromboxane A_2 mimetic) (3 µM), PAR1 AP [Ala-Phe (4fluro) Arg-Cha-homo Arg-Tyr-NH2, 18 µM] or PAR4 AP (Ala-[Phe(4-F)]-Pro-Gly-Trp-Leu-Val-Lys-Asn-Gly-NH2). PAR4 AP was tested at concentrations of 6.25, 12.5, and 25 µM. These concentrations were selected based

upon their ability to consistently induce maximal aggregation, with 6.25 μM representing the threshold concentration for this effect. Other agonists were tested at a single maximal concentration for specificity testing.

In the ex vivo assays, conscious and unsedated chaired monkeys were dosed by oral gavage with either vehicle or BMS-986120 at 0.2 mg/kg. Blood samples were obtained for platelet function testing before a dose, and at 2, 4, 24, and 48 h after a dose. Aggregation was induced by PAR4 AP at concentrations of 6.25, 12.5, and 25 μM.

Ex vivo biomarker assays in the in vivo studies in monkeys

Platelet aggregation assay in whole blood. Blood was collected by free flow from an arterial cannula into 15-ml centrifuge tubes pre-loaded with hirudin (lepirudin, 25 μg/ml final concentration). The blood was allowed to rest for 10-20 minutes. In the clopidogrel study, whole blood platelet aggregometry induced by 20 μM ADP, 5 μg/mL collagen, 18 μM high affinity PAR1 AP [Ala-Phe (4fluro) Arg-Cha-homo Arg-Tyr-NH_2] (*8*), and 12.5 μM PAR4 AP (Ala-[Phe(4-F)]-Pro-Gly-Trp-Leu-Val-Lys-Asn-Gly-NH2) was performed using a Chronolog model 592A whole blood aggregometer (Chronolog Corporation) according to the manufacturer's instructions. In the BMS-986120 study, whole blood platelet aggregometry induced by 20 μM ADP, 5 μg/mL collagen, 18 μM PAR1 AP, and 1.56 to 400 μM PAR4 AP was performed. Except where noted, single determinations were made for each concentration of agonist. Platelet aggregation was monitored and recorded for 8 minutes after the addition of agonist, and the peak aggregation response (amplitude in ohms) was used to evaluate the ex vivo effect of antagonist on platelet aggregation. Inhibition of platelet aggregation (IPA) to 20 μM ADP in whole blood was also determined.

Platelet aggregation assay in PRP. Platelet aggregation induced by 20 μM ADP was performed in a Chronolog aggregometer (model 490) using 250 μl of platelet-rich plasma according to the manufacturer's instructions (*59*). The peak aggregation response was used to evaluate the ex vivo inhibitory effect of clopidogrel.

Receptor occupancy

Percent inhibition of [^{33}P]2MeS-ADP (~2,000 Ci/mol) (PerkinElmer) binding to $P2Y_1$-blocked platelets, defined as $P2Y_{12}$ receptor occupancy, was measured in washed platelets as described previously (*59*). The only modification was in the preparation of monkey platelet-rich plasma, which was obtained from citrated whole blood centrifuged at 400 x g for 5 min. All other steps and the binding assay protocol were the same as reported (*59*).

Coagulation Assays

Arterial blood samples for the determination of ex vivo aPTT, PT, and TT (Siemens Healthcare Diagnostics) were collected in tubes containing one-tenth the volume of 0.129 M sodium citrate. Clotting times were measured with an automated coagulation analyzer (Sysmex, Dade Behring Inc.) according to the manufacturer's instructions. The aPTT, PT, and TT reagents were reconstituted and assays were performed according to the manufacturer's instructions.

Synthesis of BMS-986120 (fig. S12)

5-(Benzyloxy)-7-methoxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (2): A solution of **1** (30.00 g, 0.134 mol, see reference *60* for preparation) in *N*,*N*-dimethylformamide (DMF, 400 ml) was treated with powdered anhydrous potassium carbonate (19.41 g, 0.14 mol), added all at once. The resulting mixture was stirred *in vacuo* for 10 min. and then flushed with nitrogen. The reaction flask was placed in a water bath (22°C) and treated with benzyl bromide (24.03 g, 0.14 mol) added dropwise over 15 min. The resulting mixture was then stirred at 22°C for 18 h (no starting material remained by TLC). The solid was filtered and washed with DMF. The filtrate was evaporated *in vacuo,* and the residual oil was diluted with ethyl acetate (500 ml) and washed with cold 0.1-N HCl, saturated sodium bicarbonate, and brine. After drying over anhydrous magnesium sulfate, evaporation of the solvent gave a thick syrup. Crystallization from ethyl acetate (50 mL) and hexane (150 mL) gave 35.17 g of 5-(benzyloxy)-7-methoxy-2,2-dimethyl-4H-benzo[d][1,3]dioxin-4-one as large colorless prisms. Chromatography of the mother liquors on silica gel (4 x 13 cm column, elution with toluene - ethyl acetate 0-5%) gave 6.64 g of additional material to afford a total yield of 41.81 g (99%). HRMS (ESI) calculated for $C_{18}H_{19}O_5$ $[M+H]^+$ *m/z* 315.1227, found 315.1386. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.68 (s, 6H), 3.77 (s, 3H), 5.19 (s, 2H), 5.19 (s, 2H), 6.04 (d, J = 2.03 Hz, 1H), 6.15 (d, *J* = 2.03 Hz, 1H), 7.27 (broad t, 1H), 7.36 (broad t, 2H), 7.52 (broad d, 2H).

2-(Benzyloxy)-6-hydroxy-4-methoxybenzaldehyde (3): A solution of **2** (6.76 g, 21.5 mmol) in dichloromethane (120 mL) was cooled to -78°C and treated with 43 mL (64.5 mmol) of a 1.5 M solution of diisobutylaluminum hydride in toluene, added dropwise over 20 min. The resulting mixture was stirred at -78°C for 3 h. The reaction mixture was quenched by the careful addition

of methanol (5 mL) added dropwise over 15 min, followed by 1 N hydrochloric acid (50 mL) added dropwise over 15 min. The cooling bath was then removed, and an additional 150 mL of 1 N hydrochloric acid was added over 20 min. The mixture was stirred at 22°C for 2 h and diluted with dichloromethane (400 mL). The organic phase was collected, and the aqueous phase (pH ~ 1) was extracted with dichloromethane (3 x 50 mL). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, and concentrated *in vacuo*. The residual oil was diluted with tetrahydrofuran (70 mL), treated with 10 mL of 0.1-N HCl, and stirred at 20°C for 2 h. The reaction mixture was diluted with ethyl acetate (300 ml), washed with brine, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to give a clear oil. Chromatography on silica gel (4 x 13 cm, elution toluene) gave 4.08 g (73% yield) of the title aldehyde as a clear oil which solidified on standing. HRMS (ESI) calculated for $C_{15}H_{15}O_4$ $[M+H]^+$ *m/z* 259.0965, found 259.1153. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 3.80 (s, 3H), 5.07 (s, 2H), 5.97 (d, *Ji* = 2.1 Hz, 1H), 6.01 (d, *J*= 2.1 Hz, 1H), 7.3 - 7.4 (m, 5 H), 10.15 (s, 1H), 12.49 (s, 1H).

1-(4-(Benzyloxy)-6-methoxybenzofuran-2-yl)ethanone (4): A solution of **3** (3.46 g, 13.4 mmol) in DMF (50 mL) was treated with powdered anhydrous cesium carbonate (4.58 g, 14.05 mmol) added all at once. The resulting mixture was stirred *in vacuo* for 10 min. and then flushed with nitrogen. The reaction flask was placed in a water bath (22°C) and treated with chloroacetone (1.74 g, 18.7 mmol) added dropwise over 5 min. The resulting mixture was then stirred at 22°C for 18 h (no starting aldehyde left by TLC and formation of the intermediate alkylated aldehyde). The solid was filtered and washed with *N*,*N*-dimethylformamide. The filtrate was evaporated *in vacuo*, and the residual oil was diluted with ethyl acetate (300 mL) and washed with cold 0.1 N hydrochloric acid, saturated sodium bicarbonate, and brine. After drying

over anhydrous magnesium sulfate, evaporation of the solvent gave a thick syrup. This syrup was diluted with tetrahydrofuran (50 mL) and ethyl acetate (50 mL), treated p-toluenesulfonic acid monohydrate (0.2 g), and stirred at 20°C for 1 h (TLC indicated complete cyclization of the intermediate alkylated aldehyde to the benzofuran). The reaction mixture was diluted with ethyl acetate (300 mL) and washed with saturated sodium bicarbonate and brine. After drying over anhydrous magnesium sulfate, evaporation of the solvent gave a thick syrup. Chromatography on silica gel (4 x 12 cm, elution toluene - ethyl acetate 2-4%) gave 3.51 g (88% yield) of the title benzofuran as a yellow solid. Recrystallization from ethyl acetate (10 mL) and hexane (20 mL) gave the title material as large yellow prisms (3.15 g). HRMS (ESI) calculated for $C_{18}H_{17}O_4$ $[M+H]^+$ *m/z* 297.1121, found 297.1092. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 2.51 (s, 3H), 3.82 (s, 3H), 5.13 (s, 2H), 6.37 (d, *J* = 1.77 Hz, 1H), 6.63 (broad s, 1H), 7.34 (broad t, 1H), 7.39 (broad t, 2H), 7.44 (broad d, 2H), 7.55 (d, J = 0.7 Hz,1H).

1-(4-(Benzyloxy)-6-methoxybenzofuran-2-yl)-2-bromoethanone (5): A 250-mL, three-necked flask equipped with a magnetic stirring bar and purged with a nitrogen atmosphere was charged with anhydrous tetrahydrofuran (25 mL) followed by 9.3 mL (9.3 mmol) of a 1-M solution of lithium bis(trimethylsilyl)amide in tetrahydrofuran. The mixture was cooled to -78°C and treated with a solution of **4** (2.40 g, 8.1 mmole) in tetrahydrofuran (20 mL) added dropwise over 10 min. The resulting mixture was then stirred at -78°C for 45 min. Then chlorotrimethylsilane (1.18 mL, 9.31 mmol) was added dropwise over 5 min and the resulting solution was stirred at -78°C for another 20 min. The cooling bath was then removed and the mixture was allowed to warm to room temperature over 30 min. The reaction mixture was then quenched by addition to a cold solution of ethyl acetate (200 mL), saturated sodium bicarbonate (30 mL), and ice. The organic

phase was rapidly dried over anhydrous magnesium sulfate (magnetic stirring) and evaporated *in vacuo* to give the silyl enol ether as an oil which was co-evaporated with toluene (20 mL). The silyl enol ether was then dissolved in dry tetrahydrofuran (40 mL), cooled to -20°C, and treated with solid sodium bicarbonate (0.10 g) followed by *N*-bromosuccinimide (1.44 g, 8.1 mmol), added in small portions over 15 min. The reaction mixture was allowed to warm to 0°C over 2 h and then quenched by addition of ethyl acetate (300 mL) and saturated sodium bicarbonate. The organic phase was washed with brine, dried over anhydrous magnesium sulfate, and evaporated to give an orange oil. Chromatography on silica gel (4 x 12 cm, elution toluene - ethyl acetate 0-5%) gave 2.62 g (86% yield) of the title bromomethylketone as a yellow solid. Recrystallization from ethyl acetate (10 mL) and hexane (20 mL) gave yellow prisms (2.30 g). HRMS (ESI) calculated for $C_{18}H_{16}BrO_4$ $[M+H]^+$ *m/z* 375.0226, found 375.0277. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 3.84 (s, 3H), 4.33 (s, 2H), 5.14 (s, 2H), 6.38 (d, *J* = 1.76 Hz, 1H), 6.64 (broad s, 1H), 7.35 (broad t, 1H), 7.40 (broad t, 2H), 7.44 (broad d, 2H), 7.70 (s, 1H).

6-(4-(Benzyloxy)-6-methoxybenzofuran-2-yl)-2-bromoimidazo[2,1-*b*][1,3,4]thiadiazole (6): A mixture of **5** (3.00 g, 8.0 mmol) and 5-bromo-1,3,4-thiadiazol-2-amine (1.65 g, 9.16 mmol) in isopropanol (100 mL) was heated in a pressure flask equipped with a magnetic stirring bar at 78-80°C for 18 h (homogeneous after 20 min and then formation of a precipitate after 2 h). The cooled mixture was transferred into five 20 mL microwave vials and then heated in a microwave apparatus to 150°C for 30 min. Each vial was then diluted with dichloromethane (250 mL), washed with saturated sodium bicarbonate (25 mL) and brine (25 mL), and dried over anhydrous magnesium sulfate. The fractions were combined and concentrated *in vacuo*. Chromatography of the orange-brown residual solid on silica gel (4 x 10 cm, slow elution with dichloromethane due

to poor solubility) gave 2.96 g of the title imidazothiadiazole contaminated with some 1-(4-(benzyloxy)-6-methoxybenzofuran-2-yl)ethanone. The solid material was triturated with ethyl acetate (20 mL), filtered, washed with ethyl acetate (10 ml), and dried *in vacuo* to give 2.34 g (64% yield) of pure title imidazothiadiazole as an off white solid which was used as such for the next step. HRMS (ESI) calculated for $C_{20}H_{15}BrN_3O_3S$ $[M+H]^+$ *m/z* 456.00175, found 456.00397. ^1H NMR ($CDCl_3$, 600 MHz) δ 3.82 (s, 3H), 5.16 (s, 2H), 6.38 (d, *J* = 1.67 Hz, 1H), 6.66 (broad s, 1H), 7.15 (s, 1H), 7.31 (broad t, 1H), 7.38 (broad t, 2H), 7.45 (broad d, 2H), 8.02 (s, 1H).

6-(4-(Benzyloxy)-6-methoxybenzofuran-2-yl)-2-methoxyimidazo[2,1-*b*][1,3,4]thiadiazole (7): A solution of **6** (2.30 g, 5.04 mmol) in a mixture of dichloromethane (180 mL) and methanol (45 mL) was treated at 22°C with 4.2 mL of a 25 wt. % solution of sodium methoxide in methanol (0.2 mmol) added in one portion. More methanol (45 mL) was added, and the mixture was stirred for 1 h. The reaction mixture was quenched by the addition of 25 mL of 1 N hydrochloric acid, followed by 20 ml of saturated sodium bicarbonate. The solvent was evaporated under reduced pressure and the residue was diluted with dichloromethane (400 mL), washed with brine, dried over anhydrous magnesium sulfate, and evaporated *in vacuo*. Chromatography of the residue on silica gel (3 x 10 cm, elution with dichloromethane - ethyl acetate 0-4%) gave 1.70 g (83% yield) of the title compound as a white solid. This material was recrystallized from ethyl acetate (30 mL per gram, 80% recovery) to give white needles. HRMS (ESI) calculated for $C_{21}H_{18}N_3O_4S$ $[M+H]^+$ *m/z* 408.1013, found 408.1024. ^1H NMR ($CDCl_3$, 600 MHz) δ 3.81 (s, 3H), 4.18 (s, 3H), 5.16 (s, 2H), 6.37 (d, *J* = 1.75 Hz, 1H), 6.67 (broad s, 1H), 7.07 (s, 1H), 7.31 (broad t, 1H), 7.37 (broad t, 2H), 7.45 (broad d, 2H), 7.81 (s, 1H).

6-Methoxy-2-(2-methoxyimidazo[2,1-*b*][1,3,4]thiadiazol-6-yl)benzofuran-4-ol (8): A mixture of **7** (1.250 g, 3.06 mmol) and pentamethylbenzene (3.17 g, 21.4 mmol) in dichloromethane (200 mL) was cooled to -78°C under a nitrogen atmosphere and then treated immediately (to avoid crystallization) with 8 mL (8 mmol) of a 1 M solution of boron trichloride in dichloromethane, added dropwise over 3 min. The resulting mixture was stirred at -78°C for 1 h. The reaction mixture was then quenched by the addition of a solution of sodium bicarbonate (6 g) in water (100 mL) added in one portion. The cooling bath was removed, and the resulting mixture was stirred at room temperature for 1 h. The solid formed was filtered and washed successively with water (50 mL) and dichloromethane (50 mL). The filter cake was allowed to soak with anhydrous ethanol (15 ml) and then sucked dry. The white solid obtained was then dried under vacuum for 24 h to give 0.788 g (80% yield) of pure title material (> 95% by HPLC). The combined filtrate and washings were diluted with dichloromethane (600 mL) and stirred in a warm water bath until the organic phase was clear with no apparent solid in suspension. The organic phase was collected, dried over anhydrous magnesium sulfate, and rapidly filtered while still warm. The filtrate was evaporated and the residue (product and pentamethylbenzene) was triturated with toluene (20 mL), and the solid collected and washed with toluene (20 mL) to give 0.186 g (19% yield, 99% combined yield) of title material as a tan solid (> 95% by HPLC). HRMS (ESI) calculated for $C_{14}H_{12}N_3O_4S$ $[M+H]^+$ *m/z* 318.0543, found 318.0578. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 3.71 (s, 3H), 4.16 (s, 3H), 6.21 (d, *J* = 1.87 Hz, 1H), 6.61 (broad s, 1H), 6.95 (s, 1H), 8.29 (s, 1H), 9.96 (s, 1H).

Methyl 5-methyl-2-morpholinothiazole-4-carboxylate (10): A solution of methyl 2-bromo-5-methylthiazole-4-carboxylate (2.80 g, 11.86 mmol) and morpholine (4.5 mL, 51.7 mmol) in THF (10 mL) was heated at reflux under nitrogen for 18 h. The volatiles were then removed under

reduced pressure, and the crude product was purified by silica gel chromatography (40 g column, 0 to 40% EtOAc in DCM), to give the title compound (2.20 g, 77%) as a yellow solid. Liquid chromatography-tandem mass spectrometry (LCMS) (Atmospheric Pressure Chemical Ionization (APCI)): calculated for $C_{10}H_{15}N_2O_3S$ $[M+H]^+$ *m/z* 243.07, found 243.1. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.89 (s, 3H), 3.77-3.83 (m, 4H), 3.41-3.47 (m, 4H), 2.64 (s, 3H).

(5-Methyl-2-morpholinothiazol-4-yl)methanol (11): A solution of methyl 5-methyl-2-morpholinothiazole-4-carboxylate (4.76 g, 19.65 mmol) in tetrahydrofuran (35 mL) under nitrogen was cooled to 0°C and treated with methanol (1.6 mL, 39.5 mmol), followed by $LiBH_4$ (0.86 g, 39.5 mmol) added in small portions over 5 min. After 30 min, the cooling bath was removed and the resulting turbid solution was stirred at room temperature for 4 h. TLC indicated that the reduction was complete. The reaction mixture was cooled again in ice and quenched dropwise with a solution of acetic acid (2 mL) in water (8 mL). The reaction mixture was then diluted with dichloromethane (300 mL), washed with saturated sodium bicarbonate (10 mL), brine, and dried over anhydrous magnesium sulfate. After concentration under reduced pressure, the white solid residue was chromatographed on silica gel (elution with 0 – 10 % ethanol in dichloromethane) to give the title alcohol as a white solid (3.97 g, 95%). LCMS (APCI): calculated for $C_9H_{15}N_2O_2S$ $[M+H]^+$ *m/z* 215.08, found 215.1. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 4.48 (d, *J* = 4.7 Hz, 2H), 3.77-3.83 (m, 4H), 3.37-3.43 (m, 4H), 2.30 (t, *J* = 4.7 Hz, 1H), 2.28 (s, 3H).

4-(4-(((6-Methoxy-2-(2-methoxyimidazo[2,1-*b*][1,3,4]thiadiazol-6-yl) benzofuran-4-yl)oxy)methyl)-5-methylthiazol-2-yl)morpholine (BMS-986120): A round bottom flask was

charged with **8** (431 mg, 1.36 mmol) and **11** (320 mg, 1.49 mmol), flushed with N_2, and dry THF (20 mL) was added. To the resulting suspension was added tri-*n*-butylphosphine (0.84 mL, 3.39 mmol), and then a solution of 1,1'-(azodicarbonyl)dipiperidine (856 mg, 3.39 mmol) in dry THF (10 mL) was added dropwise over 1 h. The resulting beige suspension was stirred for an additional 2 h at room temperature. The reaction mixture was concentrated, suspended in CH_3CN, sonicated, filtered, and washed with CH_3CN (50 mL, to remove $OP(Bu)_3$). The resulting cream solid was purified by silica gel chromatography (40 g column, 0 to 40% EtOAc in CH_2Cl_2, the crude product was adsorbed on SiO_2). The desired product was isolated as a cream solid (467 mg, 0.91 mmol, 67%). HRMS (ESI): calculated for $C_{23}H_{24}N_5O_5S_2$ $[M+H]^+$ *m/z* 514.122, found 514.126. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.83 (s, 1H), 7.06 (d, *J* = 0.8 Hz, 1H), 6.69 (d, *J* = 0.8 Hz, 1H), 6.50 (d, *J* = 2.0 Hz, 1H), 5.05 (s, 2H), 4.21 (s, 3H), 3.85 (s, 3H), 3.78- 3.84 (m, 4H), 3.39- 3.46 (m, 4H), 2.37 (s, 3H). Purity >98% by analytical HPLC (column 4.6 x 30mm, Zorbax XD8-C18, 3.5 micron, solvent A: MeOH/H_2O/TFA 5/95/0.5, solvent B MeOH/H_2O/TFA 95/5/0.5**,** gradient: 100 % A 0-2 min, then 0 – 100% B 2-4 min, flow rate 3 ml/min, monitoring absorbance at 220 and 254 nm).

Synthesis of [^{3}H]BMS-986120 (fig. S13)

[^{3}H]BMS-986120: BMS-986120 (1.00 mg, 1.95 μmol) and Crabtree's Catalyst (2.45 mg, 3.04 μmol) were weighed into a 10-ml Tri-Sorber flask with stir bar under nitrogen. CH_2Cl_2 (0.5 mL) and EtOH (5.0 mL) were added. The flask was attached to a Tri-Sorber Model TS-1000 Manifold System, and tritium gas (0.6 Ci) was introduced into the reaction. The reaction was vigorously stirred at room temperature for 18 h. The reaction mixture was removed from the Tri-Sorber Manifold System, and the solvent was removed under reduced pressure. Labile tritium

was removed by dissolving the crude product in EtOH (5 mL) followed by the removal of the solvent under reduced pressure three times. The resulting crude product was purified by preparative HPLC (Agilent 1100 HPLC system with a diode array detector for UV detection. Phenomenex Luna column, 5 µm, C18, 10 x 250 mm, Mobile phase A: 60% Water (0.1% TFA) : 40% CH_3CN (0.1% TFA), Mobile phase B: 100% CH_3CN, Gradient: 0 min 100% A at 1 mL/min, 5 min 100% A at 1 mL/min, 6 min 100% A at 4 ml/min, 30 min 100% A at 4 mL/min, 35 min 100% B at 4 mL/min, UV detection at 305 nm). Collected peak with retention time = 24.1 – 26.5 min). The pooled product fractions were solvent exchanged into 90% EtOH (31 mL) to give 31 mCi (26% yield, 0.26 mg, 0.51 µmol) of product at 1.0 mCi/mL.

HPLC/Rad analysis (Agilent 1100 HPLC system with a diode array detector for UV detection and an IN/US β-Ram Model 3 detector using LauraLite Version 3.4.1.10 software for radiochemical detection. Phenomenex Luna, 5 µm, C18, 4.6 x 150 mm, Mobile phase A: Water (0.1% TFA), Mobile phase B: CH_3CN, Gradient: 0 min 40% B, 15 min 40% B, 20 min 100% B, 30 min 100% B, flow rate = 1.2 ml/min. UV detection at 305 nm showed the product to be >99.9% chemically pure and 97.9% radiochemically pure. Co-injection of the labeled product with an unlabeled BMS-986120 standard produced a single peak with a retention time of 9.60 min. LC/MS analysis (Finnigan LXQ LC/MS System with detection by ESI (+) ion.

LC/MS Method: Phenomenex Gemini 5 µm, C18, 50 x 3.0 mm, flow rate = 0.50 mL/min, UV detection by PDA at 200 - 400 nm. Mobile phase A = Water (0.1% formic acid), Mobile phase B = CH_3CN (0.1% formic acid), Gradient: 0 min 10% B, 4 min 100% B) retention time = 3.49 min, m/z = 514.33(18%) / 515.33(6%) / 516.33(62%) / 517.33(19%) / 518.25(100%) / 519.25(28%) / 520.25(83%) / 521.25(24%) 522.25(35%) / 523.25(8%) / 524.25(6%). The specific activity was measured by LC/MS at 61.1 Ci/mmol. ^3H NMR (T-H decoupled)(DMSO-

d_6, 320 MHz) δ ppm: 6.96 (s, 0.085T), 3.32 and 3.28 (s, 0.915T) (^{3}H NMR spectra were recorded on a 320 MHz Bruker Avance spectrometer.)

SUPPLEMENTARY FIGURES

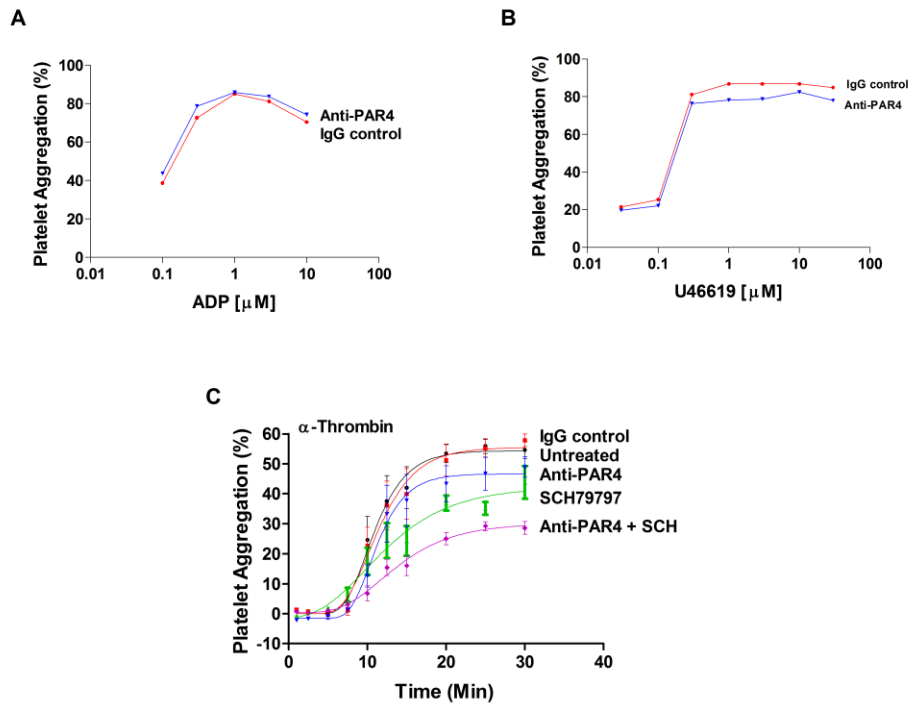
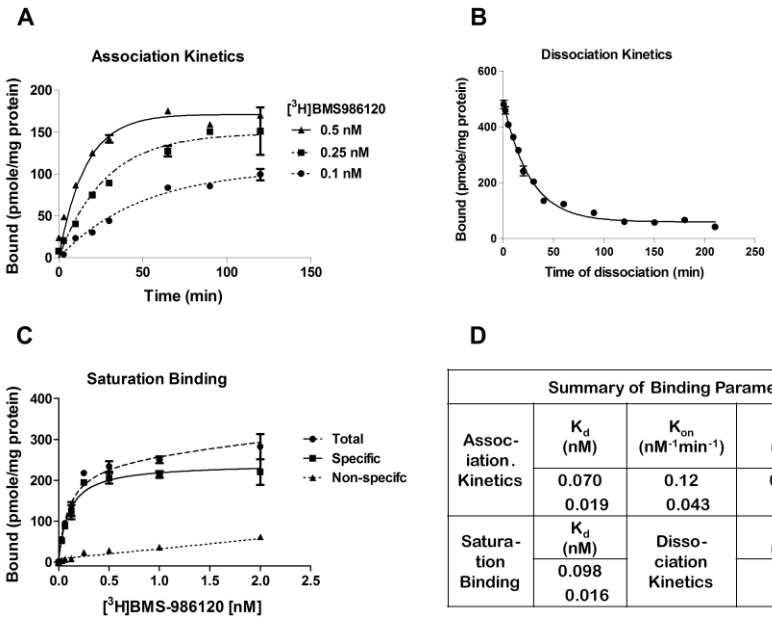


Fig. S1. In vitro characterization of anti-PAR4 antibodies in guinea pig platelet aggregation assays. Guinea pig platelet platelet-rich plasma was stimulated with different concentrations of ADP (A) or the thromboxane analog U46619 (B) in the presence of anti-PAR4 antibodies (100 μg/ml, blue) or IgG control (100 μg/ml, red). Data are representative of 2 replicate experiments. (C) Guinea pig washed platelets were stimulated with 3 nM α-thrombin in the presence of the anti-PAR4 antibodies (100 μg/ml, blue), IgG control (100 μg/ml, red), PAR1 antagonist SCH79797 (5 μM, green), or a combination of anti-PAR4 and SCH79797 (purple). Data are mean ± SEM (*n*=4).



Summary of Binding Parameters				
Assoc-iation. Kinetics	K_d (nM)	K_{on} ($nM^{-1}min^{-1}$)	K_{off} (min^{-1})	$T_{1/2\ cal}$ (min)
	0.070 0.019	0.12 0.043	0.0082 0.0016	85
Satura-tion Binding	K_d (nM)	Disso-ciation Kinetics	K_{off} (min^{-1})	$T_{1/2}$ (min)
	0.098 0.016		0.030 0.0035	24

Fig. S2. High-affinity and reversible binding of BMS-986120 to human PAR4.
Representative binding curves of [^{3}H]BMS-986120 to cell membrane extract from HEK293 cells expressing recombinant human PAR4 and summary parameters (means ± SD, *n* = 3).

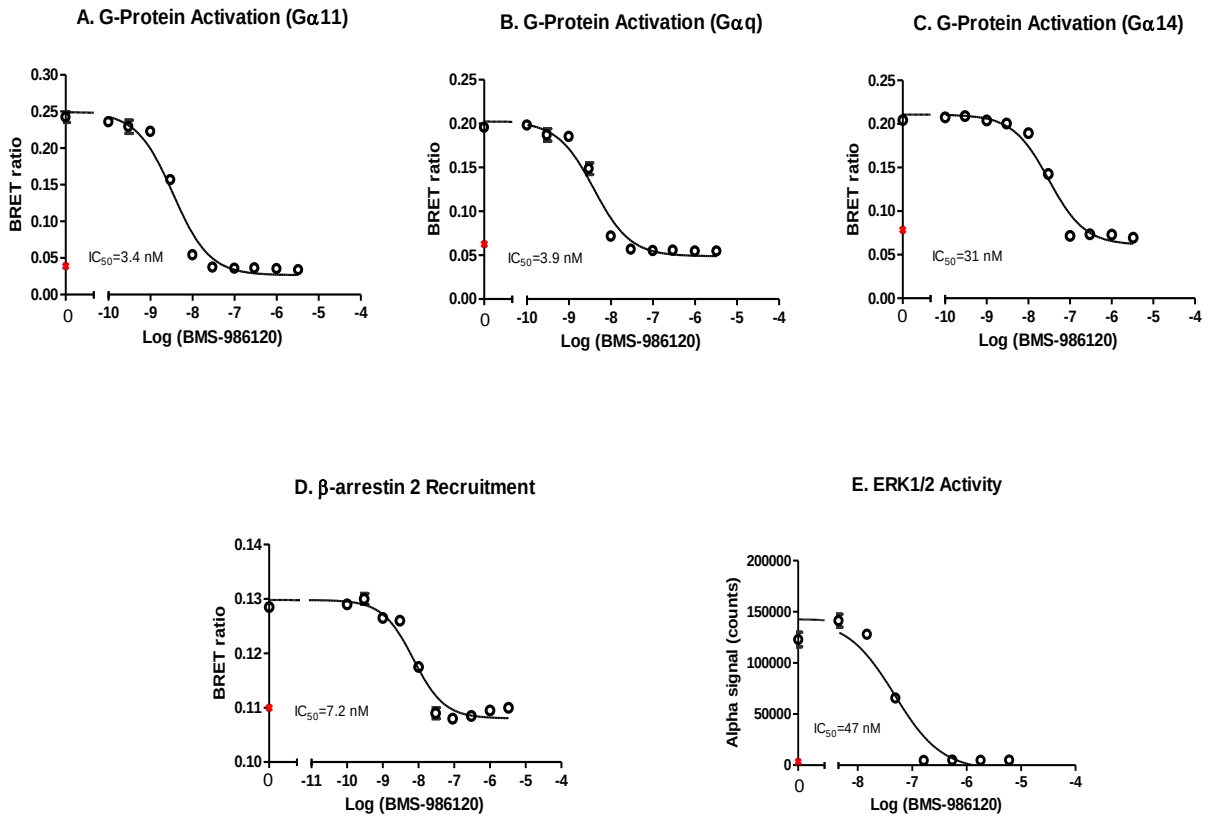


Fig. S3. Inhibition of PAR4 AP–induced signaling pathways by BMS-986120.
Bioluminescence resonance energy transfer (BRET) and AlphaScreen biosensor assays for Gα11, Gαq, Gα14, β-arrestin-2 recruitment, and ERK1/2 activation were performed using PAR4-expressing HEK293 cells. Signaling pathways were stimulated using PAR4 AP at its EC80 for each assay (10 μM for A, B, and C; 20 μM for D; and 1 μM for E). Red X symbols indicate the non-stimulated levels.

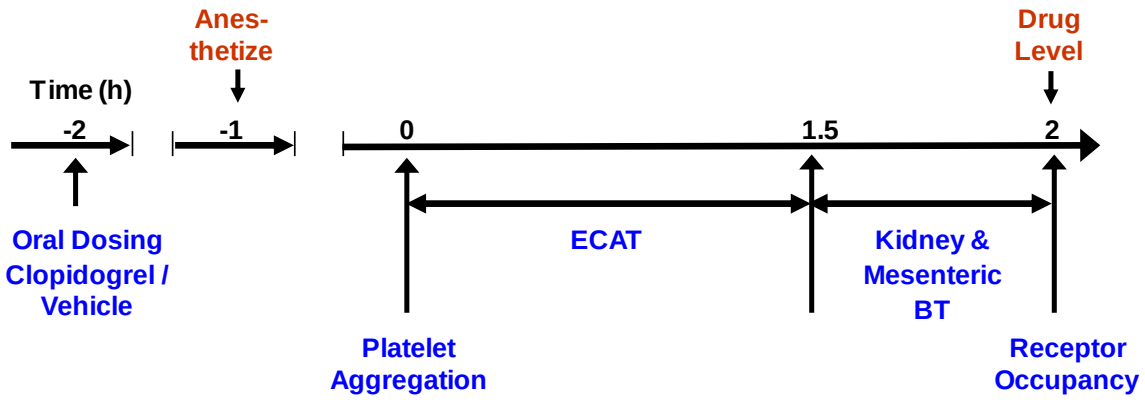


Fig. S4. Schematic representation of the clopidogrel study protocol. Clopidogrel study with *n* = 30 monkeys. Vehicle was 0.6% methocel. ECAT, electrolytic-mediated carotid arterial thrombosis; BT, bleeding time.

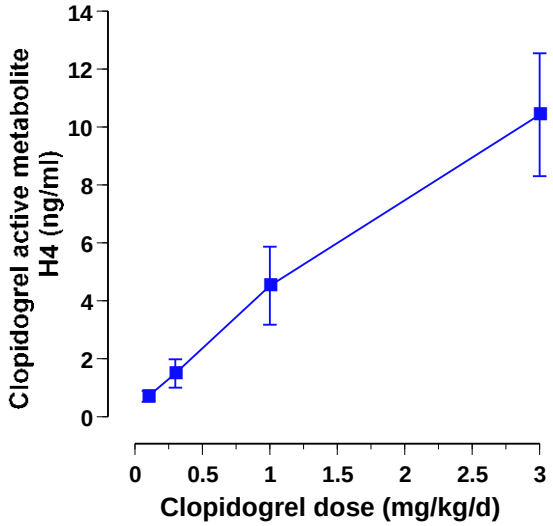


Fig. S5. Dose-dependent increases in plasma concentration of clopidogrel active metabolite in monkeys. Clopidogrel active metabolite H4 formation at 2 hours after a dose as a function of clopidogrel dose. Data are means ± SEM (*n* = 6 per group).

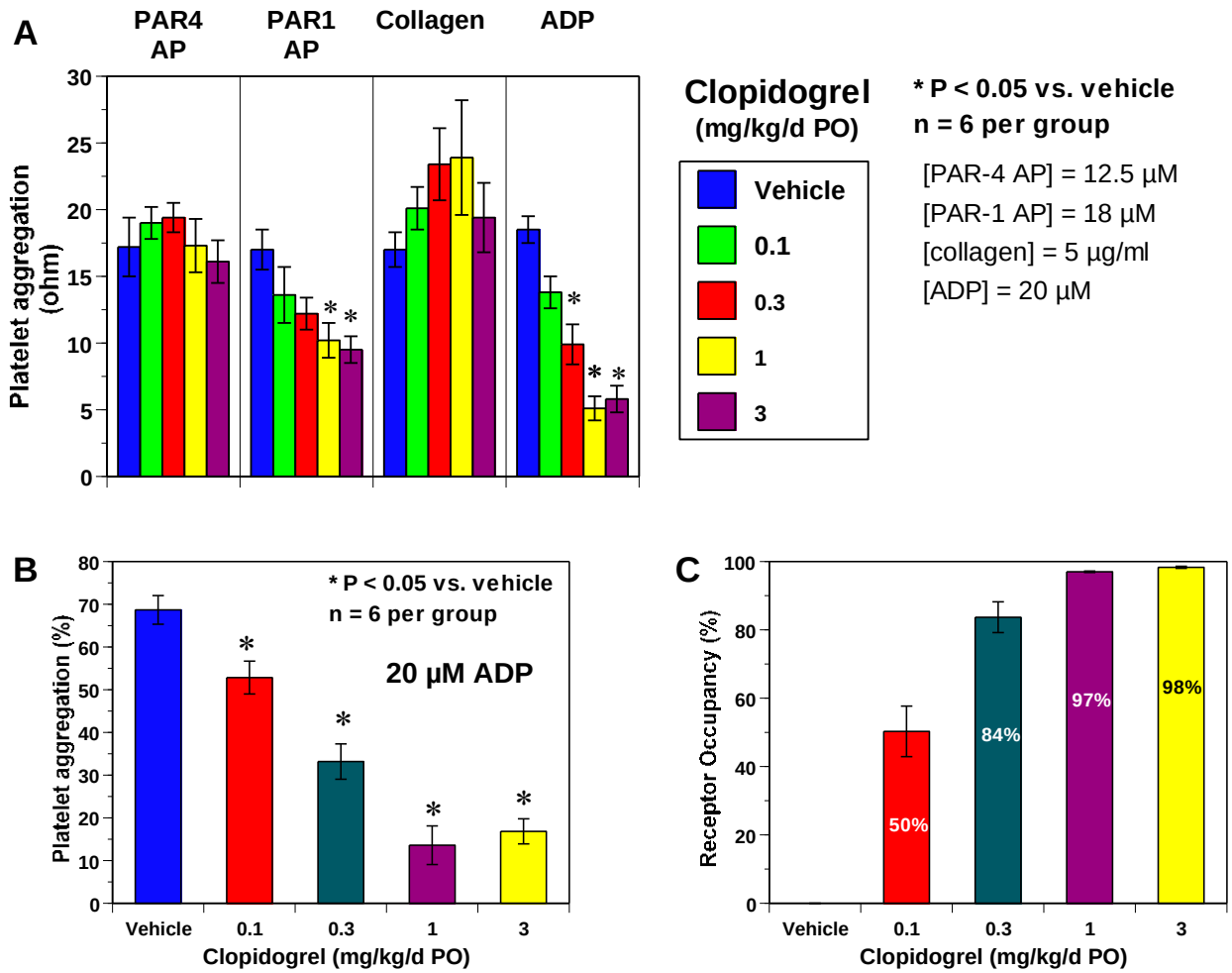


Fig. S6. Ex vivo effects of clopidogrel on platelet aggregation and P2Y$_{12}$ receptor occupancy in monkeys. (**A**) Whole-blood platelet aggregation was stimulated ex vivo by PAR4 and PAR1 AP, collagen, or ADP. (**B**) Platelet aggregation in platelet-rich plasma was induced by 20 µM ADP. (**C**) P2Y$_{12}$ receptor occupancy measured in response to varying doses of clopidogrel. Data are means ± SEM (n = 6 per group). *P < 0.05 versus vehicle, one-way ANOVA and Tukey's test. Exact P values are as follow: (A) PAR1: P = 0.0240, 1 mg/kg/d versus vehicle; P = 0.0111, 3 mg/kg/d versus vehicle. ADP: P = 0.0002, 0.3 mg/kg/d versus vehicle; P < 0.0001, 1 mg/kg/d versus vehicle; P < 0.0001, 3 mg/kg/d versus vehicle (B) P = 0.0488, 0.1 mg/kg/d versus vehicle; P < 0.0001, 0.3 mg/kg/d versus vehicle; P < 0.0001, 1 mg/kg/d versus vehicle; P < 0.0001, 3 mg/kg/d versus vehicle.

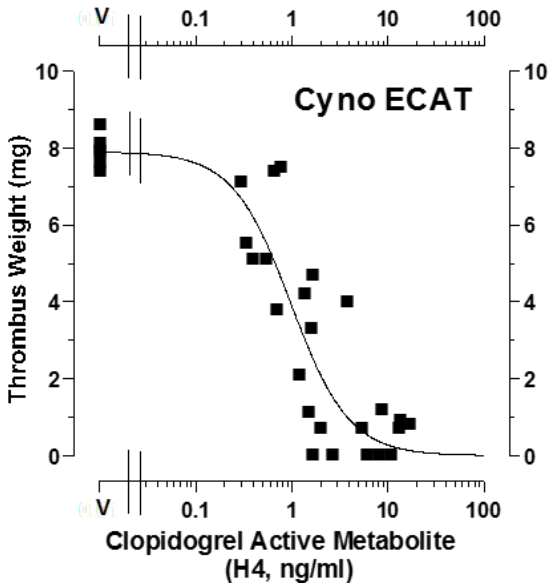


Fig. S7. Dose-response effects of clopidogrel active metabolite H4 on thrombus weight in monkeys.

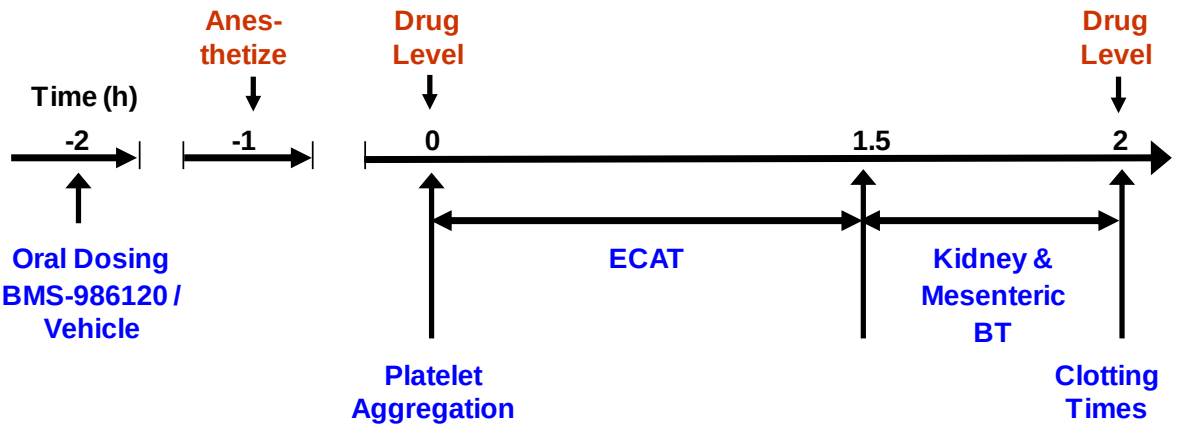


Fig. S8. Schematic representation of the BMS-986120 study protocol. BMS-986120 study with *n* = 32 monkeys. Vehicle was 40/60 w/w TPGS/PEG400. ECAT, electrolytic-mediated carotid arterial thrombosis; BT, bleeding time.

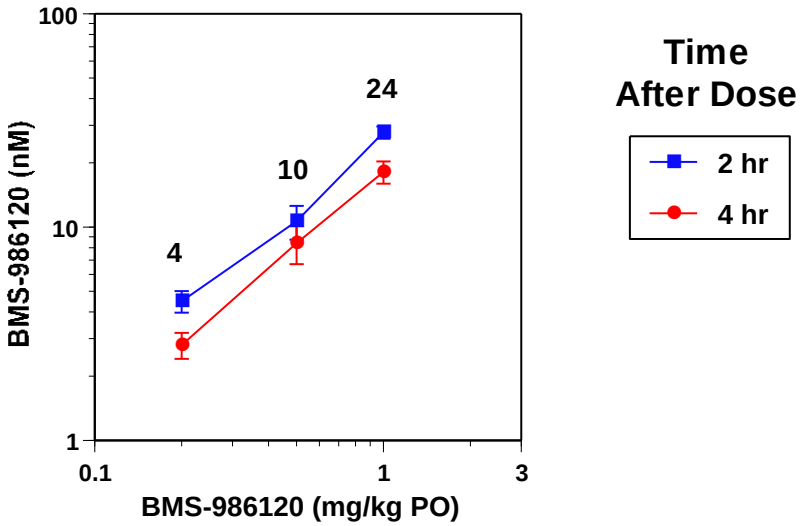


Fig. S9. Dose-dependent increases in plasma concentration of BMS-986120 in monkeys. BMS-986120 at 2 and 4 hours after a dose as a function of BMS dose. The plasma concentrations of BMS-986120 measured at 2 hours and 4 hours after a dose were similar and averaged 4, 10, and 24 nM at the doses of 0.2, 0.5, and 1 mg/kg. Data are means ± SEM (*n* = 8 per group).

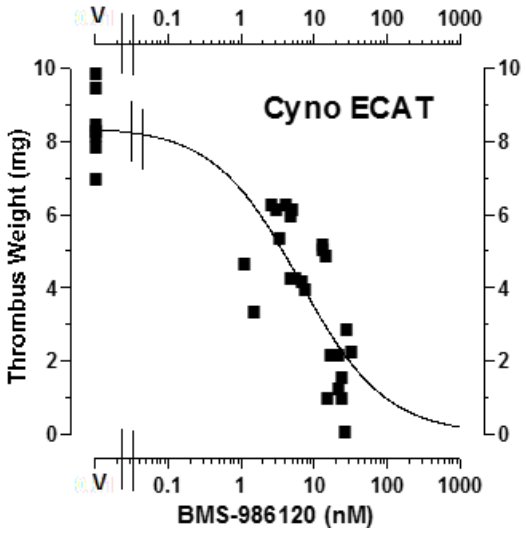


Fig. S10. Dose-response effects of BMS-986120 on thrombus weight in monkeys.

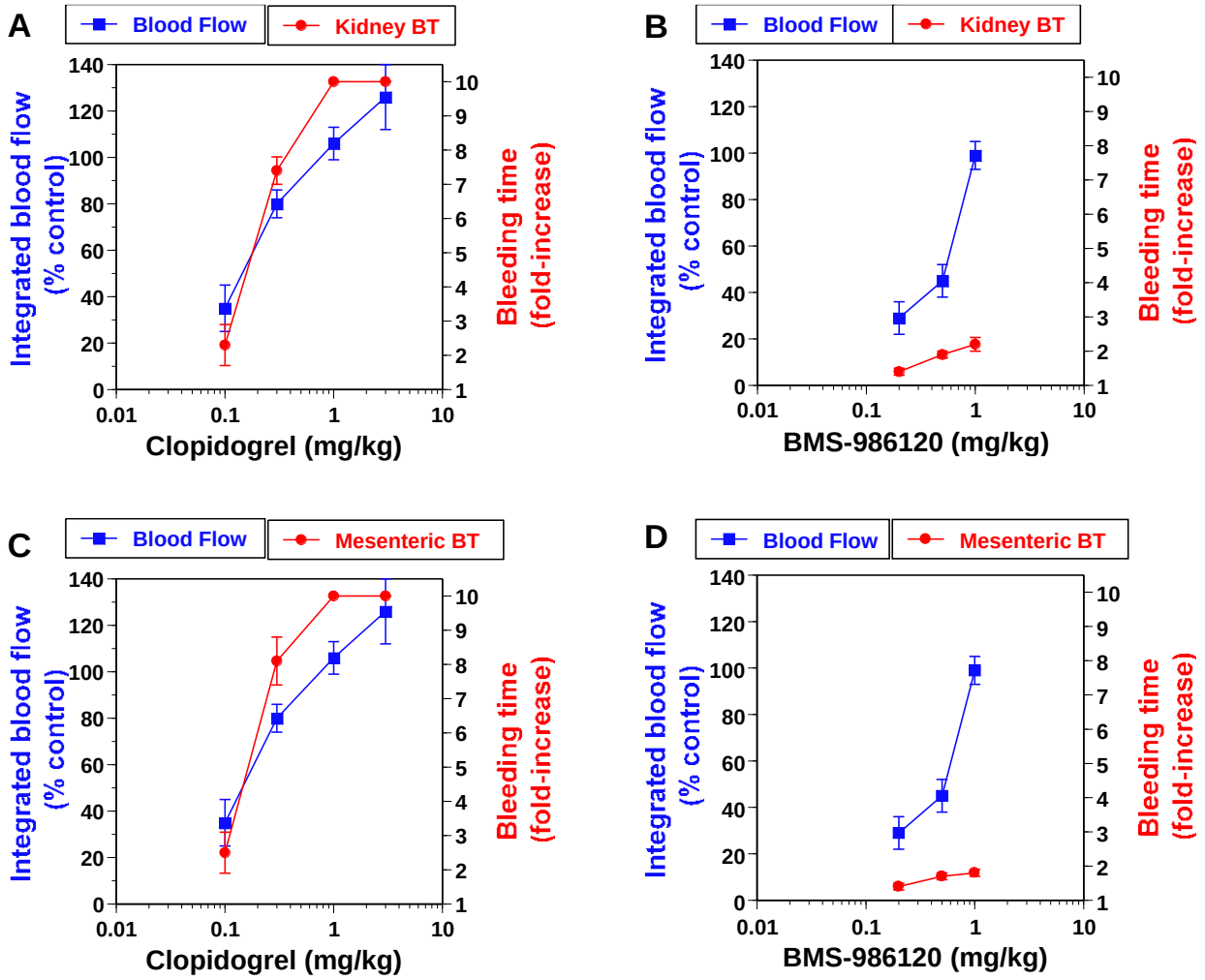


Fig. S11. Comparison of dose-dependent effect of clopidogrel and BMS-986120 on integrated blood flow and BTs. (**A** and **B**) Kidney BT. (**C** and **D**) Mesenteric BT. Clopidogrel and BMS-986120 were studied in the same cynomolgus monkey model. Integrated blood flow was expressed as a percentage of control. BT effect was expressed as a ratio of drug-treated versus the mean vehicle value. Data are mean ± SEM and n=6 per group for the clopidogrel study and n=8 per group for the BMS-986120 study.

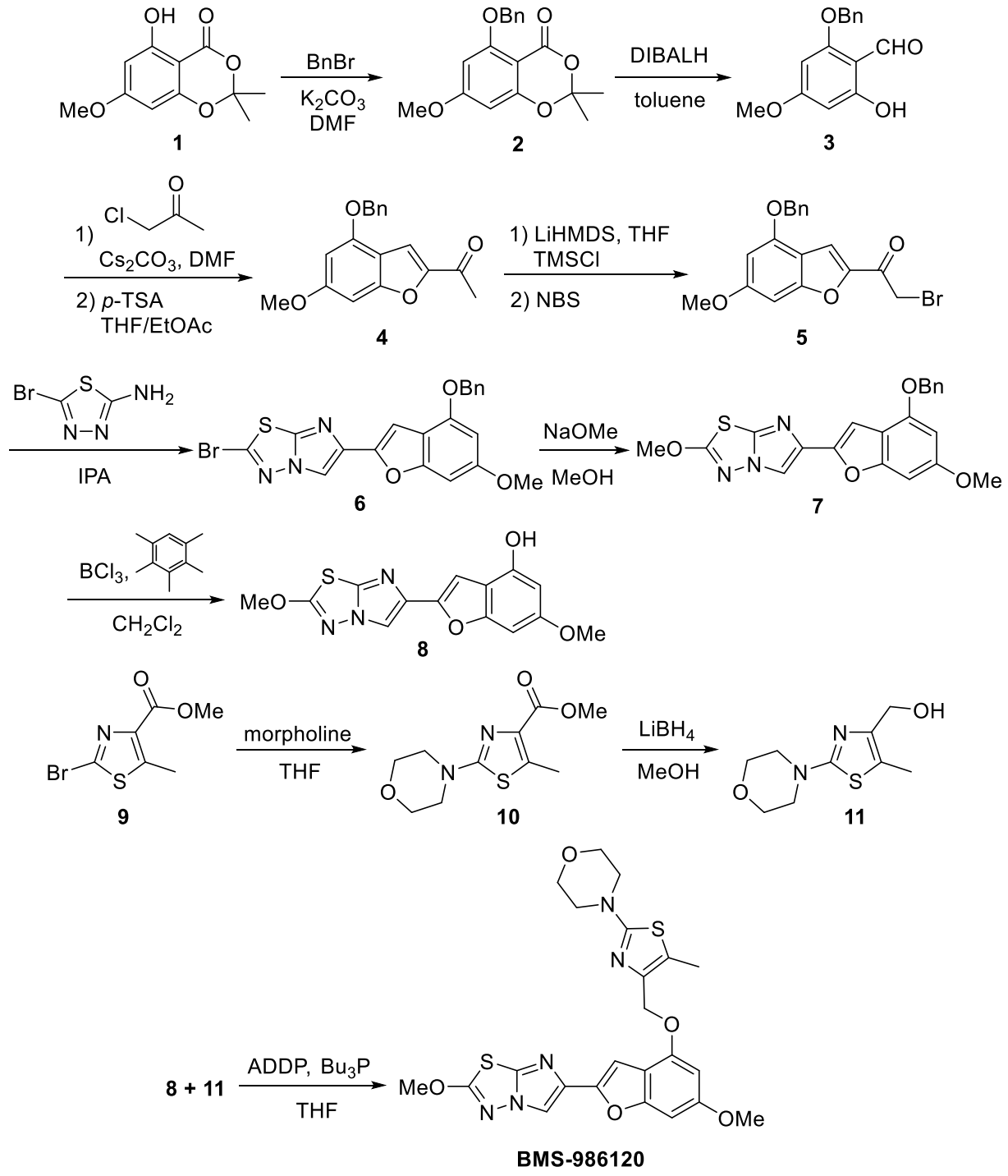


Fig. S12. Synthesis of BMS-986120.

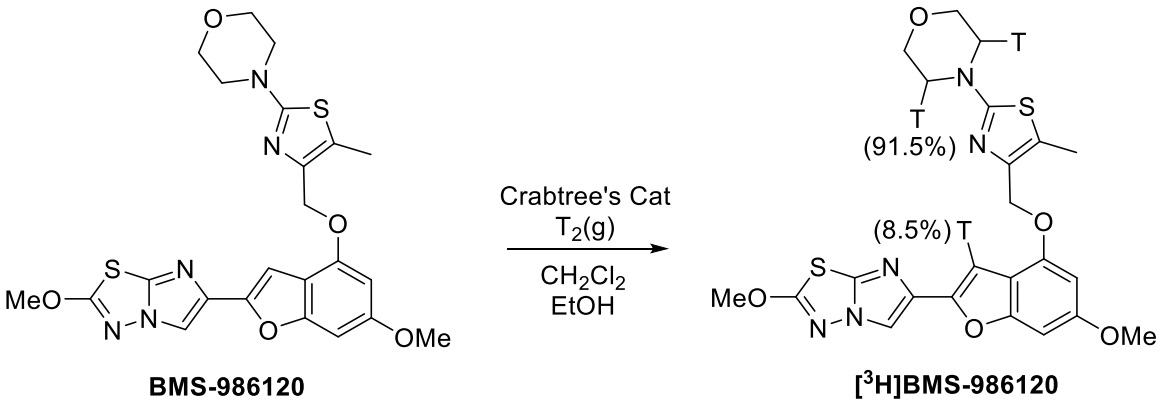


Fig. S13. Synthesis of [^{3}H]BMS-986120.

Table S1. Individual animal data from guinea pig carotid artery injury model.

Average flow over 120 min (% baseline control)			
	Anti-PAR4		
IgG control	0.34 mg/kg	1.13 mg/kg	3.4 mg/kg
18.5	13.7	25	41.7
24.2	16.7	18.5	51.5
21.2	12.6	40.9	48
14.6	10.7	26.3	24.8
15.3	14.4	53.5	54.2
18	20	19	
11.6			
22.6			
12.9			
15			
19			
21.2			
17.6			
16.2			

Table S2. Individual animal data from guinea pig bleeding model.

Bleeding Time (Fold change post vs pre doses)			
Cuticle Bleed		Renal Bleed	
IgG control	Anti-PAR4 3.4 mg/kg	IgG control	anti-PAR4 3.4 mg/kg
0.98	0.94	0.97	0.97
0.95	1.30	1.05	1.05
2.49	1.46	0.85	0.85
1.70	3.38	0.72	0.86
0.70	2.26	1.00	0.72
			1.00
			0.91
			0.05

Table S3. In vitro selectivity of BMS-986120 in the calcium mobilization assays.

	PAR4 Inhibition (IC_{50}, nM)	**PAR1 Inhibition (IC_{50}, nM)**	**PAR2 Inhibition (IC_{50} nM)**	**PAR4 Activation (EC_{50} nM)**	**PAR1 Activation (EC_{50} nM)**	**PAR2 Activation (EC_{50} nM)**
Test System	HEK293-PAR4 + PAR4 AP	HEK293-PAR1 + PAR1 AP	CHO-PAR2 + PAR2 AP	HEK293-PAR4	HEK293-PAR1	CHO-PAR2
N	6	6	1	6	6	1
Mean ± SD	0.56 ± 0.23	>5000	>42000	>5000	>5000	>42000

Table S4. BMS-986120 specificity against enzymatic activity of coagulation proteases.

Protease Assay	K_i (μM)	Protease Assay	K_i (μM)
Activated Protein C, 37ºC	>21.47	Factor XIIa, 37ºC	>3.05
Chymotrypsin, 37ºC	>13.58	γ-Thrombin	>5.83
Factor VIIa	>4.44	Plasmin, 37ºC	>15.22
Factor IXa, 37ºC	>27.12	α-Thrombin	>13.33
Factor Xa	>9.00	Trypsin	>6.22
Factor XIa	>13.33	*t*-plasminogen activator, 37ºC	>6.15
Factor XIa, 37ºC	>13.33	Urokinase, 37oC	>15.1

Table S5. Cynomolgus monkeys in the studies of clopidogrel and BMS-986120.

	Vehicle	Clopidogrel 0.1 mg/kg	Clopidogrel 0.3 mg/kg	Clopidogrel 1 mg/kg	Clopidogrel 3 mg/kg
Number of monkeys	6	6	6	6	6
Gender	4F, 2M	3F, 3M	3F, 3M	3F, 3M	3F, 3M
Age (years)	6.6 (4.8 - 11.9)	5.6 (4.7 - 7.1)	5.4 (4.6 - 6)	5.6 (4.8 - 6.2)	6 (5.3 - 8.1)
Body weight (kg)	4.9 (4 - 6.5)	6 (3.6 - 8.3)	5.2 (3.5 - 7.4)	5.4 (4.1 - 8.1)	5.1 (3.6 - 7.8)

	Vehicle	BMS-986120 0.2 mg/kg	BMS-986120 0.5 mg/kg	BMS-986120 1 mg/kg
Number of monkeys	8	8	8	8
Gender	3F, 5M	3F, 5M	3F, 5M	3F, 5M
Age (years)	7.4 (5 - 10.2)	6.9 (5.5 - 11.8)	7.3 (4.7 - 12.6)	5.6 (4 - 9.3)
Body weight (kg)	6.8 (3.1 - 13.1)	6.4 (3.5 - 7.6)	6 (4.1 - 8)	5.6 (4.2 - 8.4)