Role of a JAK3-dependent Biochemical Signaling Pathway in Platelet Activation and Aggregation*

Heather E. Tibbles‡§§, Alexei Vassilev‡‡, Heather Wendorff‡*, Dawn Schonhoff‡‡*, Dan Zhu‡, David Lorenz‡*, Barbara Waurzyniak‡, Xing-Ping Liu‡‡, and Fatih M. Uckun‡¶‡‡"§§

From the §Department of Medicine, §§Hematology, §Biochemistry, ¶¶Chemistry, and ¶¶¶Experimental Pathology, and the **Discovery Program, Parker Hughes Institute, St. Paul, Minnesota, 55113.

Published, JBC Papers in Press, February 22, 2001, DOI 10.1074/jbc.M011405200

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 21, Issue of May 25, pp. 17815–17822, 2001

Printed in U.S.A.

Received for publication, December 18, 2000, and in revised form, February 9, 2001
Published, JBC Papers in Press, February 22, 2001, DOI 10.1074/jbc.M011405200

This paper is available on line at http://www.jbc.org

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§§ To whom correspondence should be addressed: Parker Hughes Institute, 2665 Long Lake Rd., Suite 330, St. Paul, MN 55113.

1 The abbreviations used are: JAK, Janus kinase; PCR, polymerase chain reaction; bp, base pair; PEP, platelet-rich plasma; PBS, phosphate-buffered saline; STAT, signal transducers and activators of transcription; HR-LVSEM, high-resolution low-voltage scanning electron microscopy; TEM, transmission electron microscopy.

Platelet activation via cleavage of the protease-activated receptors by thrombin leads to formation of a platelet-rich thrombus, which can severely impair blood flow to vital organs, including the brain, heart, lungs, and kidneys (1). Since the serine protease thrombin is the most potent activator of platelet-mediated coagulation, a better understanding of the signaling pathways regulating thrombin-induced platelet-activated aggregation may provide the basis for new and effective strategies for prevention and/or treatment of thromboembolism.

Recent studies have revealed important roles for several protein-tyrosine kinases in platelet physiology (2–5). Notably, JAK3, a member of the Janus family of protein-tyrosine kinases (6–8), was shown to be constitutively active in human platelets, and its potential physiologic role in agonist-induced platelet activation or aggregation remains unknown. The purpose of this study was to examine the role of JAK3 in thrombin-induced platelet activation and aggregation. Here we show genetic and biochemical evidence that implicates JAK3 as one of the regulators of platelet function. Furthermore, our study uniquely identifies a small molecule chemical inhibitor of JAK3 as a novel antiplatelet agent for prevention of potentially fatal thromboembolic events.

Experimental Procedures

Tyrosine Kinase Inhibitors—The JAK3 inhibitor WHI-P131 (4-(4’-hydroxyphenyl)amino-6,7-dimethoxyquinazoline) was rationally designed using a homology model for the kinase domain of JAK3, synthesized, and characterized as previously described in detail (9, 10). WHI-P131 inhibits JAK3, but not JAK1 or JAK2, the ZAP70/SYK family tyrosine kinase SYK, the TEC family tyrosine kinase BTK, the SRC family tyrosine kinase LYN, or the receptor family tyrosine kinase insulin receptor kinase (9, 10). The physical data for WHI-P131 were as follows: m.p. 245.0–248.0 °C; 1H NMR (Me 2SO- d6) δ 11.21 (s, 1H, -NH), 9.70 (s, 1H, -OH), 8.74 (s, 1H, 2-H), 8.22 (s, 1H, 5-H), 7.40 (d, 2H, J = 8.9 Hz, 2’-6’-H), 7.29 (s, 1H, 8-H), 6.85 (d, 2H, J = 8.9 Hz, 3’-5’-H), 3.98 (3H, -OCH3), and 3.97 (3H, -OCH3); IR (KBr) 3428, 2836, 1635, 1514, 1443, and 1234 cm⁻¹; gas chromatography/mass spectrometry, m/z 298 (M⁺ + 1, 100), 297 (M⁺ + 2, 27), and 296 (M⁺ – 1, 12); and analyzed (C16H15N3O3) C, H, N. The x-ray crystallographic data for WHI-P131 were recently published (10). The physical data for the parent compound, WHI-P258 (4-(phenylamino)-6,7-dimethoxyquinazoline), were as follows: yield of 88.26%; m.p. 258.0–260.0 °C; 1H NMR (Me 2SO-d6) δ 11.41 (s, 1H, -NH), 8.82 (s, 1H, 2-H), 8.32 (s, 1H, 5-H), 7.70–7.33 (m, 5H, 2’-5’-H), 7.36 (s, 1H, 8-H), 4.02 (3H, -OCH3), and 4.00 (3H, -OCH3); IR (KBr) 2852, 1627, 1509, 1434, and 1248 cm⁻¹; gas chromatography/mass spectrometry, m/z 282 (M⁺ + 1, 11), 281 (M⁺, 55), 280 (M⁺ – 1, 100), 264 (16), and 207 (9); analyzed (C16H14N3O3) C, H, N. Mouse-Control C57BL/6 mice were purchased from Taconic Farms Inc. (Germantown, NY). A breeder pair of Jak3 knockout mice (Jak3⁻/⁻, C57BL/6 × 129/Sv, H-2d (11), A011 (male) and A1038 (female)) were obtained from Dr. J. N. Ible (St. Jude Children’s Research Hospital, Memphis, TN). These mice were created by the targeted disruption of the Jak3 gene through homologous recombination using the hygromycin resistance gene (Hyg) cassette (11). These founder Jak3⁻/⁻ mice were bred with C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), and the offspring of the F1 generation were back-crossed to C57BL/6 mice. After three generations of back-crossing to C57BL/6 mice, the offspring were intercrossed to produce homozygous Jak3⁻/⁻ and wild-type Jak3⁺/⁺ mice. The genotype of mice was confirmed by multiplex polymerase chain reaction (PCR). In brief, a 0.5-inch (1.27-cm) tail tissue section was taken from each mouse and digested at 55 °C in 600 μl of lysis buffer (50 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, and 1% SDS) with 50 μl of proteinase K (10 mg/ml). Genomic DNA was purified by phenol/chloroform extractions and ethanol precipitation (12). Three primers were employed in the PCR tests: the 30-base primer Jak3-A (5’-ACC TAG TCC CCA GGT TGG CTG TCA CTT GGG-3’), the 30-base primer Jak3-B (5’-AGC GGT GAT AGC ATG TCT CCA GAC ACC-3’), and the 30-base primer Jak3-C (5’-GTG TTT GGA TGG CTT GGG CAT GGA CCG-3’). The 30-base primer Jak3-A inhibited thrombin-induced degranulation and serotonin release as well as platelet aggregation. Highly effective platelet inhibitory plasma concentrations of WHI-P131 were achieved in mice without toxicity. WHI-P131 prolonged the bleeding time of mice in a dose-dependent manner and improved event-free survival in a mouse model of thromboplastin-induced generalized and invariably fatal thromboembolism. To our knowledge, WHI-P131 is the first anti-thrombotic agent that prevents platelet aggregation by inhibiting JAK3.

Here we provide experimental evidence that identifies JAK3 as one of the regulators of platelet function. Treatment of platelets with thrombin induced tyrosine phosphorylation of the JAK3 target substrates STAT1 and STAT3. Platelets from JAK3-deficient mice displayed a decrease in tyrosine phosphorylation of STAT1 and STAT3. In accordance with these data, pretreatment of human platelets with the JAK3 inhibitor WHI-P131 markedly decreased the base-line enzymatic activity of constitutively active JAK3 and abolished the thrombin-induced tyrosine phosphorylation of STAT1 and STAT3. Following thrombin stimulation, WHI-P131-treated platelets did not undergo shape changes indicative of activation such as pseudopod formation. WHI-P131 inhibited thrombin-induced degranulation/serotonin release as well as platelet aggregation. JAK3, a member of the Janus family of protein-tyrosine kinases in platelet physiology (2–5). Notably, Jak3, the brain, heart, lungs, and kidneys (1). Since the serine protease thrombin is the most potent activator of platelet-mediated coagulation, a better understanding of the signaling pathways regulating thrombin-induced platelet-activated aggregation may provide the basis for new and effective strategies for prevention and/or treatment of thromboembolism.

Recent studies have revealed important roles for several protein-tyrosine kinases in platelet physiology (2–5). Notably, JAK3, α member of the Janus family of protein-tyrosine kinases (6–8), was shown to be constitutively active in human platelets, but its potential physiologic role in agonist-induced
negative control.

50-mented by the presence of both 720- and 620-bp PCR products. Each Jak3

a clearing of the plasma. The IC50 values for WHI-P131-mediated

method, the formation of the large platelet aggregate is accompanied by

dual chamber instrument, Chronolog Inc.) for 5 min. When the aggre-

metric aggregation in a Whole Blood Platelet Aggregometer (Model 560

thrombin (0.1 unit/ml; Chronolog Inc., Philadelphia, PA) under stirring.

1:4 with sterile normal saline, and platelets were stimulated with

were treated with vehicle alone. The treated PRP samples were diluted

chosen from the Memorial Blood Bank (Minneapolis, MN) and used

DNA sequence using Lasergene software

accomplished by ThermoSequenase PCR (Amersham Pharmacia Bio-

(14). E23 recognizes both the

Cruz Biotechnology, Santa Cruz, CA) or STAT3 (Transduction Labora-

Following removal of the membranous fraction by centrifugation

(12,000 g/ml collagen or 0.1 unit/ml thrombin. Stimulation was

platelet aggregation was measured in wild-type and knockout mice (n =

Role of JAK3 in Platelet Activation

the homozygous wild-type Jak3+/+ genotype was documented by detection of a single 720-bp multiplex PCR

product, and the homozygous knockout jak3−/− genotype was documented by detection of a single 620-bp multiplex PCR product. Neg Con, negative control.

Fig. 1. Jak3 knockout mice. The homozygous wild-type Jak3+/+ genotype was documented by detection of a single 720-bp multiplex PCR

primer pair yielded a 720-bp wild-type PCR product in tissues from homozygous jak3−/− or heterozygous Jak3+/− mice. The homozygous

Jak3+/+ genotype was documented by a single 720-bp PCR product, and the homozygous Jak3−/− genotype was documented by a single 620-bp

PCR product (Fig. 1). The heterozygous Jak3+/− genotype was docu-

mented by the presence of both 720- and 620-bp PCR products. Each

50-μl PCR medium consisted of 1× PCR buffer II containing 2.5 mM

MgCl2 (AmpliTaq Gold kit, PerkinElmer Life Sciences), 0.2 mM dNTP

(Roche Molecular Biochemicals), 0.4 μM each primer, 6% MeSO4, and

2.5 units of AmpliTaq Gold enzyme. The PCR conditions were 94 °C for

10 min, 30 cycles (94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min with

a 5 sec extension), then 72 °C for 10 min in a Touchdown thermocycler

(Hyundai, Potomac, MD). The PCR products were cloned into the original

TA cloning vector (Invitrogen, Carlsbad, CA). Sequence analysis was

accomplished by Thermosequenase PCR (Amersham Pharmacia Bio-
tech) using Cy5-labeled T3 and T7 sequencing primers (Integrated DNA

Technologies, Inc., Coralville, IA). DNA sequences were analyzed

against published Jak3 DNA sequence using Lasergene software

(DNASTAR, Madison, WI).

Platelet Aggregation Assays—Platelet-rich plasma (PRP) was pur-

chased from the Memorial Blood Bank (Minneapolis, MN) and used

according to the guidelines of the Parker Hughes Institute Human

Subjects Committee. The PRP samples were treated with varying con-

centrations of WHI-P131 for 20 min at 37 °C. Control PRP samples

were treated with vehicle alone. The treated PRP samples were diluted

1:4 with sterile normal saline, and platelets were stimulated with

thrombin (0.1 unit/ml; Chronolog Inc., Philadelphia, PA) under stirring.

Platelet aggregation was monitored using the Born method of turbidi-

metric aggregation in a Whole Blood Platelet Aggregometer (Model 560

dual chamber instrument, Chronolog Inc.) for 5 min. When the aggre-

gating agent is added to the platelet-rich plasma according to this

method, the formation of the large platelet aggregate is accompanied

by a clearing of the plasma. The IC50 values for WHI-P131-mediated

inhibition of agonist-induced platelet aggregation were calculated by

nonlinear regression analysis using GraphPAD Prism Version 2.0 soft-

ware. In impedance aggregation studies, the increase in electrical im-

pedance caused by adherence and aggregation of platelets on an elec-

trode is measured. For these studies, blood was extracted from Jak3

knockout and control C57BL/6 mice by eye bleeds into tubes containing

0.1 unit/ml) was added at 1 min to induce aggregation. Thrombin-induced

platelet aggregation was measured in wild-type and knockout mice (n =

3 for each type) in the whole blood platelet aggregometer.

Immunoprecipitation and Western Blot Analysis—Platelets were iso-

lated from PRP as previously described (15) and resuspended at a

concentration of 3 × 109 cells/ml in modified Tyrode’s buffer (137 mM

NaCl, 2.7 mM KCl, 0.9 mM MgCl2, 5.5 mM glucose, 3.3 mM NaH2PO4,

and 3.8 mM Heps (pH 7.4)). Platelets were incubated with the indi-

cated concentrations of WHI-P131 or vehicle (PBS supplemented with

1% MeSO4) for 30 min at 37 °C. Platelets were then stimulated at 37 °C

with 2 or 10 μg/ml collagen or 0.1 unit/ml thrombin. Stimulation was

stopped, and platelets were lysed at the indicated time points by adding

ice-cold 3× Triton X-100 lysis buffer (150 mM NaCl, 15 mM EGTA, 3% Triton X-100, 3% sodium deoxycholate, 0.3% SDS, 3% phenylmeth-

ylsulfonyl fluoride, 3 mM Na3VO4, 60 μg/ml leupeptin, 60 μg/ml apro-

tin, and 50 mM Tris-HCl (pH 7.4)) and incubating for 1 h on ice.

Following removal of the membranous fraction by centrifugation

(12,000 g, 30 min), the samples were subjected to immunoprecipita-

tion utilizing antibodies raised against JAK3 and STAT1 (E23, Santa

Cruz Biotechnology, Santa Cruz, CA) or STAT3 (Transduction Labora-

tories, Lexington, KY) (14). E23 recognizes both the α (p91) and β (p84)

isoforms of STAT1. Similarly, the anti-STAT3 antibody recognizes both

the α (p92) and β (p83) isoforms of STAT3.

Immunoprecipitations, immune complex protein kinase assays, and

immunoblotting on polyvinylidene difluoride membranes (Millipore

Corp., Bedford, MA) using the ECL chemiluminescence detection sys-

tem (Amersham Pharmacia Biotech) were conducted as described pre-

viously (9, 14–17). For immunoblotting, we used antibodies against

phosphotyrosine, JAK3, STAT1, STAT3, phospho-STAT1 (recognizes

both STAT1α and STAT1β), and phospho-STAT3 (recognizes both

STAT3α and STAT3β) (New England Biolabs Inc., Beverly, MA). Horse-

radish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit

secondary antibodies were purchased from Transduction Laboratories.

Horseradish peroxidase-conjugated sheep anti-goat antibodies were

purchased from Santa Cruz Biotechnology. Following electrophoresis,

kinase gels were dried on Whatman No. 3 Filter Paper and subjected to

phosphorimaging on a molecular imager (Bio-Rad) as well as auto-

radiography on film. Similarly, all chemiluminescent JAK3 Western

blots were subjected to three-dimensional densitometric scanning with

the molecular imager and an imaging densitometer using Molecular

Analyst/Macintosh Version 2.1 software following the specifications of

the manufacturer (Bio-Rad). A JAK3 kinase activity index was deter-

mined by comparing the ratios of the kinase activity in PhosphorImager

units (PIU) and density of the protein bands in densitometric scanning

units (DSU) with those of the base-line sample using the following
Role of JAK3 in Platelet Activation

FIG. 2. JAK3-dependent tyrosine phosphorylation of STAT1 and STAT3 in thrombin-stimulated platelets. A and B, whole cell lysates from control and JAK3-deficient mouse platelets stimulated with 0.1 unit/ml thrombin were subjected to Western blot analysis utilizing antibodies raised against phosphorylated STAT1 (A, upper panels), STAT1 (A, lower panels), phosphorylated STAT3 (B, upper panels), and STAT3 (B, lower panels). C, STAT1 was immunoprecipitated from human platelets stimulated with 0.1 unit/ml thrombin. The immunoprecipitates were subjected to Western blot analysis utilizing antibodies raised against phosphorylated STAT1 (upper panel) and STAT3 (lower panel). D, STAT3 was immunoprecipitated from human platelets that were stimulated with 0.1 unit/ml thrombin. The immunoprecipitates were subjected to Western blot analysis utilizing antibodies raised against phosphorylated STAT3 (upper panel) and STAT3 (lower panel). E, JAK3 was immunoprecipitated from platelets that were stimulated with thrombin (0.1 unit/ml) after treatment with vehicle (1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline) or WHI-P131 (100 μM). The immunoprecipitates were subjected to quantitative kinase assays (upper panel) and immunoblotting with an anti-JAK3 antibody (lower panel) as described under “Experimental Procedures.” F and G, human platelets were pretreated with vehicle or WHI-P131 (100 μM) prior to thrombin stimulation. In F, STAT1 was immunoprecipitated from platelets that were stimulated with 0.1 unit/ml thrombin. The immunoprecipitates were subjected to Western blot analysis utilizing antibodies raised against phosphorylated STAT3 (upper panel) and STAT3 (lower panel). PIU, PhosphorImager units; DSU, densitometric scanning units.

formulas: activity index = (PIU of kinase band/DSU of JAK3 protein band)$_{test\ sample}$; and stimulation index = (PIU of kinase band/DSU of JAK3 protein band)$_{test\ sample}$/(PIU of kinase band/DSU of JAK3 protein band)$_{control\ sample}$.

Cytoskeletal Fractionation—Platelets (1 × 10$^8$/ml) were treated with WHI-P131 (100 μM, 30 min, 37 °C) or vehicle (1% Me2SO) and stimulated with thrombin (0.1 unit/ml) or collagen (10 μg/ml). Isolation of the cytoplasmic and Triton X-100-soluble and -insoluble fractions was performed as previously described (18, 19). Fractions were analyzed by Western blot analysis utilizing antibodies raised against JAK3, STAT1, SYK (Santa Cruz Biotechnology), STAT3, tubulin, and actin (Sigma).

Serotonin Release—Platelet samples were prepared as previously described (20). Release of serotonin from thrombin (0.1 unit/ml)-stimulated platelets was measured using a serotonin detection kit (ImmunoTech, Marseilles, France) according to the manufacturer’s specifications. Sonicated platelets were used for measurement of the total serotonin content of platelets.

High-resolution Low-voltage Scanning Electron Microscopy (HR-LVSEM)—HR-LVSEM was utilized for topographical imaging of the platelet surface membrane as previously reported (21). Aliquots of human platelets were incubated with 100 μM WHI-P131 or vehicle alone for 30 min. Treated platelets were then stimulated with thrombin (0.1 unit/ml) for 10 s. 3% glutaraldehyde was added to stop the reaction. Samples were prepared for HR-LVSEM as previously described (21) and analyzed using a Hitachi S-900 SEM instrument at an accelerating voltage of 2 kV.

Transmission Electron Microscopy (TEM)—Aliquots of human platelets were incubated with 100 μM WHI-P131 or vehicle alone for 30 min and then stimulated with thrombin (0.1 unit/ml) for 10 s. Samples were prepared for TEM as previously described (22). Briefly, 0.1% glutaraldehyde was added to stop the reaction. Following a brief centrifugation, the sample pellets were layered with 3% glutaraldehyde for 40 min at room temperature. The samples were then post-fixed in 1% OsO$_4$ for 1 h at 4 °C, rinsed three times in distilled water at room temperature, and dehydrated in a graded ethanol series (25, 50, 75, 90, 95, and 100%) and 100% propylene oxide. The samples were embedded in Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). Silver sections were picked up on mesh grids and stained for 10 min in 1% uranyl acetate and 70% ethanol and for 10 min in Reynold’s lead citrate. Sections were viewed in a Jeol 100× electron microscope at 60 kV. True magnifications were determined by photographing a calibration grid at each magnification step on the microscope and using this scale to determine final print enlargements.

Measurement of Bleeding and Clotting Times in Mice—Mice were treated intravenously with a single intraperitoneal bolus injection of 200 μl of vehicle (PBS supplemented with 10% Me$_2$SO) or varying doses of WHI-P131 in 200 μl of vehicle 30 min prior to and with an intravenous bolus of the same dose 5 min before the bleeding time measure-
Role of JAK3 in Platelet Activation

RESULTS

JAK3-dependent Tyrosine Phosphorylation of STAT1 and STAT3 Proteins in Thrombin-stimulated Platelets—We first set out to examine the effects of thrombin stimulation on the phosphorylation status of STAT1 and STAT3 proteins in platelets from wild-type C57BL/6 mice. Notably, treatment of platelets with 0.1 unit/ml thrombin induced tyrosine phosphorylation of the α isoform (p91) of STAT1 (Fig. 2A) and the β isoform (p85) of STAT3 (Fig. 2B). Thrombin-induced tyrosine phosphorylation of STAT1 and STAT3 was JAK3-dependent because thrombin stimulation failed to induce tyrosine phosphorylation of these STAT proteins in JAK3-deficient platelets from Jak3 knockout mice (Jak3−/−). Similarly, stimulation of human platelets with 0.1 unit/ml thrombin increased the tyrosine phosphorylation of STAT1 and STAT3 proteins (Fig. 2, C and D). Pretreatment of human platelets with the JAK3 inhibitor WHI-P131 (100 μM) decreased the baseline enzymatic activity of constitutively active JAK3 by 81% as measured by autophosphorylation (Fig. 2E) and abolished the thrombin-induced tyrosine phosphorylation of STAT1 and STAT3 (F and G). It is noteworthy that at 60 s after thrombin stimulation, the enzymatic activity of JAK3 was reduced by 41% (activity index: 0.35 = 59% of the activity index before thrombin stimulation) (Fig. 2E, first and second lanes). The significance of this observation is currently unknown.

Effects of the JAK3 Inhibitor WHI-P131 on Thrombin-induced Platelet Activation—Activation of platelets after exposure to thrombin is associated with actin polymerization and rapid translocation of the tyrosine kinase SYK (24, 25) as well as tubulin to the Triton X-100-insoluble fraction that is associated with the actin filament network. As shown in Fig. 3A, Western blot analysis of the cytoplasmic and Triton X-100-soluble and -insoluble fractions from unstimulated platelets confirmed the presence of abundant amounts of actin in the Triton X-100-insoluble fraction and of SYK as well as tubulin in the Triton X-100-soluble (but not -insoluble) fraction. Within 60 s after thrombin stimulation, a significant amount of SYK and tubulin translocated to the membrane-associated cytoskeleton as evidenced by the Western blot detection of SYK and tubulin in the actin-containing Triton X-100-insoluble fractions. Notably, thrombin stimulation also induced the translocation of Jak3, STAT1α/β, and STAT3β proteins to the Triton X-100-insoluble fraction. As shown in Fig. 3B, pretreatment of platelets with the JAK3 inhibitor WHI-P131 prevented the thrombin-induced relocalization of SYK, tubulin, Jak3, STAT1, STAT3, and STAT3 to the Triton X-100-insoluble fractions.

Platelet activation after thrombin stimulation was accompanied by marked changes in platelet shape and ultrastructural organization. Topographical imaging of the surface membrane of thrombin (0.1 unit/ml)-stimulated human platelets by HR-LVSEM at 40× magnification showed development of pseudo pod extensions indicative of activation (Fig. 4, A and B). WHI-P131 (100 μM) inhibited thrombin-induced pseudopod formation (Fig. 4, C and D). Examination of thrombin-stimulated platelets by TEM at 40,000× magnification showed a rapid shape change from discoidal cells to spheres with pseudopods extending from the surface and coalescence of granules as well as canalicular cisternae in the center of the platelet as a pre-
Role of JAK3 in Platelet Activation

Role of JAK3 in Thrombin-induced Platelet Aggregation—We next sought to examine the role of JAK3 in thrombin-induced platelet aggregation. To this end, we first compared the thrombin-induced aggregatory responses of platelets from wild-type and Jak3 knockout mice. As shown in Fig. 6, the magnitude of the thrombin (0.1 unit/ml)-induced aggregatory response of Jak3^{+/+} platelets from wild-type mice was greater than that of Jak3^{-/-} platelets from Jak3 knockout mice. In accordance with these results, pretreatment of human platelets with the JAK3 inhibitor WHI-P131 for 30 min inhibited thrombin (0.1 unit/ml)-induced platelet aggregation in a concentration-dependent fashion, with an average IC_{50} value of 1.5 μM (Fig. 7, A and B). By comparison, WHI-P258, a structurally similar compound that does not inhibit JAK3, did not affect the thrombin-induced aggregation of platelets even at 100 μM.

WHI-P131 Prolongs Bleeding Time in Vivo and Protects Mice against Thromboplastin-induced Fatal Thromboembolism—WHI-P131 is not toxic to mice or monkeys when administered systemically at dose levels ranging from 1 to 100 mg/kg. WHI-P131 prolonged the tail bleeding times of mice in a dose-dependent manner: the average tail bleeding times were 1.5 ± 0.1 min for vehicle-treated controls (n = 12), 9.4 ± 0.6 min for 20 mg/kg WHI-P131 (n = 10; p < 0.001), >10 min for 40 mg/kg WHI-P131 (n = 10; p < 0.001), and >10 min for 80 mg/kg WHI-P131 (n = 10; p < 0.001).

Notably, WHI-P131 also improved the survival outcome in a mouse model of thromboplastin-induced generalized and invariably fatal thromboembolism (Fig. 8). This model, 100% of the challenged mice develop dyspnea, ataxia, and seizures and die within 10 min after the thromboplastin challenge from widespread thrombosis in multiple organs and massive pulmonary thromboembolism. All of the 20 vehicle-treated mice died after the thromboplastin challenge, with a median survival time of 2.5 min. WHI-P131 more than doubled the median survival time and produced an event-free survival outcome of...
FIG. 5. Effects of WHI-P131 on thrombin-induced ultrastructural changes and degranulation in platelets. A–C, aliquots of human platelets were incubated with 100 μM WHI-P131 or vehicle alone for 30 min and then stimulated with thrombin (0.1 unit/ml) for 10 s. Samples were prepared for TEM as described under "Experimental Procedures." Sections were viewed in a Jeol 100× electron microscope at 60 kV. A, TEM images of untreated unstimulated control (CON) platelets with a typical discoid appearance and disperse distribution of granules; B, TEM images of vehicle-treated, thrombin-stimulated platelets with spike-like pseudopodia and coalescence of granules in the center; C, TEM images of WHI-P131-treated unstimulated platelets; D, TEM images of WHI-P131-treated, thrombin-stimulated platelets with the largely discoid appearance of resting platelets; E, serotonin release from platelets stimulated with 1 unit/ml thrombin for 60 s measured using the serotonin detection kit according to the manufacturer’s specifications.
30 ± 15% (Fig. 8). The cause of death in WHI-P131-pretreated thromboplastin-challenged mice was generalized thrombembolism. No drug-related toxic lesions were detected in any of the organs of these mice. All of the 20 control mice treated with 80 mg/kg WHI-P131 without a subsequent thromboplastin challenge survived beyond the 48-h observation period without any evidence of impaired health status or bleeding.

DISCUSSION

In summary, our findings reveal an essential role for JAK3 in thrombin-induced platelet activation and aggregation. As a serine protease, thrombin activates protease-activated receptors 1 and 4 (26) by cleaving the N-terminal portion of the receptor. The cleaved peptide then acts as a tethered ligand that activates the G-protein-coupled receptor independent of receptor cleavage (27). JAK3 may bind to the cytoplasmic C-terminal portion of the protease-activated receptor(s) and play a pivotal role in transduction of the thrombin-induced biochemical signal once the receptor is cleaved. Further studies are needed to decipher the molecular mechanism of JAK3-mediated regulation of platelet function.

WHI-P131 inhibited thrombin-induced tyrosine phosphorylation of STAT1 and STAT3 proteins as well as activation-associated translocation of SYK and tubulin to the Triton X-100-insoluble fraction. In agreement with these results, platelets from JAK3-deficient mice displayed a decrease in thrombin-induced platelet aggregation and tyrosine phosphorylation of STAT1 and STAT3. Following thrombin stimula-

![Fig. 6. Role of JAK3 in thrombin-induced platelet aggregation.](image)

![Fig. 7. Effects of the JAK3 inhibitor WHI-P131 on thrombin-induced platelet aggregation.](image)
tion, WHI-P131-treated platelets did not undergo shape changes indicative of activation such as pseudopod formation. WHI-P131 inhibited thrombin-induced degranulation/serotonin release as well as platelet aggregation. Highly effective platelet inhibitory plasma concentrations (>10 μM) of WHI-P131 were achieved in mice without toxicity. WHI-P131 prolonged the bleeding time of mice in a dose-dependent manner and improved event-free survival in a mouse model of thromboplastin-induced generalized and fatal thromboembolism, involving the lungs, liver, heart, and central nervous system. Thus, this study uniquely identifies WHI-P131 as a novel antiplatelet agent targeting JAK3 for prevention of potentially fatal thromboembolic events. To our knowledge, WHI-P131 is the first anti-thrombotic agent that prevents platelet aggregation by inhibiting JAK3. WHI-P131 is also being developed as an apoptosis-promoting anticancer agent (28). JAK3 inhibitors such as WHI-P131 may be useful as a new class of anticagulants for treatment of hypercoagulable metastatic cancer patients as well as patients with a primary cardiovascular, cerebrovascular, or hematologic disease at risk for thromboembolic complications.

**REFERENCES**

1. Teng, C. M., Wu, C. C., Ko, F. N., Lee, F. Y., and Kuo, S. C. (1997) *Eur. J. Pharmacol.* **320**, 161–166
2. Pasquet, J. M., Quex, L., Pasquet, S., Poole, A., Mathews, J. R., Lowell, C., and Watson, S. P. (2000) *J. Biol. Chem.* **275**, 28526–28531
3. Ezumi, Y., Shindoh, K., Tsuji, M., and Takayama, H. (1999) *J. Biol. Chem.* **274**, 25039–25044
4. Sada, K., Minami, Y., and Yamamura, H. (1997) *J. Biol. Chem.* **272**, 191–197
5. Melford, S. K., Turner, M., Briddon, S. J., Tybulewicz, V. L., and Watson, S. P. (1997) *Mol. Cell. Biol.* **17**, 5304–5311
6. Uckun, F. M. (1999) *Clin. Cancer Res.* **5**, 1569–1582
7. Mahajan, S., Ghosh, S., Sudbeck, E. A., Liu, X. P., Narla, R. K., Mahajan, S., Ghosh, S., Mao, C., and Uckun, F. M. (1998) *J. Biol. Chem.* **273**, 17473–17478
8. Goodman, P. A., Niehoff, L. B., and Uckun, F. M. (1998) *J. Biol. Chem.* **273**, 17473–17478
9. Quek, L. S., Bolen, J., and Watson, S. P. (1998) *Curr. Biol.* **8**, 899–902
10. Mahajan, S., Ghosh, S., Sudbeck, E. A., Zheng, Y., Downs, S., Hupke, M., and Uckun, F. M. (1999) *J. Biol. Chem.* **274**, 9587–9599

**FIG. 8. Protective effects of WHI-P131 in a mouse model of fatal thromboembolism.** Mice were treated intravenously with 200 μl of vehicle (PBS supplemented with 10% Me2SO; n = 20) or WHI-P131 (20 mg/kg twice; n = 10) in 200 μl of vehicle. The mice were challenged with 25 mg/kg thromboplastin via an intravenous bolus injection into the tail vein. Shown are the cumulative proportions of mice surviving event-free 3 min, 6 min, and 48 h after the injection of thromboplastin. Error bars represent the S.E. values. *p < 0.05, log-rank test.