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Discovery and characterization of a selective, nonpeptidyl thrombopoietin receptor agonist

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***Objective*. Peptide and other small molecule agonists have been described for several cytokines and growth factors. Hydrazone compounds described here as thrombopoietin receptor agonists were identified as activating STAT proteins in a Tpo responsive cell line.**

***Methods*. STAT activation and analysis of signal transduction pathways in cell lines and normal human platelets was elucidated by Western blot and electrophoretic mobility shift assays. Proliferation assays in cell types responsive to other cytokines determined specificity for Tpo receptor. Flow cytometry quantified differentiation of $CD34^+$ cells into $CD41^+$ megakaryocytes and platelet production in vitro.**

***Results*. Activation of STAT5, mitogen-activated protein kinase, p38, and early response genes by SB 394725 was similar to that induced by Tpo. SB 394725 induced a reporter gene response under a STAT activation promoter as well as the megakaryocyte-specific gpIIb promoter. The compound induced proliferation of Tpo responsive lines but demonstrated no activity in cell lines responding to other cytokines, i.e., erythropoietin, granulocyte-colony stimulating factor, interleukin-3, interferon-γ. The response of normal human Tpo receptors was elucidated by measuring growth and differentiation of human bone marrow in vitro. Activation of endogenous Tpo receptors by SB 394725 was demonstrated in human and chimp platelets, but not in platelets of other species including mouse, dog, rabbit, or cynomolgus monkey.**

***Conclusions*. SB 394725, a small molecule with a molecular weight of 452 Da, is capable of activating Tpo-specific signal transduction, proliferation, and differentiation responses similar to the responses and functions of the protein growth factor, Tpo.**

Proliferation and differentiation along the megakaryocytic pathway is tightly controlled by circulating levels of thrombopoietin (Tpo), a cytokine produced primarily by the liver [1,2]. Absence of Tpo leads to profound thrombocytopenia; conversely, administration of recombinant Tpo to rodents and humans leads to significant increases in circulating platelet levels [3–7]. As a result, various forms of recombinant Tpo are currently undergoing clinical trials for the treatment of iatrogenic thrombocytopenia. Tpo binds to a specific cell surface receptor, TpoR (Tpo receptor), present on the surface of platelets, megakaryocytes, and megakaryocytic precursor cells. Binding of Tpo to its receptor triggers the activation of the cytoplasmic tyrosine kinases JAK2 and Tyk2, which in turn results in activation of STAT5, PI-3 kinase and the Ras-mitogen-activated protein kinase (MAPK) pathways [8–11]. Activation of these signal transduction pathways results in changes in gene expression, which are thought to promote progression along the megakaryocytic pathway. Interestingly, stimulation of these signal transduction pathways in response to Tpo is retained in platelets, and appears to sensitize platelets to activation by agonists [12,13].

The TpoR is a member of the type I family of cytokine receptors and is most closely related to erythropoietin (Epo), growth hormone, and granulocyte-colony stimulating factor (G-CSF) receptors [14]. Considerable evidence suggests that these receptors are activated by a ligand-induced dimerization of receptor chains. Although the structure of the Tpo/TpoR complex is not known, mutagenesis and molecular modeling data support a similar model, in which a single

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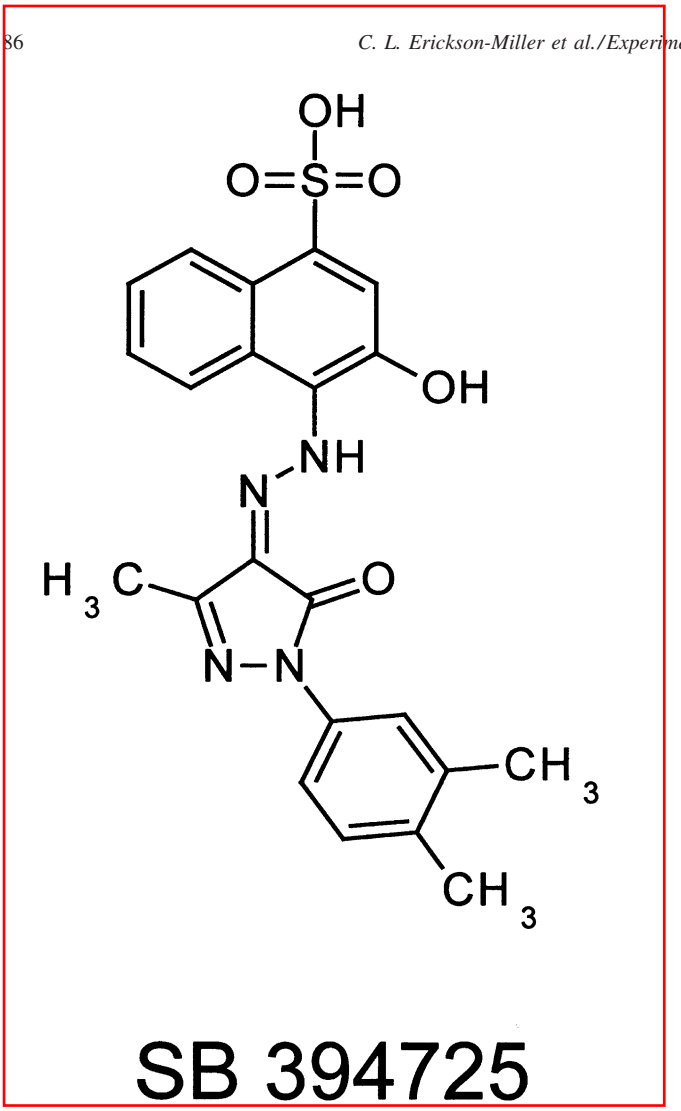

Figure 1. Chemical structure of the nonpetidyl, TpoR agonist, SB 394725, molecular weight of 424.5 Da.

Tpo molecule triggers receptor dimer formation by binding to two receptor chains [15]. Dimerization of TpoR chains by alternate routes can also lead to receptor activation. A monoclonal antibody to the receptor that acts as a receptor agonist has been identified [16]. This antibody promotes megakaryocytopoiesis of primary cells in vitro, but the corresponding Fab fragment is inactive, indicating that antibody-mediated receptor dimerization is probably the origin of the agonist effect. When cysteines are introduced into the extracellular domain of the TpoR at positions that allow disulphide bond formation between chains to occur, the resulting receptors are constitutively active, but only under nonreducing conditions [15,17].

Here we describe the identification and characterization of a selective, nonpeptidyl small molecule agonist of TpoR using a cell-based screen that detects induction of gene expression in response to STAT activation. The compound identified is active only in cells expressing the TpoR, and promotes differentiation of primary human bone marrow cells along the megakaryocytic pathway with an efficacy equivalent to that of recombinant human Tpo (rhTpo).

Materials and methods

Animal blood and marrow samples were obtained using the appropriate International Animal Care and Use Committee-approved protocols. Human blood and marrow was obtained from normal donors after informed consent under an Institutional Review Board-approved protocol.

All experiments were performed on at least three different occasions.

Cytokines and cells

rhTpo, recombinant human stem cell factor (rhSCF) and recombinant mouse interleukin-3 (rmIL-3) were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human Epo and G-CSF were obtained from Amgen (Thousand Oaks, CA, USA) UT7-Tpo cells were derived from the parental UT7 cells by growth in rhTpo 100 ng/mL and were generously provided by Dr. Camille N. Abboud of the University of Rochester Medical Center. The BAF3/hTpoR (BAF3-3B5) cell line was made by stable transfection of both the hTpoR and IRF promoter coupled to a luciferase reporter gene and grown in rmIL-3 (5 ng/mL). The BAF3 parental control line has no transfected TpoR. HepG2 cells were transiently transfected with receptor DNA and luciferase reporter DNA using FuGENE (Roche, Indianapolis, IN, USA) when 60% confluent [18,19]. Twenty-four hours after transfection, the medium was replaced with fresh Dulbecco's modified Eagle's medium/10% fetal calf serum (FCS) prior to use.

The 32D-mpl cells were stably transfected with hTpoR and gpIIb promoter linked to luciferase and maintained in rmIL-3 (5 ng/mL).

Luciferase reporter gene assay

BAF3-Tpo or 32D-mpl cells were washed and starved overnight of rmIL-3 prior to treatments. And 1×10^5 cells/mL of starved BAF3-Tpo or 32D-mpl cells in Iscove's Modified Dulbecco's Medium (IMDM)/0.5% fetal bovine serum (FBS) and 30 uM $ZnCl_2$ were treated with compound or rhTpo (100 ng/mL) at 37°C, 5% CO_2 for 4 hours for BAF3-Tpo or 5 hours for 32D-mpl cells. Suspension cells were centrifuged before removing the medium. Cells were lysed in 100 uL lysis buffer (25 mM Tris, 15% glycerol, 2% Chaps, 1% lecithin, 1% bovine serum albumin (BSA), 4 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, 8 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.4 mM polymethylsulfonylfluoride (PMSF), pH 7.8) for 15 minutes. Lysates (30 uL) were transferred to a 96-well plate. Promega Steady Glow (100 uL; Promega, Madison, WI, USA) was added to each well immediately before reading on a Dynatech Model 1000 chemiluminometer.

Western blots for MAPK and p38

BAF3-Tpo cells (1×10^6) per data point were cytokine-starved overnight in IMDM containing glutamine and 0.5% FBS. Cells were then washed once in phosphate-buffered saline (PBS), pelleted at 1,000 × *g*, and resuspended in 1 mL fresh cytokine-free medium, to which compound or TPO was added to a final concentration up to 30 uM or 100 ng/mL, respectively, for up to 30 minutes. Cells were centrifuged at 4°C for 1 minute, the supernatant discarded, and the pellets placed on dry ice until all samples were processed. The

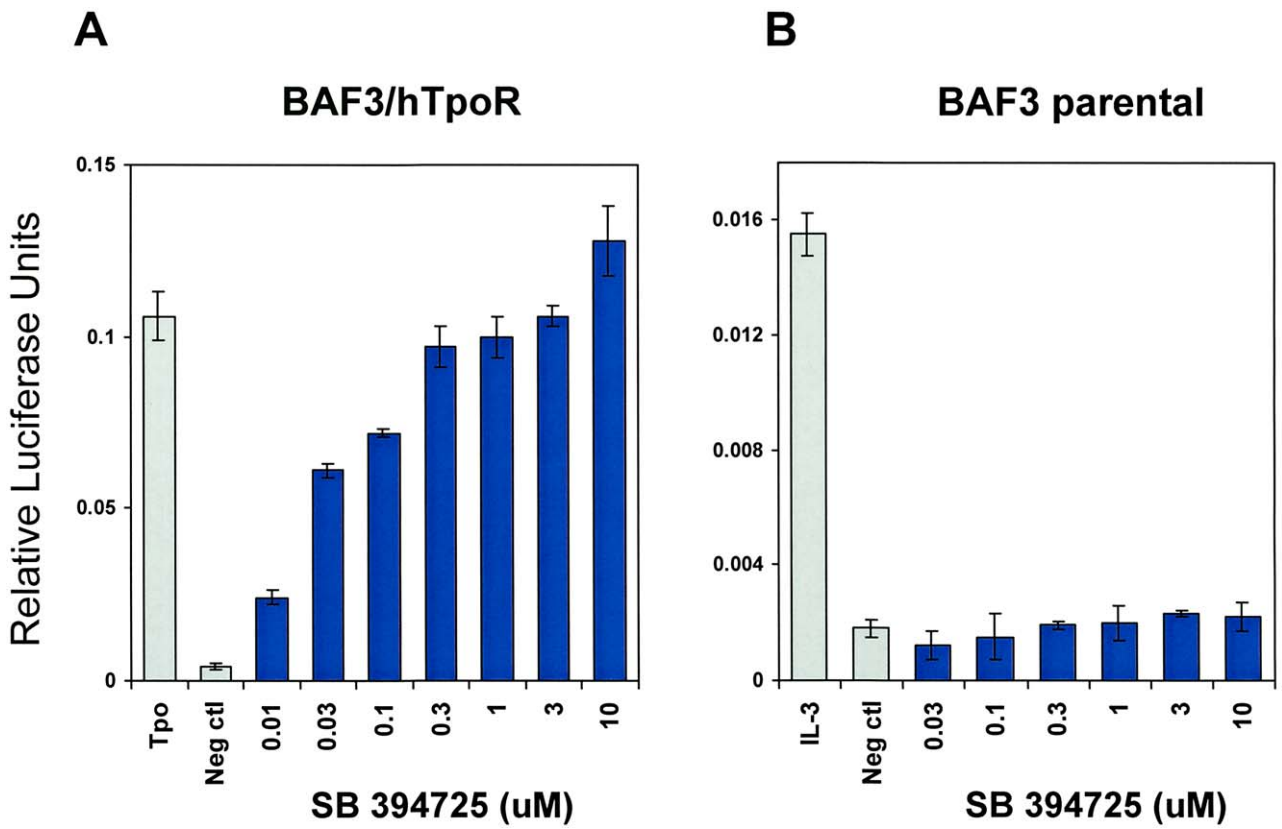

Figure 2. Reporter gene activity of SB 394725 in BAF3/TpoR cells. The BAF3/hTpoR cell line with the IRF promoter coupled to a luciferase reporter gene detects activity of SB 394725 with an EC_{50} of 30 nM (**A**). The BAF3 parental control line has no transfected TpoR and while, rhIL-3 stimulated luciferase production, SB 394725 did not induce activity (**B**). Cells (1×10^5/well) and sample are incubated in RPMI/1% FCS for 3 hours prior to lysis and detection by luminometer.

pellets were resuspended in 120 uL of reduced Laemmli buffer, boiled for 3 minutes and sonicated for 5 minutes in a sonicating bath. After centrifugation, 25 uL of supernatant/lane were loaded on a precast Novex Tris/glycine/sodium dodecyl sulfate mini-gel (10,12, or 4–12% acrylamide) and run at 100 volts for 100 minutes. The gel was then transferred to 0.2 μm nitrocellulose (Schleicher and Schule, Keane, NH, USA) in 10% MeOH in Tris/glycine buffer at 30 volt for 45 minutes. Blots were rinsed in Tris buffered saline (TBS) after transfer and blocked for 1 hour at room temperature in TBS-T/5% dry fat-free milk.

Immunoblotting was performed with phospho-p44/42 MAPK, p44/42 MAPK and phospho-p38 and p38 antibodies (New England Biolabs, Inc, Beverly, MA, USA) according to the manufacturer's recommendation. Briefly, blots were incubated with the primary antibody in TBS-T + 5% BSA (antibodies were diluted 1:1,000) overnight at 4°C, washed three times for 5 minutes each in TBS-T at room temperature, incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature and washed three times as previously. The blot was developed using electrochemiluminescence (Amersham, Piscataway, NJ, USA) and exposed to film for 10 to 30 seconds.

Detection of early response genes

UT7-TPO cells were washed in PBS and starved of Tpo overnight in IMDM/10% FBS. Cells/data point (2×10^6) were resuspended in 1 mL fresh cytokine-free medium containing 30 uM $ZnCl_2$ and treated with compound or Tpo (75 ng/mL) for 15 to 120 minutes. Cells were centrifuged and the RNA extracted from the pellets by the RNAeasy method (Qiagen, Almeda, CA, USA). cDNA was synthesized from 2 ug RNA using Superscript (GibcoBRL, Grand Island, NY, USA).

Each 50 uL polymerase chain reaction (PCR) contained: 10 uL of diluted cDNA template, 10 uL 5× Invitrogen Taq buffer, 1 uL PCR primer mix containing 120 ng/mL each of forward and reverse primers, 1 uL of internal standard (18S:competimer in a ratio of 4:6), 2 uL dNTP mix composed of 10 mM dCTP, dGTP, dTTP, and 1 mM dATP, 0.4 uL Taq enzyme, 0.2 uL 33P-ATP and water to 50 uL. The cycling conditions were 94°C for 3 minutes for the first cycle, followed by 22 cycles at 94°C for 30 seconds, 60°C for 1 minute, 72°C for 2 minutes, followed a final extension at 72°C for 7 minutes. PCR products were loaded onto a Invitrogen TBE 4% to 20% acrylamide gel (Carlsbad, CA, USA). GAPDH was used to normalize for concentration.Commercially available oligos were used for cFos (Stratagene, La Jolla, CA, USA). The sequences of the CIS and EGR-1 PCR primers were:

cCis
(sense) 5′ GTA CAG GGA TCT TGT CCT TTG 3′
(antisense) 5′ TTG GCT ATG CAC AGC AGA TC 3′
EGR1
(sense) 5′ CCT GAC CGC AGA GTC TTT TC 3′
(antisense) 5′ CAT GCT CAC TAG GCC ACT GA 3′

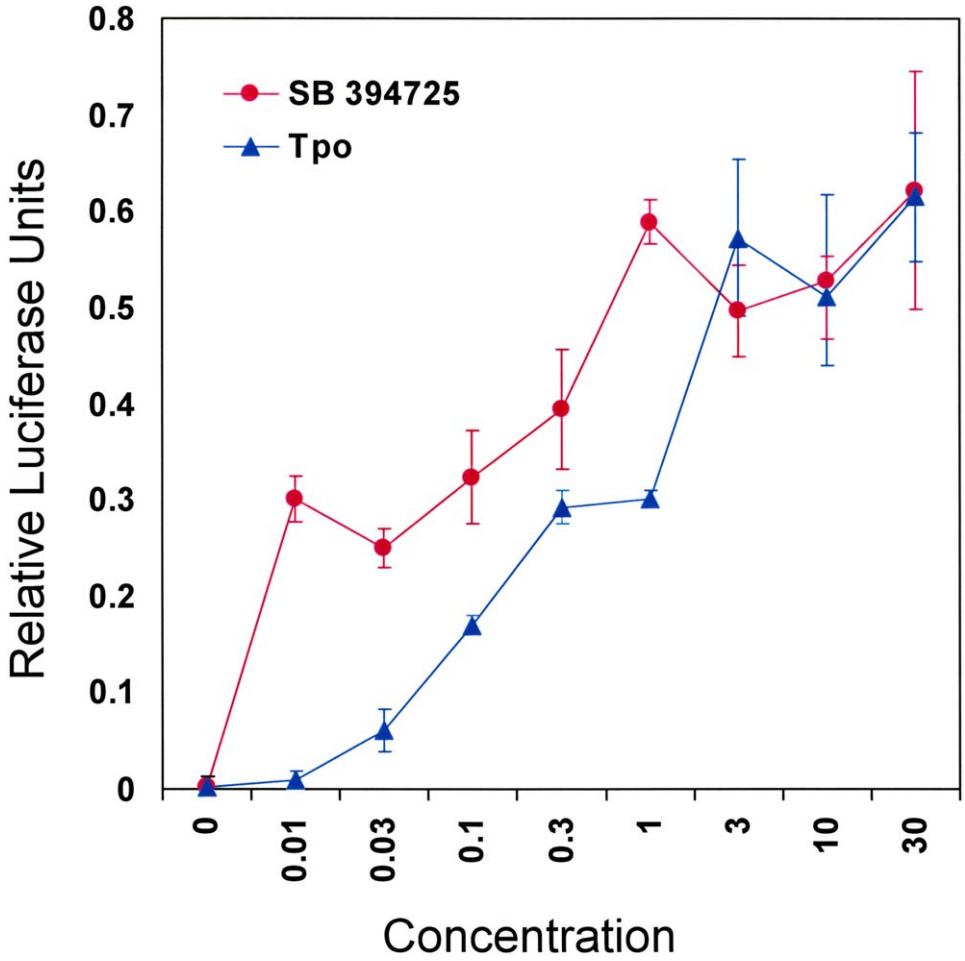

Figure 3. Reporter gene activity of SB 394725 on the gpIIb promoter in 32D-mpl cells. The megakaryocyte-specific gpIIb promoter was coupled to luciferase and SB 394725 (uM) and rhTpo (ng/mL) assayed. SB 394725 demonstrates activity with an EC_{50} of 10 to 100 nM. Control 32D parental cells without the TpoR (mpl) transfected demonstrate no activity (data not shown).

Proliferation assays

Cytokine-starved UT7-Tpo or BAF3-Tpo cells were plated in 96-well plates at 4×10^4 cells/well. Tpo (100 ng/mL) or compound was added to triplicate wells and the cells incubated in 5% CO_2 at 37°C. BAF3-TpoR cells were grown for 44 hours, at which time cells were labeled with 5-bromo-2′-deoxyuridine (BrdU) and returned to the incubator for the remaining 4 hours. Plates were developed using a BrdU cell proliferation kit commercially available from Roche and results read on an enzyme-linked immunosorbent assay plate reader at 380 nm. UT7-Tpo cells were incubated for 72 hours prior to measuring proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with results read at 570/750 nm.

Human marrow proliferation, viability, and differentiation

Light density cells from normal human bone marrow aspirates were incubated with anti-CD34 antibody-conjugated magnetic beads. $CD34^+$ cells were eluted from a magnetic separation column in 1 mL of 0.5% BSA, PBS, 2 mM ethylenediaminetetraacetic acid (EDTA) buffer, according to manufacturer's directions (MACS; Milltenyi, Auburn, CA). Purity of $CD34^+$ cells was 93% to 97% as determined by flow cytometry.

$CD34^+$ cells (1×10^5 cells/mL) purified from normal human marrow were plated in IMDM with 20% FCS and 100 ng/mL rhSCF. Compound (0.003–10 uM) or rhTpo (100 ng/mL) was added. Proliferation of cells in culture was determined by viable cells counted by trypan blue exclusion in the $CD34^+$ cell cultures at day 10, prior to megakaryocyte analysis by flow cytometry. Cells were stained for flow cytometry by dividing them equally into three groups and staining with either fluorescein isothiocyanate (FITC)-anti-CD41a (gpIIb), FITC-isotype control antibody, or PBS as an autofluorescence control. Flow cytometric analysis was performed on a Becton-Dickinson FACScan. Gates of $CD41^+$ cells were based on the negative control, SCF-treated cultures. Only assays with >8% difference in SCF and SCF + Tpo samples were considered for analysis.

Data is represented as a percent of the $Tpo_{max} = (\%CD41_{sample} - CD41_{SCF})/(\%CD41_{Tpo} - \%CD41_{SCF})$.

Detection of $CD34^+$ cell-derived platelets

$CD34^+$ cells were purified and cultured as described in the presence of rhSCF (100 ng/mL) with rhTpo (100 ng/mL) or SB 394725 (30 uM). At day 15, supernatants were stained with anti-CD61;

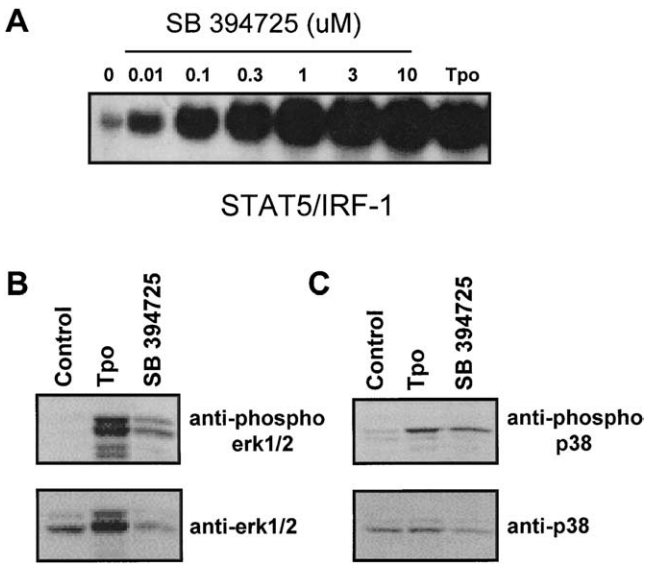

Figure 4. Signal transduction pathways activated by SB 394725. BAF3/hTpoR cell line treated with Tpo or compound for 30 minutes. (**A**) Lysates are incubated with ^{32}P-labeled IRF promoter for 30 minutes and run on a 5% acrylamide gel for the STAT5 electrophoretic mobility shift assay; (**B**) MAPK p42/44 (erk1/2) and (**C**) p38 are activated in response to SB 394725. Total cell lysates were run on a 15% acrylamide gel and Western blotted with anti-phospho erk1/2 to measure MAPK activation.

20,000 events were collected in the forward/side scatter gate set by peripheral blood platelets. The CD61$^+$ events were collected.

Peripheral blood platelet preparation

Blood was obtained from human, chimp, cynomolgus macaques, mouse, pig, ferret, cotton rat, tupaia (tree shrew), and cat. Blood

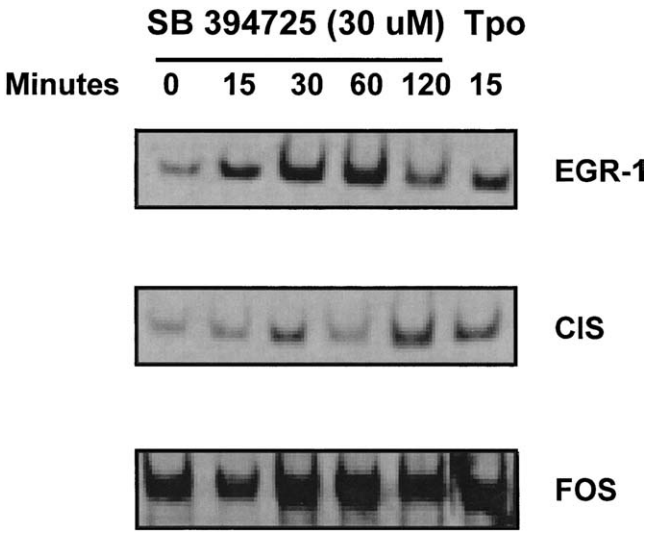

Figure 5. Early response genes induced by SB 394725. Expression of EGR-1, CIS, and FOS in mRNA prepared from UT7-Tpo cells stimulated with 30 uM SB 394725 for 15 to 120 minutes or 100 ng/mL Tpo for 15 minutes.

was collected in 3.8% citrate and centrifuged at 57*g*, 4°C for 20 minutes. The platelet rich plasma (PRP) layer was transferred to a 50-mL conical tube containing salicylic acid (final concentration of 2 mM) and incubated at room temperature for 30 minutes. Prostaglandin E_1 (final concentration of 1 uM) was then added. The platelets were pelleted from the PRP at 500*g* for 20 minutes at 4°C and resuspended to the initial volume in prewarmed RPMI medium containing glutamine and 0.5% FBS. Platelets were used within 8 hours.

Electrophoretic mobility shift assay (EMSA)

Platelets or starved BAF-TpoR cells were incubated with compound or rhTpo for 20 minutes at 37°C, pelleted and pellets resuspended in TAPAS buffer (20 mM HEPES pH 7.9, 300 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 10 mM NaF, and 2 mM Na_3VO_4, 0.5 mM DTT, 1× AEBSF-Pefabloc) and lysed on ice for 20 minutes. Probe mix (20 uL) containing 5 uL lysate, 0.2 uL of ^{32}P probe (IRF-1; labeling method appears later) DNA, 1 mM DTT, 1 ug d(I-C), 3% glycerol in 80 mM HEPES (pH 7.9) was incubated at room temperature for 20 minutes. Labeled DNA/lysate mix (12 uL) was loaded onto a 5% acrylamide gel and results were visualized by autoradiography.

DNA probe preparation for STAT EMSA

Annealed IRF-1 probe was diluted to 100 ng/uL with sodium/Tris/EDTA buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA) and stored frozen at −20°C. For radiolabeling, 1 uL of annealed oligo was incubated with 27 uL dH_2O, 4 uL of 10× M buffer (Roche Applied Science), 1 uL 2 mM dCTP + dTTP, 3 uL ^{32}P dATP (10 uCi/uL), 3 uL ^{32}P dGTP (10 uCi/uL), and 1 uL Klenow for 30 minutes at 37°C. The probe was purified on a Stratagene column before use.

IRF-1 oligos:
5′-GATCGATTTCCCCGAAAT-3′
5′-ACTGATTTCGGGGAAATC-3′

Data analysis

Luciferase and proliferation assays report the mean and standard error of quadruplicate wells. Quantification of EMSA results was accomplished using ImageQuant software and comparing the intensity of bands on gels from SB 394725 treated platelets to that of 100 ng/mL Tpo-treated platelets.

Results

Identification of active compound

SB 394725, described in these experiments, is a compound of the hydrazine class with a structure shown in Figure 1. Compound banks were first screened in the BAF3/TpoR luciferase assay based on STAT activation and stimulation of the IRF-1 promoter linked to a luciferase reporter gene. SB 394725 demonstrated an EC_{50} of 40 nM in this assay with the maximum level of activity equivalent to 125% of the maximum Tpo (100 ng/mL) (Fig. 2a). The activity of the compound was dependent on TpoR as there was no activity with either Tpo or SB 394725 on the parental BAF3 cells

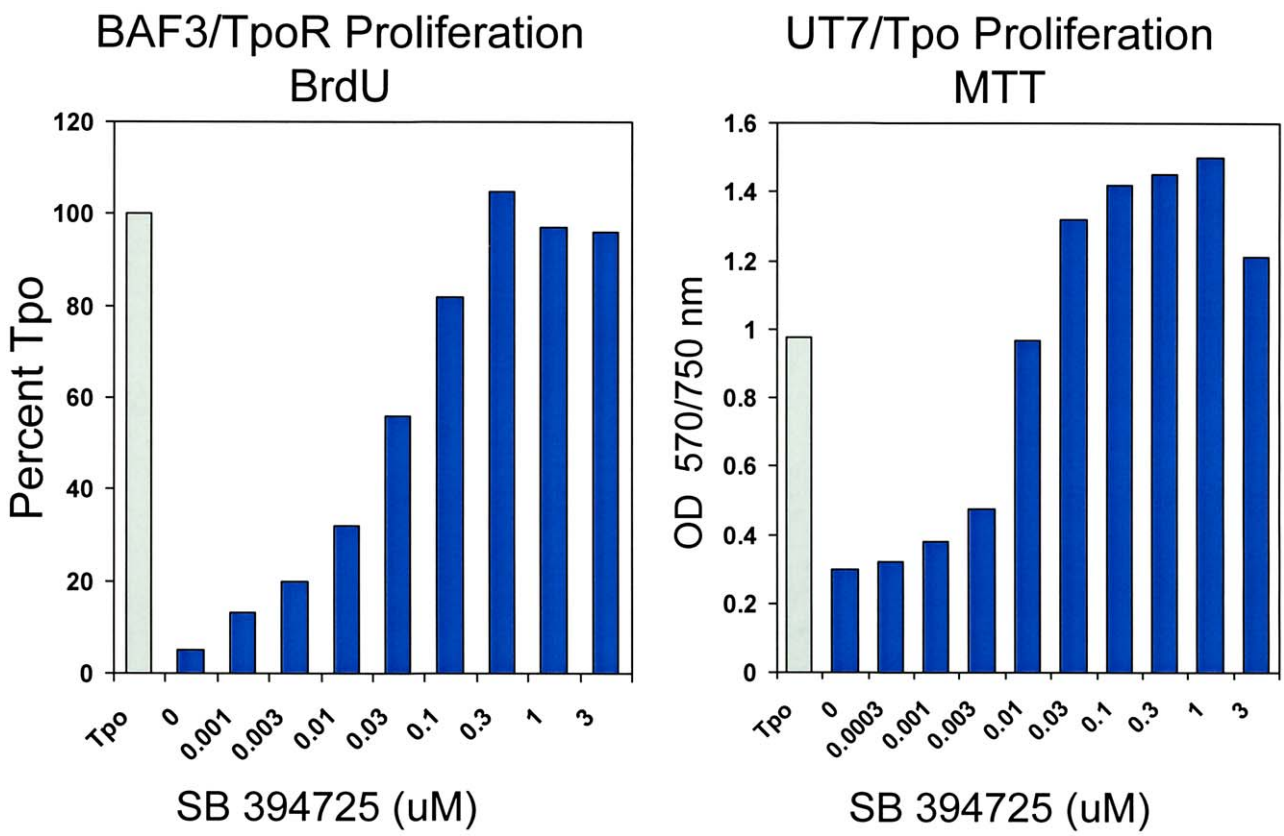

Figure 6. Proliferation of BAF3/TpoR (left panel) and UT7-Tpo (right panel) cells induced by SB 394725. BAF3/TpoR cell proliferation was measured with a BrdU assay after 48 hours. Proliferation of UT7-Tpo was measured by an MTT assay at 72 hours.

in this reporter-gene assay, although luciferase production could be induced by IL-3 (Fig. 2b).

Luciferase production was also induced by SB 394725 in cell lines in which the luciferase reporter gene was linked to the megakaryocyte specific gpIIb promoter. As in the assays using the IRF-1 promoter, 3 uM SB 394725 reached 100% the efficacy of Tpo, with an EC_{50} of 30 nM in the 32D-mpl luciferase assay utilizing the gpIIb promoter (Fig. 3).

Signal transduction

To confirm that the activity induced by the compound was acting through the JAK/STAT signal transduction pathway, EMSA assays were done on the BAF3/TpoR screening cell line, as well as on UT7-Tpo, a Tpo-dependent human cell line that endogenously expresses TpoR. Figure 4A demonstrates that as little as 0.01 uM SB 394725 activated STAT5; higher concentrations of compound shifted the IRF-1 probe in the EMSA in a dose-dependent manner. The compound activated STATs with similar kinetics to Tpo; peak activity was demonstrated at 30 minutes and decreased to background levels at approximately 60 to 120 minutes (data not shown). The p42/44 (erk1/2) and p38 MAP kinases are also reported to be activated by Tpo [9] and phosphorylation of p42/44 and p38 was demonstrated in lysates of BAF3/TpoR cells following 30 minutes of treatment with rhTpo or 30 uM SB 394725 (Fig. 4B,C).

In addition to activation of the STAT and MAPK signal transduction paths, expression of certain early response genes was also demonstrable with SB 394725. Fos, EGR-1, and CIS were detectable in the message of UT7-Tpo cells treated with both Tpo (100 ng/mL) and SB 394725 (30 uM) as early as 30 minutes and peaked at 30 to 60 minutes and diminished by 120 hours, CIS was more delayed than EGR-1 and Fos and peaked at 120 minutes (Fig. 5).

Proliferation, differentiation, and selectivity for TpoR

In addition to the signal transduction pathways traditionally stimulated by Tpo, the cellular functions of proliferation and differentiation were examined. Proliferation assays were performed by measuring BrdU or MTT in BAF3/TpoR and UT7-Tpo cell lines. SB 394725 had an EC_{50} of 30 nM and 40 nM in BAF3/TpoR and UT7-Tpo proliferation assays, respectively (Fig. 6). The maximum activity of the compound reached a plateau at 100 to 300 nM and was consistently 100% to 140% of Tpo maximum in these assays.

An assay to measure the capacity of the compound to induce differentiation of human marrow megakaryocyte progenitors utilized $CD34^+$ cells purified by magnetic separation from normal human marrow. The cells were treated with Tpo or SB 394725 for 10 days after which the total number of cells and the percent of cells differentiating into

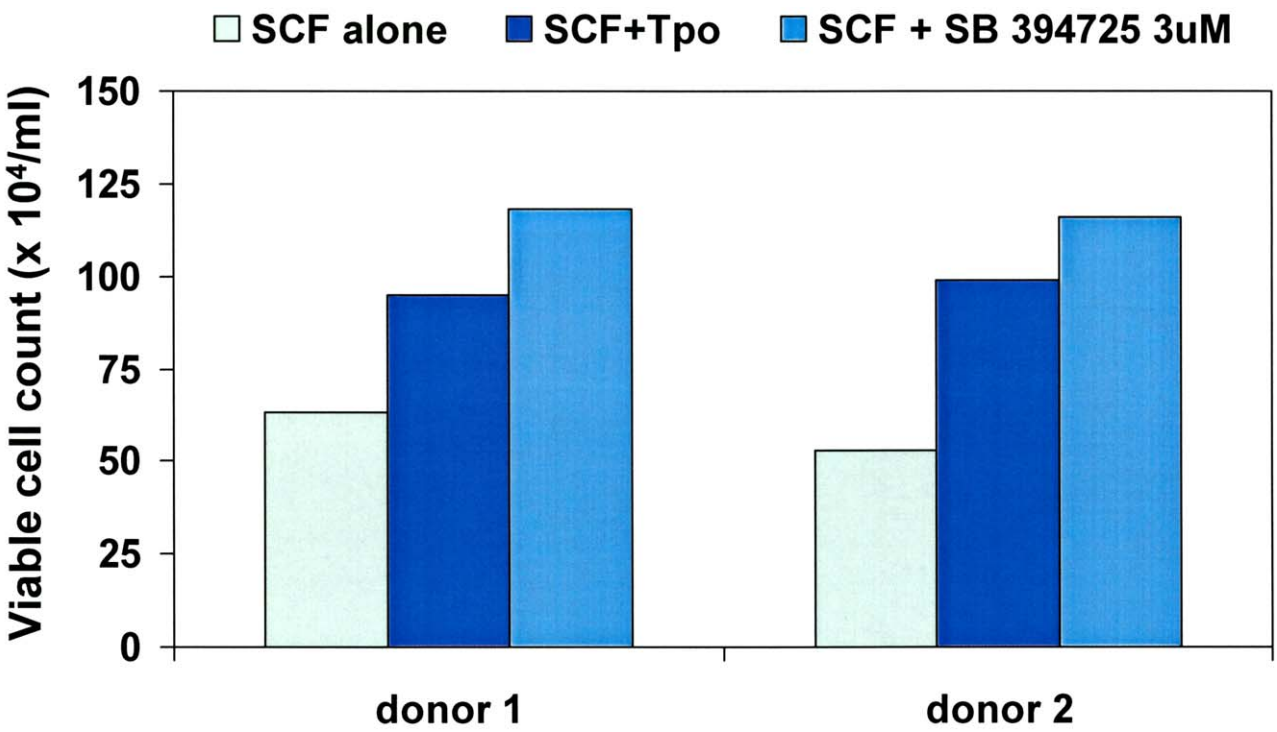

Figure 7. Viable cell counts of CD34$^+$ cells after 10 days in culture. Data represents two different donors of marrow from which CD34$^+$ cells were purified and cultured for 10 days in the presence of SCF (100 ng/mL) alone, SCF + Tpo (100 ng/mL), or SCF + SB 394725 (3 uM). Hemacytometer counts of viable cells, excluding trypan blue, are expressed.

megakaryocytes were determined. Figure 7 shows the increase in total cell number after 10 days with Tpo, an increase of 1.5-fold while SB 394725 increased the number of cells twofold in several different donors. The percent of CD41$^+$ megakaryocytic cells (expressed as a percent of that induced by 100 ng/mL Tpo) detected at day 10 increased in a dose-dependent manner following treatment with SB 394725 with an average EC_{50} of 200 nM in multiple experiments (Fig. 8). The efficacy of the compound relative to Tpo max averaged 120% at 3 uM in more than 20 individual marrow experiments.

Selectivity for Tpo receptor

The selectivity of SB 394725 for the TpoR was demonstrated by assays measuring proliferation, STAT activation or reporter gene expression in various transfected and non-transfected cell lines. Table 1 lists the cell lines in which no activation or proliferation in response to either Tpo (100 ng/mL) or SB 394725 (0.001–100 uM) was detected, although these cells did respond as expected to control cytokines specific for each cell type. These assays confirm the requirement of SB 394725 for cells expressing the TpoR, but not interferon-γ, G-CSF, granulocyte macrophage-CSF, Epo, or IL-3 receptors.

In vitro platelet response and species specificity

Dose-dependent STAT activation was detected 20 minutes after treatment of normal human platelets with 0.3 to 30 uM SB 394725. This is further evidence that SB 394725 has functional activity on normal, endogenously expressed human TpoR.

A murine differentiation assay utilizing SCA$^+$ cells demonstrated differentiation of 70% of the cells into CD41$^+$

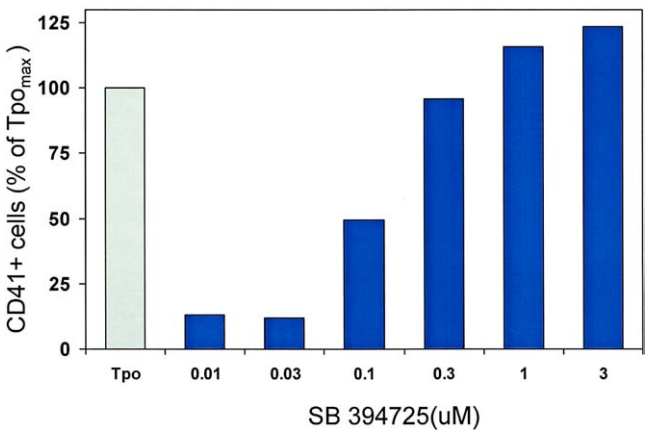

Figure 8. Megakaryocyte differentiation of CD34$^+$ cells after 10 days in culture with SB 394725. CD34$^+$ cells were purified and cultured for 10 days in SCF (100 ng/mL) + Tpo (100 ng/mL) or SCF + SB 394725 (0.01–3 uM). The percent of CD41$^+$ megakaryocytic cells was determined by flow cytometry. The data is presented relative to the CD41$^+$ cells induced by maximum Tpo (100 ng/mL).

Table 1. Selectivity of SB 394725 for Tpo Receptor

Cell line	Cytokine response	Assay type	Positive control	Tpo	SB 394725
BAF3/Tpo	Tpo/IL-3	Proliferation, STAT 5 EMSA	++	++	++
UT7-Tpo	Tpo	Proliferation, STAT5 EMSA	++	++	++
hMPL-HepG2	Tpo	STAT5 EMSA	++	++	+
BAF3/G-CSF	G-CSF/IL-3	STAT5 EMSA	++	−	−
NFS-60	G-CSF	Proliferation	+++	−	−
Kasumi 1	G-CSF	Proliferation	++	−	−
TF-1	GM-CSF/Epo	Proliferation	+++	−	−
UT7-Epo	Epo	Proliferation STAT5 EMSA	+++	−	−
HEPG2	IFN-γ	STAT1 EMSA	+++	−	−

cells 9 days after Tpo treatment, however there was no response to SB 394725 over a wide range of concentrations (0.001–100 uM). This result was confirmed by a lack of STAT activation in murine platelets following SB 394725 treatment. In fact, using platelets from a number of animal species, it was determined that the activity of SB 394725 was limited to human and chimpanzee platelets (Fig. 9). The platelets of additional species tested and found to lack a response to SB 394725 include, cynomolgus macaques, mouse, pig, ferret, and tupaia (tree shrew) (Fig. 9) as well as rabbit, rat, dog, rhesus, and titi monkeys (data not shown).

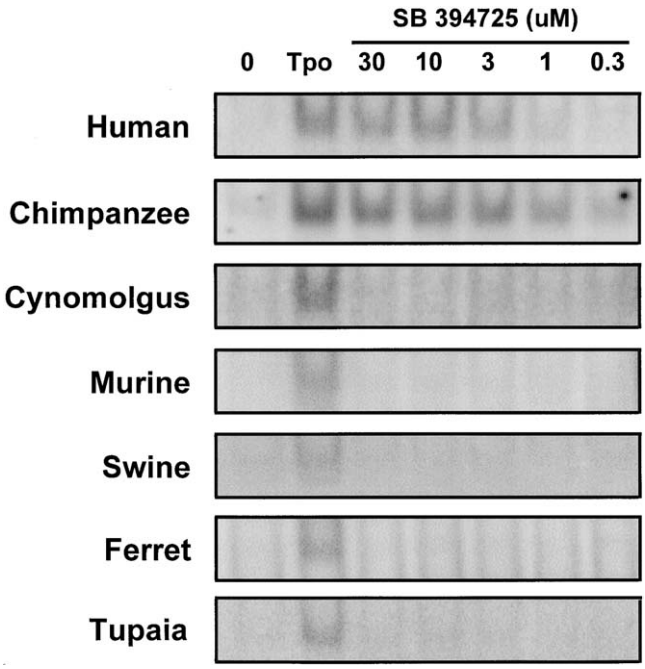

Figure 9. STAT activation induced by SB 394725 in platelets of different species. Normal human blood platelets are purified from peripheral blood collected in sodium citrate. The lysates are incubated with ^{32}P-labeled IRF promoter for 20 minutes and run on a 5% acrylamide gel. The autoradiograph demonstrates a level of activation of STATs from human platelets equivalent to Tpo at 10 uM SB 394725 and even at 0.01 uM, activity is detectable above background. Only chimpanzee and human platelets were activated by the compound.

Discussion

Several peptides, GW395058 and EMP-1 identified through phage display have been reported to be functional agonists of Tpo [20,21] and Epo [22,23], respectively. Remarkably, in each case short peptides were obtained that both bound and activated growth factor receptor, despite the fact that the peptides show no amino acid sequence homology to the cytokines themselves. Covalent dimerization of one of these peptides resulted in a substantial increase in potency, consistent with a mechanism of receptor activation involving peptide-induced dimerization [20]. These studies demonstrated that peptides much smaller than the natural ligand are capable of efficient activation of the TpoR.

More recent reports have suggested that nonpeptide molecules are also capable of being agonists of growth factor receptors, including TpoR. Nonpeptide agonists were identified for Tpo (JTZ-132) [24], G-CSF (SB 247464 and SSCL02446-8) [25,26], insulin [27,28], and Epo [29]. Nonpeptide agonists have an advantage over peptide agonists in that they could be orally bioavailable and would be unlikely to induce an immune response.

Duffy et al. [30] described the chemical modifications made to the initial STAT-based luciferase assay screening hit leading to increased potency of the hydrazone, SB 39472. In repeated assays, SB 394725 demonstrates a similar potency, EC_{50} of approximately 10 to 40 nM in luciferase production and proliferation in cell lines, and 200 nM in differentiation assays in marrow progenitors. The maximum level of activity, of the compound in the proliferation and differentiation assays is equivalent to that seen with Tpo. Also of significance is the similarity in the kinetics of responses between Tpo and SB 394725.

The measurement of activity of SB 394725 on a number of cell lines and primary marrow cells has demonstrated that the compound requires TpoR expression for activity. No other growth factor receptors, even those acting through the JAK/STAT signaling pathway are sufficient for activity. Conclusive demonstration of binding to TpoR, i.e., by BIAcore, has not been possible due to difficulties in expressing sufficient quantities of TpoR.

Assays to measure proliferation and differentiation from $CD34^+$ progenitor cells demonstrated that SB 394725 was

capable of inducing these Tpo-like functions on the key natural TpoR expressing cell. Thus, SB 394725 is a true agonist of the TpoR and would be able to act like Tpo in relieving thrombocytopenia.

Activity of SB 394725 on only human and chimpanzee platelet TpoR, while unlike that of the recently reported TpoR agonist, JTZ-132 [25], is not surprising. Species specificity for a small molecule growth factor agonist was previously reported by Tian et al. [26] for SB 247464, a murine-specific G-CSF agonist, discovered using a similar screening assay. Identification of the TpoR regions responsible for the specificity of SB 394725 for humans and chimpanzees will be later addressed by Tian et al. (manuscript in preparation). Unfortunately, the specificity of SB 394725 has precluded a demonstration of in vivo biological activity for this molecule. A human TpoR knock-in mouse model or studies in chimpanzees or humans will be required to fully demonstrate the biological activity of this molecule.

These data demonstrate the activity of a small, less than 500 Da, molecular weight compound that acts as a Tpo agonist with EC_{50} values of approximately 20 nM, further supporting the feasibility of identifying orally active growth factor agonists with their resulting ease of administration that would provide an advantage in treating patients suffering from thrombocytopenia, anemia, or neutropenia.

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