The effect of a novel, small non-peptidyl molecule butyzamide on human thrombopoietin receptor and megakaryopoiesis

Wataru Nogami,[1,2] Hiroshi Yoshida,[2] Kenzo Koizumi,[2] Hajime Yamada,[2] Kenji Abe,[2] Akinori Arimura,[2] Noriko Yamane,[2] Koji Takahashi,[2] Akiko Yamane,[1] Atsushi Oda,[3] Yoshikazu Tanaka,[2] Hiroshi Takemoto,[2] Yasuyuki Ohnishi,[4] Yasuo Ikeda,[1] and Yoshitaka Miyakawa[1]

[1]Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo; [2]Discovery Research Laboratories, Shionogi & Co., Ltd., Osaka; [3]Department of Preventive Medicine, Hokkaido University School of Medicine, Hokkaido, and [4]Central Institute for Experimental Animals, Kanagawa, Japan

Acknowledgments: we thank T. Nakamura from our laboratory for his helpful discussion and A. Ikejima for her technical assistance.

Manuscript received January 15, 2008. Revised version arrived on May 16, 2008. Manuscript accepted June 5, 2008.

Correspondence:
Yoshitaka Miyakawa, M.D., Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan.
E-mail: yoshi@sc.itc.keio.ac.jp

ABSTRACT

Background

Thrombocytopenia is a common problem in the management of patients with cancer and other conditions that affect hematopoietic cells. In previous clinical trials, the polyethylene-glycol-conjugated recombinant human megakaryocyte growth and development factor increased platelet counts in patients with idiopathic thrombocytopenic purpura and solid tumors undergoing chemotherapy. However, antibodies to polyethylene-glycol-conjugated recombinant human megakaryocyte growth and development factor develop in healthy volunteers and patients undergoing chemotherapy and cross-react with endogenous thrombopoietin. As a result, clinical development of polyethylene-glycol-conjugated recombinant human megakaryocyte growth and development factor was discontinued in 1998. The aim of this study was to identify an orally bioavailable human Mpl activator that does not develop autoantibodies against endogenous thrombopoietin.

Design and Methods

We screened our chemical library and created a novel non-peptidyl thrombopoietin receptor, Mpl activator named butyzamide. We evaluated the effect of butyzamide on megakaryopoiesis *in vitro* using Ba/F3 cells expressing Mpl and human hematopoietic stem cells. For the evaluation of its *in vivo* effect, we administered butyzamide orally to immunodeficient NOD/Shi-*scid*,IL-2Rγ^{null} (NOG) mice transplanted with human fetal liver-derived CD34$^+$ cells and investigated the production of human platelets.

Results

Butyzamide specifically reacted with human Mpl and activated the same signal transduction pathway as thrombopoietin. However, unlike thrombopoietin, butyzamide did not react with murine Mpl and was shown to require the histidine residue in the transmembrane domain of Mpl for its agonistic activity. Butyzamide induced colony-forming unit-megakaryocytes and polyploid megakaryocytes from human CD34$^+$ hematopoietic progenitor cells, and its effects were comparable to those of thrombopoietin. When butyzamide was administered orally at the doses of 10 and 50 mg/kg for 20 days to NOG mice transplanted with human fetal liver-derived CD34$^+$ cells, the human platelet count increased by 6.2- and 22.9-fold, respectively.

Conclusions

Butyzamide is an orally bioavailable human Mpl activator, and appears to have potential for clinical development as a therapeutic agent for patients with thrombocytopenia.

Key words: megakaryocyte, platelet, thrombocytopenia, thrombopoietin, thrombopoietin receptor.

Citation: Nogami W, Yoshida H, Koizumi K, Yamada H, Abe K, Arimura A, Yamane N, Takahashi K, Yamane A, Oda A, Tanaka Y, Takemoto H, Ohnishi Y, Ikeda Y, and Miyakawa Y. The effect of a novel, small non-peptidyl molecule butyzamide on human thrombopoietin receptor and megakaryopoiesis. Haematologica 2008; 93:1495-1504. doi: 10.3324/haematol.12752

Introduction

Thrombopoietin (TPO) is a critical hematopoietic cytokine for regulating megakaryopoiesis.[1–5] It exerts its function through binding and activation of the TPO receptor Mpl, which is one of the type I transmembrane receptors expressed on hematopoietic stem cells, megakaryocytes and platelets.[6,7] After binding with TPO, the Mpl receptor is believed to change its conformation.[8] This event induces transphosphorylation and activation of two tethered Janus kinases, JAK2 and TYK2,[9–11] which in turn results in the activation of various proteins such as STAT3, STAT5,[12] MAPK,[13] PI3K[14,15] and protein kinase C.[16] Activation of these signal transduction pathways results in changes of gene expression and induces megakaryopoiesis and thrombopoiesis. The genetic elimination of either TPO or Mpl in mice reduces the number of circulating platelets by 80-90%.[17–19] This indicates that the TPO-induced signaling pathway is critical for maintaining a normal number of platelets *in vivo*.

In clinical situations, thrombocytopenia is a common problem in the management of patients with cancer and other conditions that affect hematopoietic cells. Thrombocytopenia is also a frequent problem in patients with myelodysplastic syndrome, idiopathic thrombocytopenic purpura (ITP), chronic liver disease and acquired immunodeficiency syndrome. In previous clinical trials, the polyethylene-glycol-conjugated recombinant human megakaryocyte growth and development factor (PEG-MGDF) increased platelet counts in patients with ITP and solid tumors undergoing chemotherapy.[20] However, antibodies to PEG-MGDF develop in healthy volunteers and patients undergoing chemotherapy and cross-react with endogenous TPO.[21,22] This antibody also neutralizes the physiological activity of endogenous TPO, which results in persistent thrombocytopenia. As a result, clinical development of PEG-MGDF was discontinued in 1998. However, most investigators recognize Mpl as an ideal target for developing new agents to increase the number of platelets. The aim of this study was to identify an orally bioavailable human Mpl activator that does not develop autoantibodies against endogenous thrombopoietin. In order to do this we screened our chemical library and created a novel non-peptidyl Mpl activator named butyzamide.

Design and Methods

Reagents

Butyzamide (molecular weight=591.55), (E)-3-[2,6-dichloro-4-[4-[3-[2,2-dimethyl-1-propoxypropyl]-phenyl]-thiazol-2-ylcarbamoyl]-phenyl]-2-methylacrylic acid, was chemically synthesized by Shionogi (Osaka, Japan) (Figure 1A). Recombinant human TPO (rhTPO; PeproTech, Rocky Hill, NJ, USA) and recombinant human granulocyte colony-stimulating factor (rhG-CSF; Calbiochem, La Jolla, CA, USA) were obtained as indicated. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), recombinant mouse GM-CSF (rmGM-CSF), recombinant human interleukin-3 (rhIL-3), recombinant mouse IL-3 (rmIL-3) and recombinant human erythropoietin (rhEPO) were purchased from R&D Systems (Minneapolis, MN, USA).

Cells

The IL-3-dependent murine pro B-cell line Ba/F3 was engineered to express the human (Ba/F3-hMpl) or murine (Ba/F3-mMpl) Mpl receptor. A human Mpl mutant, whose histidine at position 499 was substituted with leucine, was expressed in Ba/F3 cells and named Ba/F3-hMpl(H499L). A murine Mpl mutant, whose leucine at position 490 was substituted with histidine, was expressed in Ba/F3 cells and named Ba/F3-mMpl(L490H). As a basal growth medium, we used RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 10 mM HEPES (Invitrogen, San Diego, CA, USA), 55 μM 2-mercaptoethanol (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen) and 1% MIL34-conditioned medium, as a source of mouse IL-3. The growth medium for Ba/F3-hMpl, Ba/F3-mMpl and Ba/F3-hMpl(H499L) cells was also supplemented with 500 μg/mL geneticin (Nacalai Tesque, Kyoto, Japan). Ba/F3 cells were engineered to express the human EPO receptor (Ba/F3-EPOR). The human myeloid leukemia cell line TF-1 (American Type Culture Collection, Manassas, VA, USA), human erythroleukemia cell line F-36P (RIKEN Cell Bank, Ibaraki, Japan), human myeloid cell line NOMO-1 (Health Science Research Resources Bank, Tokyo, Japan), and murine myeloid cell line DA-3 (RIKEN Cell Bank) were obtained as indicated. NOMO-1 cells expressing human G-CSF receptor were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. TF-1 and F-36P cells, hIL-3 and hGM-CSF-dependent cell lines, were maintained in the presence of 5 ng/mL rhGM-CSF. Ba/F3-EPOR cells, a hEPO-dependent cell line, or DA-3 cells, a mIL-3 and mGM-CSF-dependent cell line, were maintained in the presence of 10 U/mL rhEPO or 5 ng/mL rmGM-CSF, respectively.

Plasmid construction

Full-length hMpl cDNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA extracted from KU812 cells as a template. Amplified PCR fragments were cloned into the pSPORT1 vector (Invitrogen). The resultant plasmid was named pSPORT1-hMpl. Full-length hMpl(H499L) cDNA was cloned into the pGEM3 vector (Promega, Madison, WI, USA). Full-length mMpl cDNA was amplified by RT-PCR using total RNA extracted from bone marrow cells of BALB/c mice as a template. Amplified PCR fragments were cloned into the pcDNA3.1(-) vector (Invitrogen). The resultant plasmid was named pcDNA3.1-mMpl. Full-length mMpl (L490H) cDNA was subcloned into pQCXIP retrovirus vector (Invitrogen). All plasmids were sequenced to confirm the presence of the cloned genes.

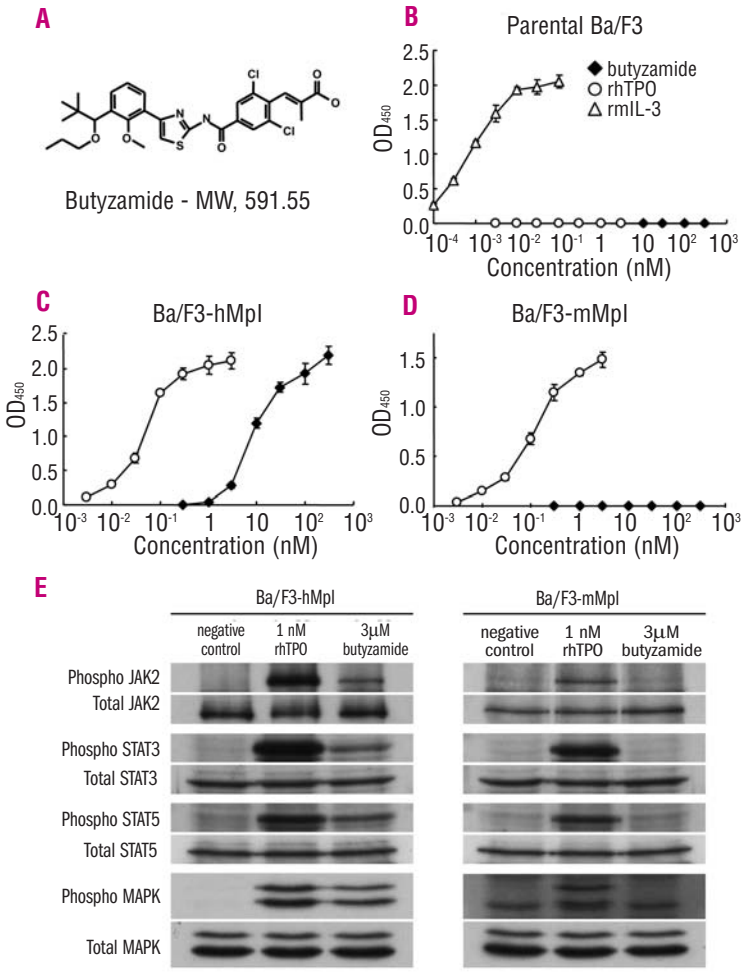

Figure 1. Butyzamide reacts with human but not murine Mpl. (A) The molecular weight (MW) of butyzamide is 591.55 kDa. (B) Neither butyzamide (□) nor rhTPO (○) induced proliferation of parental Ba/F3 cells that did not express Mpl. Recombinant mouse IL-3 (Δ) was used as a positive control. (C) Butyzamide and rhTPO induced proliferation of Ba/F3-hMpl cells in a dose-dependent manner. (D) Unlike rhTPO, butyzamide did not induce proliferation of Ba/F3-mMpl cells. Results are representative of at least three independent experiments run in triplicate and expressed as the mean±SD. (E) Butyzamide and rhTPO induced phosphorylation of JAK2, STAT3, STAT5 and MAPK in Ba/F3-hMpl cells (left panel). Recombinant human TPO, but not butyzamide, induced their phosphorylation in Ba/F3-mMpl cells (right panel). Results are representative of at least three independent experiments.

Establishment of stable cell lines

For the establishment of Ba/F3-hMpl, Ba/F3-mMpl and Ba/F3-hMpl(H499L) cells, Ba/F3 cells were transfected with each constructed plasmid by electroporation. The cells were cloned by a limiting dilution method in the presence of 2 mg/mL geneticin. For the establishment of Ba/F3-mMpl(L490H) cells, 293GP2 packaging cells were transfected with pQCXIP-mMpl(L490H) and pVSV-G vector (Invitrogen) by Fugene 6 (Roche, Mannheim, Germany), according to the manufacturer's protocol. The culture supernatant was collected and used to infect Ba/F3 cells. These established stable cell lines proliferated in response to rmIL-3, as well as parental Ba/F3 cells, and appeared to maintain the character of parental Ba/F3 cells.

Proliferation assay

Cells were cultured at a density of 7.5×10^3 cells/200 μL in 96-well plates with various concentrations of butyzamide or recombinant cytokines. The plates were incubated for 48 h at 37°C in a humidified chamber with 5% CO_2, and 10 μL WST-8 reagent (Kishida Chemical, Osaka, Japan) were added to each well during the last 4 h of culture. The absorbance was measured at a wavelength of 450 nm using a 96-well microplate reader, Model 680 (Bio-Rad, Hercules, CA, USA).

Western blotting

Cells were washed with phosphate-buffered saline (PBS; Sigma) and suspended at a density of 1×10^6 cells/mL in RPMI-1640 medium supplemented with 0.5% bovine serum albumin (BSA; Wako, Osaka, Japan). After incubation for 5 h, the cells were stimulated by 3 μM butyzamide or 1 nM (18.6 ng/mL) rhTPO at 37°C for 15 min, washed once with ice-cold PBS and lysed on ice for 30 min in lysis buffer consisting of 20 mM Tris/HCl, 150 mM NaCl, 1 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM phenyl methane sulfonyl fluoride, 1 mM sodium orthovanadate (Na_3VO_4), 1% Triton X-100 and a protease inhibitor cocktail (Roche), pH 7.4. The lysates were centrifuged at 10,000 g at 4°C for 10 min, and the protein concentration of the collected supernatant was measured using a Protein Dc assay kit (Bio-Rad). After addition of an equal volume of 2×Laemmli's sample buffer, the samples were boiled at 95°C for 5 min. Protein samples were separated by 10% SDS-PAGE and transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The blots were blocked by 5% BSA in TBS-T (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h, and then incubated overnight at 4°C with a primary antibody. The primary antibodies were anti-phospho JAK2 (Tyr1007/1008), anti-phospho STAT3 (Tyr705), anti-phospho STAT5 (Tyr694), and

anti-phospho p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology, Danvers, MA, USA). After washing three times with TBS-T, the blots were probed at room temperature for 1 h with anti-rabbit IgG, horseradish peroxidase-linked whole antibody (GE Healthcare, Uppsala, Sweden) as a secondary antibody. After washing three times, the blots were incubated with ECL detection reagent (GE Healthcare) and exposed to Hyperfilm (GE Healthcare). For comparisons of loaded proteins, the blots were stripped of bound antibodies by incubation with Re-blot plus mild solution (Chemicon, Temecula, CA, USA), and reprobed with anti-JAK2, anti-STAT3, anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-p44/42 MAPK (Cell Signaling Technology) as a primary antibody.

Immunoprecipitation

For the detection of phosphorylated JAK2, 500-750 μg cell extracts were incubated with anti-JAK2 antibody at 4°C overnight, and immunoprecipitates were adsorbed by protein G-coupled Sepharose beads (GE Healthcare) for several hours. After washing three times with lysis buffer, 20 μL of 2×Laemmli's sample buffer were added, and the samples were boiled at 95°C for 5 min. The samples were separated by 10% SDS-PAGE and subjected to western blotting as described above.

Assay for CFU-MK

Colony assays for quantifying human megakaryocytic progenitors were performed using the MegaCult-C kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol. $CD34^+$ cells from human fetal liver and bone marrow were purchased from Cambrex Bio Science Walkersville (currently Lonza Walkersville, East Rutherford, NJ, USA). Cambrex obtained the human tissue under informed consent and purified $CD34^+$ cells using positive immunomagnetic selection. Purity was >95%. The gestational age of fetal liver (lot #050373) was 19 weeks. We obtained approval from the internal review board for this project. $CD34^+$ cells were suspended at a density of 5.0×10^3 cells/well in a double-chamber slide, with various concentrations of butyzamide or rhTPO. After incubation for 10 days at 37°C in a humidified chamber with 5% CO_2, CFU-MK were detected by staining the cells with anti-human CD41 antibody. The number of CFU-MK, counted and subdivided by colony size, was classified as small (3-20 cells/colony), medium (21-49 cells/colony), and large (≥50 cells/colony).

Liquid culture of human bone marrow-derived $CD34^+$ cells

Human bone marrow-derived $CD34^+$ cells were cultured at a density of 5.0×10^4 cells/mL in 24-well plates. As a serum-free medium, we used Iscove's modified Dulbecco's medium (Sigma) supplemented with 20% BIT9500 (StemCell Technologies). Cells were treated with 1 nM rhTPO or 3 μM butyzamide in triplicate for 10 days at 37°C in a humidified chamber with 5% CO_2.

Megakaryocyte ploidy and morphological analysis

Cultured human bone marrow-derived cells were stained with phycoerythrin-labeled anti-human CD41 antibody (BD PharMingen, San Diego, CA, USA) and fixed with 1% paraformaldehyde (Wako), followed by permeabilization with PBS containing 10% DMSO and 1% Tween 20. After washing with PBS, the cells were stained with 7-amino-actinomycin D (Beckman Coulter, Franklin Lakes, NJ, USA) for 30 min at room temperature, and analyzed using a BD FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were obtained by electronically gating 7-amino-actinomycin D-stained $CD41^+$ cells, and at least 30,000 cells were analyzed for each sample. The ploidy distribution was determined by setting markers at the nadirs between the peaks. For morphological analysis, aliquots of cells were centrifuged onto glass slides using a Cytospin 2 centrifuge (Shandon Instruments, Sewickley, PA, USA) and stained with Wright–Giemsa stain. Cells were observed using an Olympus BX51 microscope equipped with a 40×0.75 NA objective, connected to an Olympus DP12 camera, using DP12 software (Olympus, Tokyo, Japan).

Xenotransplantation assay

Immunodeficient NOD/Shi-*scid*,IL-2Rγ^{null} (NOG) mice (8-10 weeks old) were irradiated with 2.4 Gy (125 kV, 10 mA, 0.45 Gy/min) by MBR-1520R-3 (Hitachi Medico, Tokyo, Japan), and on the next day, 6.7×10^4 human fetal liver (FL)-derived $CD34^+$ cells were transplanted intravenously. Two months after transplantation, the NOG mice were randomized into three groups, based on the number of human platelets and body weight. The vehicle or butyzamide at a dose of 10 or 50 mg/kg was administered orally for 21 days. The numbers of human platelets and megakaryocytes were calculated as described below.

Flow cytometry

To detect human platelets in the peripheral circulation of NOG mice transplanted with human FL-derived $CD34^+$ cells, multicolor cytometric analysis was performed using an EPICS-XL flow cytometer (Beckman Coulter). Blood samples were incubated with fluorescein isothiocyanate-labeled anti-mouse CD41 antibody (BD PharMingen) and PE-labeled anti-human CD41 antibody at room temperature. After fixation with 1% paraformaldehyde, samples were subjected to flow cytometry. The actual number of human $CD41^+$ cells was calculated as:

[human $CD41^+$ cell count/(human $CD41^+$ cell count + murine $CD41^+$ cell count)] × whole platelet count

The whole number of platelets was measured using a Sysmex K-4500 instrument (Sysmex, Hyogo, Japan). Reticulated platelets were counted by staining with thiazole orange (Sigma) as described previously.[23] After administration of butyzamide for 21 days, the mice were sacrificed and the femora were removed. Bone marrow cells were collected by flushing with PBS and staining with phycoerythrin-labeled anti-human CD41

antibody. The samples were then subjected to flow cytometry. The actual number of human megakaryocytes was calculated as:

[human CD41⁺ cell count/(human CD41⁺ cell count + murine CD41⁺ cell count)] x whole bone marrow cell count.

Immunohistochemistry

The femur on the other side was used for staining of megakaryocytes. The tissue was fixed in neutral-buffered formalin and decalcified, then embedded in paraffin and sectioned. Immunohistochemical staining was performed with anti-human CD42b (Chemicon) and an EnVision+ peroxidase staining kit (Dako, Carpinteria, CA, USA).

Platelet preparation and aggregation studies

Human blood from healthy volunteers was drawn by venipuncture into 1/10 vol of 3.8% trisodium citrate and gently mixed. Platelet-rich plasma was prepared by centrifugation of whole blood at 200 g for 10 min and adjusted to a concentration of 2×10^8 cells/mL with platelet-poor plasma. Platelet aggregation was measured with an aggregometer (Hema Tracer TM Model 601; Niko Bio Science, Tokyo, Japan) with continuous stirring (1,000 rpm) at 37°C. For western blotting, platelet-rich plasma was aspirated and incubated with 2 mM aspirin for 30 min at room temperature. After the addition of 1 μM PGE1 from a stock solution in absolute ethanol (1 mM), the platelet-rich plasma was spun at 800 g for 10 min to form a soft platelet pellet. The pellet was resuspended in 1 mL of modified HEPES/Tyrode buffer (129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM dextrose, and 10 mM HEPES, pH 7.4), which also contained 2 U/mL apyrase, and was washed twice. Platelets were suspended at a concentration of 1.5×10^9 cells/mL in the same buffer, which contained 2 U/mL apyrase at 37°C, and were stimulated with 100 ng/mL rhTPO or 10 μM butyzamide for 5 or 10 min at 37°C. The reaction was stopped by adding an equal volume of 2xLaemmli's sample buffer. Phosphorylation of JAK2, STAT3, STAT5 and MAPK was analyzed as described above. All the agents were purchased from Sigma, unless otherwise indicated.

Statistical analysis

The results are expressed as the mean±SD. Statistical differences were determined using a two-sided Student's *t* test. p values <0.05 are considered statistically significant.

Results

Butyzamide is a non-peptidyl agonist for human TPO receptor Mpl

We screened our chemical library of 53,000 compounds by proliferation assays using Ba/F3-hMpl cells and obtained some precursor compounds. We modified their structure using combinatorial chemistry and newly synthesized some Mpl activators. Briefly, the insertion of a tertiary butyl group to the lead compound improved the biological activities *in vitro* and bioavailability in mice (*Takayama M. et al., unpublished data*). One of the synthesized compounds was named butyzamide and is shown in Figure 1A. Butyzamide promoted the proliferation of Ba/F3-hMpl cells in a dose-dependent manner (Figure 1C). The 50% effective concentration (EC_{50}) for cell proliferation with butyzamide or rhTPO was approximately 10 and 0.05 nM, respectively. Neither butyzamide nor rhTPO promoted the proliferation of parental Ba/F3 cells that did not express human Mpl. Recombinant mIL-3 promoted the proliferation of parental Ba/F3 cells, as a positive control (Figure 1B). To investigate the effects of butyzamide on murine Mpl, we engineered Ba/F3-mMpl cells expressing murine Mpl, and compared the cell proliferation activity of butyzamide and rhTPO (Figure 1D). Butyzamide did not show any stimulating activity for Ba/F3-mMpl cells, although rhTPO showed dose-dependent cell-proliferating activity. Unlike TPO, which is cross-reactive with human and murine Mpl, butyzamide was shown to have species specificity for human but not murine Mpl.

Intracellular signaling is induced by butyzamide

TPO binds to Mpl and triggers phosphorylation of the cytoplasmic tyrosine kinases JAK2 and TYK2, which in turn induces phosphorylation of various proteins, such as STAT3, STAT5, MAPK and PI3K. As the major components of TPO-mediated signal transduction pathways, we focused on JAK2, STAT3, STAT5 and MAPK, and investigated whether these molecules were phosphorylated by butyzamide. Ba/F3-hMpl and Ba/F3-mMpl cells were starved of cytokines for 5 h and stimulated with 3 μM butyzamide or 1 nM rhTPO for 15 min. Butyzamide or rhTPO was used at the concentration which showed the maximum effect in the cell proliferation and colony-forming assays. After stimulation with butyzamide or rhTPO, phosphorylated JAK2, STAT3, STAT5 and MAPK were detected using each specific antibody. Butyzamide and rhTPO phosphorylated JAK2, STAT3, STAT5 and MAPK in Ba/F3-hMpl cells (Figure 1E, left panel). However, unlike rhTPO, butyzamide did not phosphorylate JAK2, STAT3, STAT5 and MAPK in Ba/F3-mMpl cells (Figure 1E, right panel).

The histidine residue in the Mpl transmembrane domain is critical for the activity of butyzamide

Butyzamide had species specificity and reacted only with human Mpl and not with murine Mpl. The butyzamide derivatives also had the same species specificity and this specificity was dependent on histidine residue 499 (His499) in the transmembrane domain of human Mpl.[24] The same species specificity has been reported for other non-peptidyl Mpl activators such as eltrombopag (SB 497115)[25], NIP-004[26] and AKR-501 (YM477),[27] and all of these require a histidine residue in the transmembrane domain of Mpl to display their biological activity. Therefore we constructed Ba/F3-hMpl(H499L) and Ba/F3-mMpl(L490H) cells which have an H499L or L490H mutation in the transmembrane

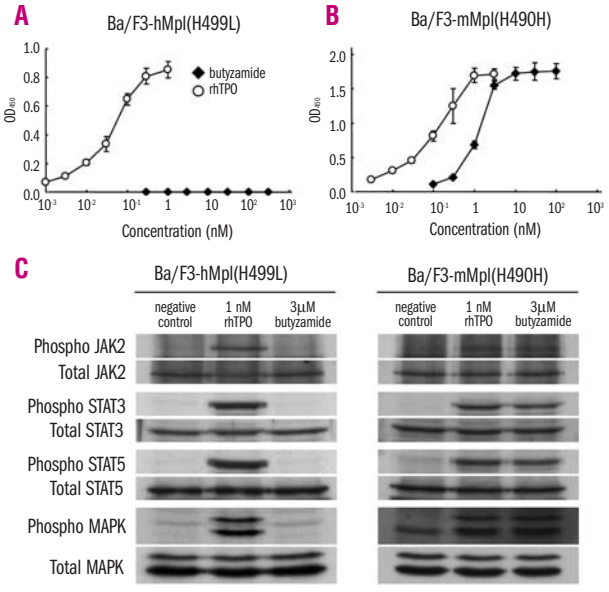

Figure 2. Histidine residue in the transmembrane domain of Mpl was critical for the activity of butyzamide. (A) Butyzamide (u) did not stimulate proliferation of Ba/F3-hMpl(H499L) cells. Recombinant human TPO (°) was used as a positive control. (B) Butyzamide and rhTPO induced proliferation of Ba/F3-mMpl(L490H) cells in a dose-dependent manner. Results are representative of at least three independent experiments run in triplicate and expressed as the mean±SD. (C) Butyzamide did not induce phosphorylation of JAK2, STAT3, STAT5 and MAPK in Ba/F3-hMpl(H499L) cells (left panel). Butyzamide induced tyrosine phosphorylation of intracellular proteins in Ba/F3-mMpl(L490H) cells (right panel). In each experiment, two clones were studied to ensure that the results were internally consistent. Results are representative of at least three independent experiments.

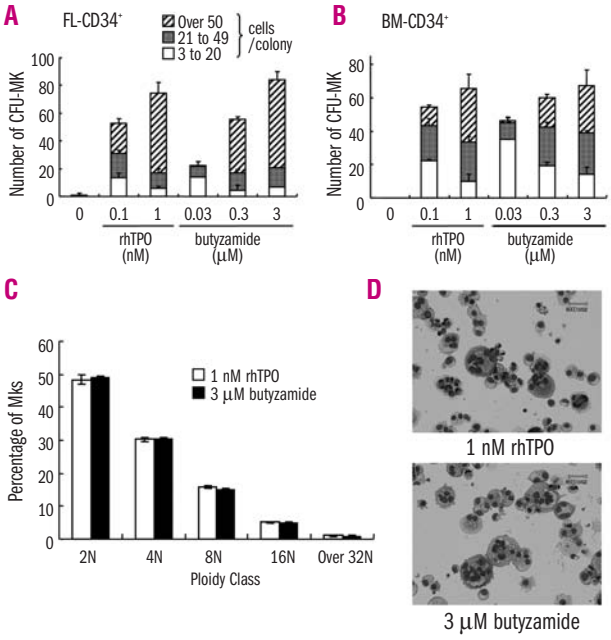

Figure 3. Butyzamide induced colony-forming unit-megakaryocyte and polyploid megakaryocytes from human CD34+ hematopoietic progenitor cells. (A, B) Butyzamide dose-dependently increased the number of CFU-MK from fetal liver (FL)- and bone marrow (BM)-derived CD34+ cells. The number of CFU-MK was counted, subdivided by colony size, and classified as follows: small (3-20 cells/colony); medium (21-49 cells/colony); and large (over 50 cells/colony). Results are representative of at least three independent experiments run in duplicate and expressed as the mean±SD. (C) Human bone marrow-derived CD34+ cells were cultured for 10 days in serum-free medium containing 1 nM rhTPO (®) or 3 μM butyzamide (n). Megakaryocytes (MK) were stained with phycoerythrin-labeled human CD41 antibody and 7-amino actinomycin D. DNA ploidy was analyzed by flow cytometry. Results are representative of at least three independent experiments run in triplicate and expressed as the mean±SD. (D) Aliquots of cultured cells were stained with Wright-Giemsa stain. Bars, 30 μm.

domain of human or murine Mpl, respectively. Although rhTPO maintained proliferation of Ba/F3-hMpl(H499L) cells, butyzamide did not show any such activity (Figure 2A). Corresponding to the loss of proliferative activity, butyzamide did not induce phosphorylation of JAK2, STAT3, STAT5 and MAPK, while rhTPO did (Figure 2C, left panel). On the other hand, in Ba/F3-mMpl(L490H) cells, butyzamide showed cell-proliferative activity and induced phosphorylation of signal-transduction molecules (Figure 2B and 2C, right panel). These results show that the agonistic activity of butyzamide on Mpl is dependent on the histidine residue in the transmembrane domain of Mpl.

Butyzamide induced megakaryopoiesis in CD34+ hematopoietic cells

As TPO is essential for megakaryopoiesis, we compared the effect of butyzamide and rhTPO on increasing the number of CFU-MK in hematopoietic stem cells. Human fetal liver and human bone-marrow-derived CD34+ cells (FL-CD34+ and BM-CD34+, respectively) were used as sources of hematopoietic stem cells and cultured with various concentrations of butyzamide or rhTPO in semisolid culture systems, using the MegaCult-C kit. The number of CFU-MK, counted and subdivided by colony size, was classified as follows: small (3-20 cells per colony); medium (21-49 cells per colony); or large (≥50 cells per colony). In each assay using FL-CD34+ or BM-CD34+, butyzamide increased the number of CFU-MK dose-dependently, and the activity of 3 μM butyzamide was comparable to that of 1 nM rhTPO (Figure 3A, B). The number of large colonies was markedly higher with FL-CD34+ than with BM-CD34+. These results suggest that FL-CD34+ have more immature progenitors for megakaryopoiesis or have a higher potency for cell division compared to BM-CD34+.

Butyzamide increased megakaryocyte ploidy in vitro

Megakaryocyte ploidy is correlated with the degree of endomitosis and reflects the maturation stage of megakayocytes. To investigate whether butyzamide increases mature megakaryocytes *in vitro*, we analyzed the DNA ploidy of megakaryocytes cultured with butyzamide. Human BM-CD34+ cells were cultured for 10 days in serum-free medium containing 3 μM butyzamide or 1 nM rhTPO as a positive control. The treatment with 3 μM butyzamide produced a similar distribution of DNA ploidy to that with 1 nM rhTPO (Figure 3C). For morphological analysis, aliquots of each set of cultured cells were stained with Wright-Giemsa stain.

Corresponding to the results of flow cytometry, 3 μM butyzamide increased polyploid megakaryocytes in a similar manner to 1 nM rhTPO (Figure 3D). These results indicate that butyzamide has as much potential as TPO to induce mature megakaryocytes *in vitro*.

Butyzamide increased human platelets in FL-CD34⁺-transplanted NOG mice

To evaluate the ability of butyzamide to produce human platelets *in vivo*, FL-CD34⁺ cells were transplanted into immunodeficient NOG mice which do not have lymphocytes or natural killer cells, and have low complement activity.[28] Several investigators have used NOG mice to analyze hematopoiesis of stem cells, mast cells and megakaryocytes, because the engraftment rate of human cells is higher in NOG mice than in NOD/SCID mice.[26,29] When butyzamide was administered at a dose of 10 or 50 mg/kg once daily for 20 days to NOG mice transplanted with FL-CD34⁺ cells, it increased the number of human platelets but did not affect the number of murine platelets (Figure 4A, B). Before the increase in human platelets, reticulated human CD41⁺ platelets, which were stained by thiazole orange, were also increased by butyzamide (Figure 4C). After treatment with butyzamide for 21 days, the mice were sacrificed and bone marrow cells were collected by flushing the femora. The number of human CD41⁺ megakaryocytes in the bone marrow was increased by the administration of butyzamide (Figure 4D). The femora on the other side were stained with anti-human CD42b antibody to visualize human megakaryocytes in paraffin sections (Figure 4E). Treatment with butyzamide increased human CD42b⁺ cells compared with the controls, which indicates that butyzamide stimulates the proliferation and maturation of human megakaryocytes *in vivo*. These data show that oral administration of butyzamide is effective at increasing the number of human platelets *in vivo*.

Butyzamide did not enhance agonist-induced platelet aggregation in vitro

To evaluate the effects of butyzamide on human platelet function, we studied aggregation of human platelets. Platelet-rich plasma was preincubated with 100 ng/mL rhTPO or various concentrations of butyzamide at 37°C for 5 min. Butyzamide did not enhance the platelet aggregation induced by 1 μM ADP and 1 μg/mL collagen (Figure 5A, B). In contrast, 100 ng/mL rhTPO enhanced this agonist-induced platelet aggregation as a positive control. Butyzamide, at a dose of 10 μM, induced phosphorylation of JAK2, STAT3, STAT5 and MAPK proteins in human platelets (Figure 5C). rhTPO, as a positive control, induced phosphorylation of all these proteins more strongly than butyzamide did in human platelets.

Cytokine receptor specificity of butyzamide

To study the cytokine receptor specificity of butyzamide, we performed a proliferation assay using various cytokine-dependent cell lines. Butyzamide did not induce proliferation of Ba/F3-EPOR (hEPO, mIL-3-dependent), NOMO-1 (hG-CSF-dependent), TF-1 (hGM-CSF, hIL-3-dependent), F-36P (hGM-CSF, hIL-3-dependent) or DA-3 (mGM-CSF, mIL-3-dependent) (Figure 6A–E). rhEPO, rhG-CSF, rhGM-CSF, rhIL-3, rmGM-CSF and rmIL-3 were used as positive controls for the proliferation assay. These data indicate that butyzamide did not stimulate other cytokine receptors of EPO, G-CSF, GM-CSF and IL-3.

Discussion

In Ba/F3-hMpl cells, butyzamide induced cell proliferation and phosphorylation of JAK2, STAT3, STAT5 and MAPK. However, unlike rhTPO, butyzamide did not induce either proliferation or signal transduction in Ba/F3-mMpl cells. The intensity of phosphorylation of JAK2, STAT3, STAT5 and MAPK protein induced by 3 μM butyzamide was weaker than that induced by 1 nM rhTPO in Ba/F3-hMpl cells. The 50% effective concentration (EC_{50}) for cell proliferation of Ba/F3-hMpl with butyzamide or rhTPO was approximately 10 and 0.05

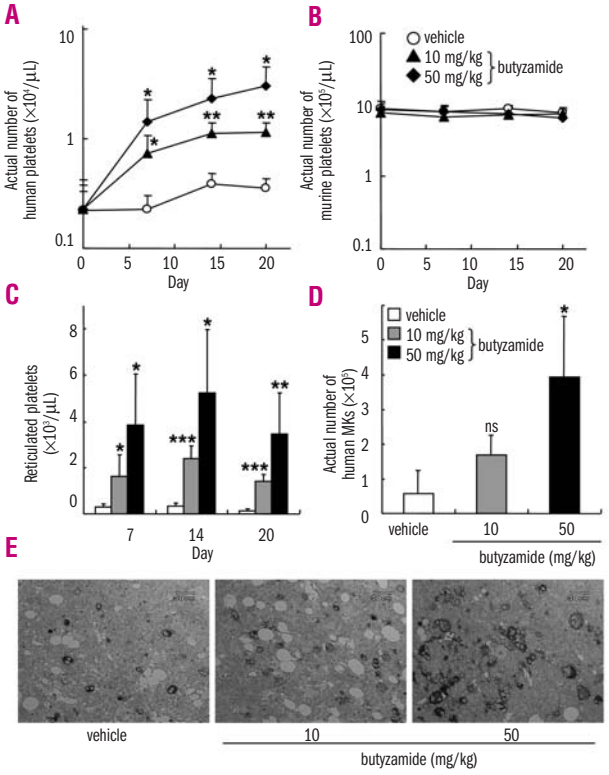

Figure 4. Butyzamide increased circulating human platelets and megakaryocytes in FL-CD34⁺-transplanted NOG mice. Butyzamide was administered orally once daily at a dose of 10 (▲) or 50 mg/kg (u) for 20 days. Butyzamide increased the number of human platelets (A), but did not affect the number of murine platelets (B). Before any increase in human platelets was observed, human reticulated platelets were increased by butyzamide (C). Results are representative of two independent experiments and are expressed as the mean±SD (n=4). (D) Butyzamide increased human CD41⁺ megakaryocytes in the bone marrow of NOG mice. (E) The femur on the other side was stained with human CD42b antibody. Compared with the vehicle, butyzamide increased human CD42b⁺ mature megakaryocytes. Bars, 30 μm. The statistical difference was determined by a two-sided Student's *t* test. ns, not significant; *p<0.05, **p<0.01 and ***p<0.001.

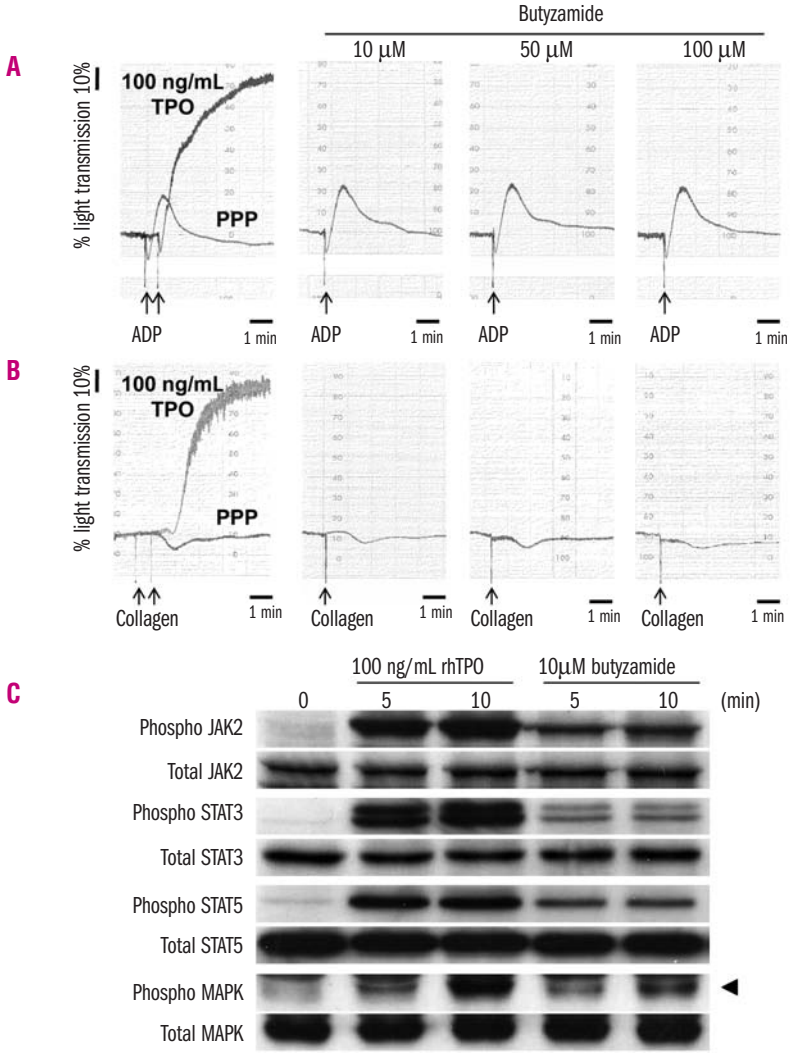

Figure 5. Butyzamide did not enhance agonist-induced platelet aggregation, but induced intracellular signaling in human platelets. Platelet-rich plasma was preincubated with 100 ng/mL rhTPO or various concentrations of butyzamide at 37°C for 5 min. Platelet-poor plasma was used as a negative control. After the addition of 1 μM ADP (A) or 1 μg/mL collagen (B), platelet aggregation was measured in the aggregometer for 5 min under constant stirring. (C) Washed platelets were stimulated with 100 ng/mL rhTPO or 10 μM butyzamide at 37°C for the times indicated. Phosphorylation of JAK2, STAT3, STAT5 and MAPK was detected with phospho-specific antibodies. Each membrane was reprobed with anti-JAK2, STAT3, STAT5 and MAPK antibody to confirm equal protein loading. Results are representative of at least three experiments. The arrow head indicates phospho-MAPK.

nM, respectively. We speculate that the intensity of phosphorylation of intracellular proteins is correlated with proliferation. We cannot, however, exclude the possibility that butyzamide induces alternative signaling pathways that are different from those induced by TPO.

As reported elsewhere, other small non-peptidyl Mpl activators, eltrombopag, NIP-004 and AKR-501, require a histidine residue in the transmembrane domain of Mpl to demonstrate their effects. Butyzamide is a 2-methyl-3-phenylacrylic acid derivative with thiazole scaffolds and eltrombopag is a [1,1'-biphenyl]-3-carboxylic acid derivative with pyrazole scaffolds. Although they have different structures, both of them specifically stimulate human Mpl and need a histidine residue in the transmembrane domain of Mpl to induce intracellular signaling and reveal their biological activities. Very recently, Kim *et al*. performed nuclear magnetic resonance structural studies of the interactions of a small non-peptidyl TPO mimetic, SB 394725, with Mpl.[24,30] They demonstrated that SB 394725 specifically interacts with the extracellular juxtamembrane region and the transmembrane domain of Mpl. SB 394725 interacts with His499 in the transmembrane domain and a few distinct residues in the juxtamembrane region, and affects several specific C-terminal transmembrane residues. As all the small non-peptidyl TPO mimetics are dependent on His499 and have species specificity, we speculate that butyzamide might directly interact with His499 and induce megakaryopoiesis by a mechanism similar to that of SB 394725. Further studies are, however, needed to clarify the activation mechanism of butyzamide. Interestingly, we found that Ba/F3-mMpl(L490H) cells became more sensitive to butyzamide than Ba/F3-hMpl *in vitro*. The phosphorylation of JAK, STAT and MAPK proteins by butyzamide was definitely greater in Ba/F3-mMpl(L490H) cells compared to that in wild-type Ba/F3-hMpl cells. The proliferation of Ba/F3-mMpl(L490H) was clearly better than that of Ba/F3-hMpl cells in the presence of butyzamide. These data raise the possibility that it is a histidine residue in mMpl(L490H) that improves the affinity of mouse Mpl for butyzamide, changes the conformation of Mpl, and increases the sensitivity to induce intracellular signaling. We expect that nuclear magnetic reson-

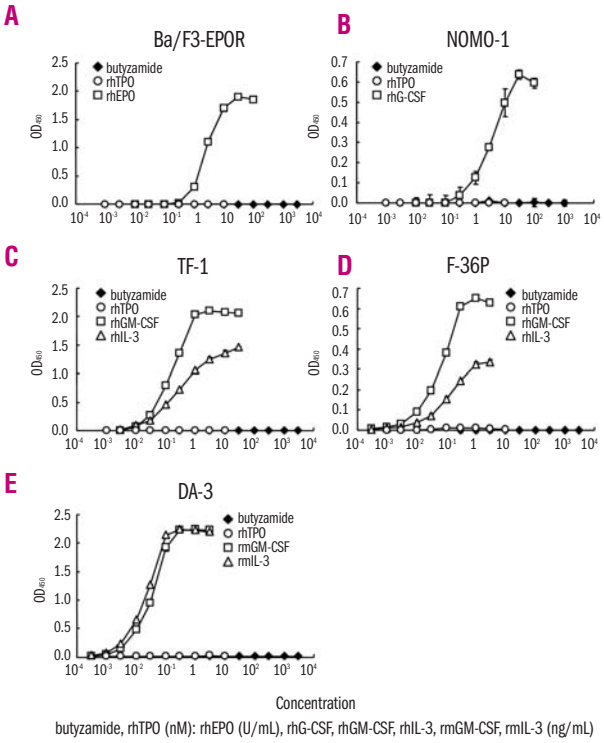

Figure 6. Cytokine receptor specificity of butyzamide. Butyzamide did not induce proliferation of hEPO-dependent Ba/F3-EPOR (A), hG-CSF-dependent NOMO-1 (B), hGM-CSF and hIL-3-dependent TF-1 (C) and F-36P (D), or mGM-CSF and mIL-3-dependent DA-3 (E) cells. Results are representative of at least three independent experiments run in triplicate and are expressed as the mean±SD.

nance studies will answer the question of how butyzamide acts more efficiently with mouse Mpl(L490H) than human Mpl.

We confirmed that butyzamide by itself can induce CFU-MK and polyploid megakaryocytes from human CD34$^+$ hematopoietic progenitor cells under serum-free conditions *in vitro*. As there are no reliable *in vitro* assays to evaluate the effects of the compounds on platelet production, we prepared an experimental animal model using immunodeficient NOG mice transplanted with human FL-derived CD34$^+$ cells after irradiation. We found that around 2-3% of the circulating platelets were derived from human megakayocytes. Using this experimental animal model, we successfully demonstrated that, compared to the vehicle, butyzamide administered orally at the doses of 10 and 50 mg/kg for 20 days increased human platelets by 6.2- and 22.9-fold, respectively, *in vivo*. We also analyzed the effects of butyzamide on other cell lineages in hu-NOG mice. Butyzamide did not increase the number of CD3$^+$ T cells, CD19$^+$ B cells, or CD33$^+$ granulocytes in the peripheral blood or CD45$^-$CD71$^+$ erythroid cells in the bone marrow of NOG mice (*data not shown*).

To evaluate the specificity of butyzamide, we examined its effect on the proliferation of various cytokine-dependent cell lines such as NOMO-1, TF-1, F36-P, DA-3, Ba/F3-EPOR (Figure 6). Expectedly, butyzamide showed no action on these cells, which indicates that butyzamide has high specificity for hMpl, similar to other Mpl activators such as eltrombopag, NIP-004 and AKR-501.

TPO activates signal transduction pathways even in human platelets,[31] and this enhances the platelet aggregation induced by several agonists such as ADP, collagen, epinephrine and thrombin *in vitro*.[32] We, therefore, analyzed the activation of signal transduction pathways in human platelets and the priming effects of butyzamide. *In vitro*, butyzamide phosphorylated signal transduction molecules in human platelets but did not promote human platelet aggregation induced by ADP or collagen. These results are compatible with those of a previous study demonstrating that eltrombopag does not have a priming effect on human platelet aggregation *in vitro*.[25] We found that butyzamide induced phosphorylation of various signaling molecules such as TPO, in human platelets. However, the degree of phosphoylation induced by butyzamide was much smaller than that induced by TPO. This weak effect on signaling might be related to the lack of a priming effect on platelet aggregation by butyzamide. It would be interesting to examine the effects of other Mpl activators on platelet signal transduction. The relatively weak effect on signaling was also noted when we examined Ba/F3-hMpl cells. In marked contrast, butyzamide induced CFU-MK and polyploid megakaryocytes from human CD34$^+$ hematopoietic progenitor cells, as efficiently as TPO. The effects of butyzamide on megakaryopoiesis exceeded those estimated by the intensity of Mpl activation. This point has not been addressed by previous studies on similar compounds, and will be an interesting area for further study.

With regards to toxicity, we did not find any obvious damage in the tissue sections from lungs, liver or kidneys or to murine whole blood counts in NOG mice after 21 days of treatment with butyzamide (*data not shown*). The effects and side effects of butyzamide will, however, need to be evaluated carefully in future clinical trials. Several peptide and non-peptide TPO mimetics are undergoing preclinical and clinical trials.[33–37] Our findings suggest that butyzamide offers some advantages as a small non-peptidyl TPO mimetic because it can be administered orally to patients. In brief, butyzamide appears to have potential for future clinical development.

Authorship and Disclosures

YM, WN, HY, KK, HY, KA, AY, AO and AA designed the study; WN, HY and KK performed experiments and data analyzed; NY, KT, YT and HT constructed the cell lines; YO provided live mice; YM, HY, KA and AA supervised the experiments and data analysis; WN and YM wrote the paper; YI reviewed and commented on the draft of the report; all authors confirmed the contents of this manuscript. WN, HY, KK, HY, KA, AA, NY, KT, YT, HT are employees of Discovery Research Laboratories, Shionogi, Osaka, Japan. YM received the research support from Shionogi. The other authors reported no potential conflicts of interest.

References

1. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature 1994;369:533-8.
2. Lok S, Kaushansky K, Holly RD, Kuijper JL, Lofton-Day CE, Oort PJ, et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature 1994;369:565-8.
3. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature 1994;369:568-71.
4. Wendling F, Maraskovsky E, Debili N, Florindo C, Teepe M, Titeux M, et al. c-Mpl ligand is a humoral regulator of megakaryocytopoiesis. Nature 1994; 369:571-4.
5. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F, et al. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 1994;77: 1117-24.
6. Vigon I, Mornon JP, Cocault L, Mitjavila MT, Tambourin P, Gisselbrecht S, et al. Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. Proc Natl Acad Sci USA 1992;89:5640-4.
7. Debili N, Wendling F, Cosman D, Titeux M, Florindo C, Dusanter-Fourt I, et al. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. Blood 1995;85:391-401.
8. Alexander WS, Metcalf D, Dunn AR. Point mutations within a dimer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity. EMBO J 1995;14: 5569-78.
9. Sasaki K, Odai H, Hanazono Y, Ueno H, Ogawa S, Langdon WY, et al. TPO/c-mpl ligand induces tyrosine phosphorylation of multiple cellular proteins including proto-oncogene products, Vav and c-Cbl, and Ras signaling molecules. Biochem Biophys Res Commun 1995;216:338-47.
10. Drachman JG, Griffin JD, Kaushansky K. The c-Mpl ligand (thrombopoietin) stimulates tyrosine phosphorylation of Jak2, Shc, and c-Mpl. J Biol Chem 1995;270:4979-82.
11. Ezumi Y, Takayama H, Okuma M. Thrombopoietin, c-Mpl ligand, induces tyrosine phosphorylation of Tyk2, JAK2, and STAT3, and enhances agonists-induced aggregation in platelets in vitro. FEBS Lett 1995;374: 48-52.
12. Bacon CM, Tortolani PJ, Shimosaka A, Rees RC, Longo DL, O'Shea JJ. Thrombopoietin (TPO) induces tyrosine phosphorylation and activation of STAT5 and STAT3. FEBS Lett 1995; 370:63-8.
13. Rojnuckarin P, Drachman JG, Kaushansky K. Thrombopoietin-induced activation of the mitogen-activated protein kinase (MAPK) pathway in normal megakaryocytes: role in endomitosis. Blood 1999;94:1273-82.
14. Miyakawa Y, Rojnuckarin P, Habib T, Kaushansky K. Thrombopoietin induces phosphoinositol 3-kinase activation through SHP2, Gab, and insulin receptor substrate proteins in BAF3 cells and primary murine megakaryocytes. J Biol Chem 2001;276:2494-502.
15. Sattler M, Salgia R, Durstin MA, Prasad KV, Griffin JD. Thrombopoietin induces activation of the phosphatidylinositol-3' kinase pathway and formation of a complex containing p85PI3K and the protooncoprotein p120CBL. J Cell Physiol 1997;171:28-33.
16. Hong Y, Dumenil D, van der Loo B, Goncalves F, Vainchenker W, Erusalimsky JD. Protein kinase C mediates the mitogenic action of thrombopoietin in c-Mpl-expressing UT-7 cells. Blood 1998;91:813-22.
17. de Sauvage FJ, Carver-Moore K, Luoh SM, Ryan A, Dowd M, Eaton DL, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med 1996;183:651-6.
18. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. Science 1994;265:1445-7.
19. Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. Blood 1996;87:2162-70.
20. Kuter DJ, Begley CG. Recombinant human thrombopoietin: basic biology and evaluation of clinical studies. Blood 2002;100:3457-69.
21. Li J, Yang C, Xia Y, Bertino A, Glaspy J, Roberts M, et al. Thrombocytopenia caused by the development of antibodies to thrombopoietin. Blood 2001;98:3241-8.
22. Basser RL, O'Flaherty E, Green M, Edmonds M, Nichol J, Menchaca DM, et al. Development of pancytopenia with neutralizing antibodies to thrombopoietin after multicycle chemotherapy supported by megakaryocyte growth and development factor. Blood 2002;99:2599-602.
23. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood 1990;75:116-21.
24. Yamane N, TanakaY, Ohyabu N, Yamane S, Maekawa K, Ishizaki J, et al. Characterization of novel non-peptide thromobopoietin mimetics, their species specificity and the activation mechanism of the thrombopoietin receptor. Eur J Pharmacol 2008;586:44-51.
25. Erickson-Miller CL, Delorme E, Giampa L, Hopson C, Valoret E, Tian SS, et al. Biological activity and selectivity for Tpo receptor of the orally bioavailable, small molecule Tpo receptor agonist, SB-497115. Blood 2004;104:2912a.
26. Nakamura T, Miyakawa Y, Miyamura A, Yamane A, Suzuki H, Ito M, et al. A novel nonpeptidyl human c-Mpl activator stimulates human megakaryopoiesis and thrombopoiesis. Blood 2006;107:4300-7.
27. Suzuki K, Abe M, Fukushima M, Sugasawa K, Hirayama F, Kawasaki T, et al. YM477, a novel orally-active thrombopoietin receptor agonist. Blood 2005;106:2298a[abstract].
28. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, et al. NOD/SCID/γ(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002;100:3175-82.
29. Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chain(null) mice. Blood 2005;106: 1565-73.
30. Kim MJ, Park SH, Opella SJ, Marsilje TH, Michellys PY, Seidel HM, et al. NMR structural studies of interactions of a small, nonpeptidyl Tpo mimic with the thrombopoietin receptor extracellular juxtamembrane and transmembrane domains. J Biol Chem 2007; 282:14253-61.
31. Miyakawa Y, Oda A, Druker BJ, Miyazaki H, Handa M, Ohashi H, et al. Thrombopoietin induces tyrosine phosphorylation of Stat3 and Stat5 in human blood platelets. Blood 1996;87: 439-46.
32. Oda A, Miyakawa Y, Druker BJ, Ozaki K, Yabusaki K, Shirasawa Y, et al. Thrombopoietin primes human platelet aggregation induced by shear stress and by multiple agonists. Blood 1996;87:4664-70.
33. Jenkins JM, Williams D, Deng Y, Uhl J, Kitchen V, Collins D, et al. Phase I clinical study of eltrombopag, an oral, non-peptide thrombopoietin receptor agonist. Blood 2007;109:4739-41.
34. Bussel JB, Cheng G, Saleh M, Kovaleva L, Stone N, Mayer B, et al. Analysis of bleeding in patients with immune thrombocytopenic purpura (ITP): a randomized, double-blind, placebo-controlled trial of eltrombopag, an oral platelet growth factor. Blood 2006;108:475a [abstract].
35. Bussel JB, Kuter DJ, George JN, McMillan R, Aledort LM, Conklin GT, et al. AMG 531, a thrombopoiesis-stimulating protein, for chronic ITP. N Engl J Med 2006;355:1672-81.
36. Kuter DJ, Bussel JB, George JN, Aledort LM, Lichtin AE, Lyons RM, et al. Long-term dosing of AMG 531 in thrombocytopenic patients with immune thrombocytopenic purpura: 48-week update. Blood 2006; 108:476a [abstract].
37. Desjardins RE, Tempel DL, Lucek R, Kuter DJ. Single and multiple oral doses of AKR-501 (YM477) increase the platelet count in healthy volunteers. Blood 2006;108:477a[abstract].