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Characterization of novel non-peptide thrombopoietin mimetics, their species specificity and the activation mechanism of the thrombopoietin receptor

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Abstract

A series of non-peptide small compounds discovered to be thrombopoietin receptor agonists showed species specificity to humans. Compound I could induce megakaryocyte lineage from human bone marrow cells, but not from mouse, guinea pig or cynomolgus monkey bone marrow cells. To elucidate the mechanism, we identified the pivotal amino acid residue for the receptor activation by compound I by taking advantage of its species specificity. The response of compound I to three human/mouse chimeric receptors indicated the importance of the transmembrane domain. Comparison of amino acid sequences of the transmembrane domain of the thrombopoietin receptor between human and three animal species led us to hypothesize that histidine 499 is necessary for the reactivity to the thrombopoietin mimetics. We verified the hypothesis using two mutant receptors: the human thrombopoietin receptor mutant His499Leu and the mouse thrombopoietin receptor mutant Leu490His. These results should be helpful for structure–activity relationship studies and conducting *in vivo* studies of thrombopoietin mimetics.

Keywords: Thrombopoietin; Human thrombopoietin mimetics; Species specificity; Chimera/mutant receptor; Loss/gain of function; Megakaryocytosis

1. Introduction

In trying to develop a drug to treat thrombocytopenic patients in place of platelet transfusion (Kuter, 2007), we found a novel series of non-peptide thrombopoietin receptor agonists. They directly stimulated the thrombopoietin-dependent cell line Ba/F3-huMPL to proliferate in a dose-dependent manner and promoted *in vitro* megakaryocytopoiesis from human bone marrow cells (Yamane, N. et al. in submission). They could activate the thrombopoietin signal transduction pathway in Ba/F3-huMPL, such as the thrombopoietin receptor, Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, the Ras-mitogen-activated protein kinase (MAPK) pathway, and phospholipase Cγ (PLCγ), as well as recombinant human thrombopoietin. However, as these compounds are specific for the human receptor, we could not evaluate their *in vivo* activity in common experimental animals. We tried to elucidate the mechanism of their thrombopoietin-like activities and species specificity.

The thrombopoietin receptor is a type I cytokine receptor consisting of an extracellular domain (485 amino acids), a transmembrane domain (22 amino acids), and a cytoplasmic domain (122 amino acids). The extracellular domain contains two cytokine receptor homology regions (CRH1, CRH2). The cytoplasmic domain (lacking kinase activity) is defined by BOX1 and BOX2 motifs, through which various signaling molecules are regulated (Bazan, 1990; Kaushansky, 2005; Vigon et al., 1992).

In this study, we engineered three human/mouse chimeras and two mutants of the thrombopoietin receptor to identify the

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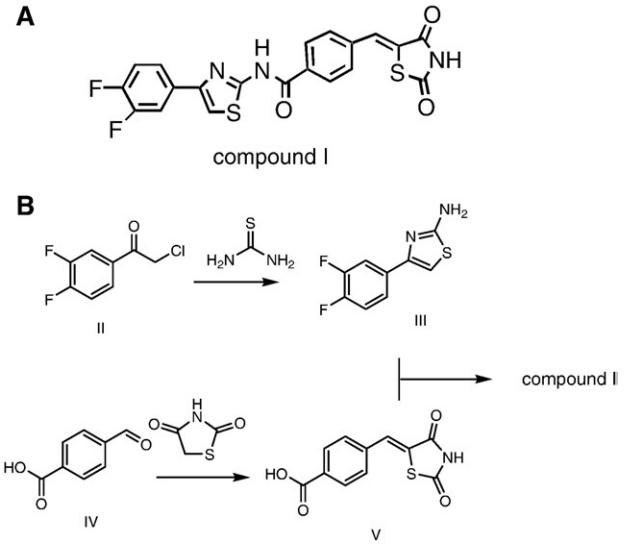

Fig. 1. (A) Chemical structure of compound I (*N*-[4-(3,4-Difluoro-phenyl)-thiazol-2-yl]-4-[2,4-dioxo-thiazolidin-(5Z)-ylidenemethyl]-benzamide), a tool compound of non-peptide thrombopoietin mimetics used in this study. Molecular weight is 443.45 Da. (B) Synthesis of Compound I.

pivotal site for the receptor activation by these compounds, which should help clarify their *in vivo* activity.

2. Materials and methods

2.1. Reagents

Recombinant human thrombopoietin and recombinant mouse thrombopoietin were purchased from Genzyme (MA, U.S.A). Anti-human Glycoprotein IIb/IIIa (CD41a) monoclonal antibody was purchased from StemCell Technologies (Vancouver, Canada). Compound I, with the structure shown in Fig. 1A, was synthesized by Shionogi (Osaka, Japan). Briefly, it was synthesized from the amidation reaction with intermediate III and intermediate V. Intermediate III was synthesized from the commercially available compound II (2-chloro-1-(3,4-difluorophenyl)-ethanone, Enamine Ltd., Kiev, Ukraine), which was allowed to react with thiourea. Similarly intermediate V was synthesized from commercially available compound IV (terephthalaldehydic acid, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), which was allowed to react with 2, 4-thiazolidinedione. The structure and the synthesis method are shown in Fig. 1B.

2.2. Cells and cultures

An interleukin (IL)-3-dependent mouse pre-B cell line Ba/F3 (Riken Cell Bank, Tsukuba, Japan) was maintained in RPMI1640 medium containing L-glutamine, 10% fetal bovine serum (HyClone), and 10% WEHI-3 conditioned medium as a source of mouse interleukin-3 (mIL-3). A packaging cell line 293GP was purchased from Clontech Laboratories Inc. (CA, USA). Human $CD34^+$ bone marrow hematopoietic progenitor cells were purchased from Cambrex Bio Science Walkersville, Inc. (MD, U.S.A). This study was approved by the institutional ethics committee, and all specimens were obtained with informed consent.

2.3. Animals

Balb/c mice and Hartley guinea pigs were purchased from Charles River Japan, and cynomolgus monkeys from Japan E.D.M., Inc. (Tokyo, Japan). The experimental protocol for this study was approved by the local animal ethics research

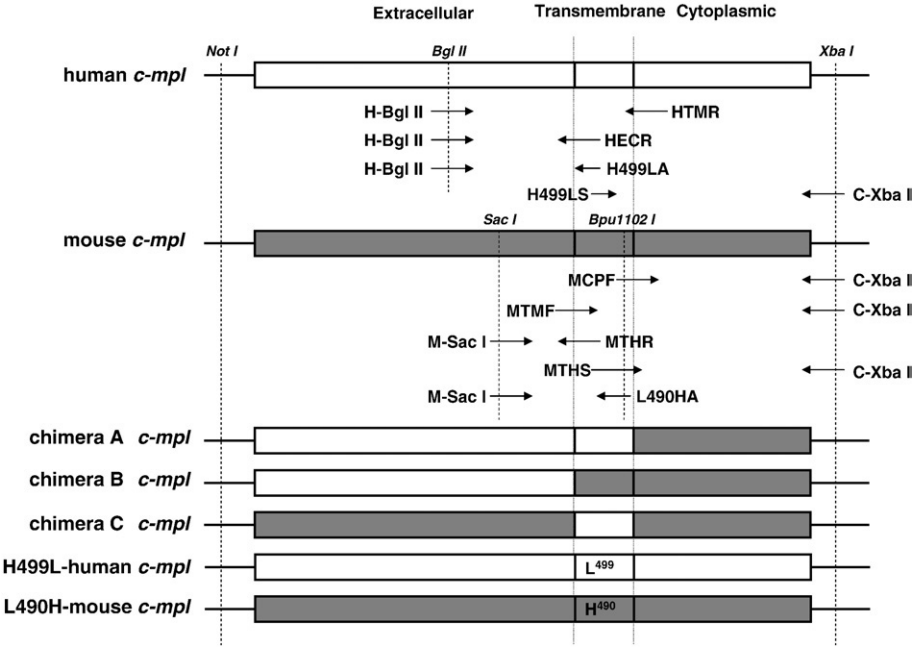

Fig. 2. Schematic presentation of the constructions of cDNA encoding chimeric and mutant thrombopoietin receptors. This figure shows primers used in their construction. The construction procedures are described in detail in Materials and methods. The nucleotide sequences of the primers are shown in Table 1.

committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4. Construction of expression plasmids of wild-type thrombopoietin receptors

Human thrombopoietin receptor gene, *c-mpl* cDNA was amplified from human leukemia cell cDNA and cloned into a mammalian expression vector pGEM3-SRα-neo (pGEM3-huMPL) in our recent study (Yamane, N. in submission). Mouse *c-mpl* cDNA was amplified from total cDNA of Balb/c mouse bone marrow cells using PCR primers: 5′-ATG CCC TCT TGG GCC CTC TTC A-3′ and 5′-CTG CCT TCA GGG CTG CTG CCA A-3′, and cloned into the expression vector pcDNA3.1(−) containing a Cytomegalovirus (CMV) promoter (Invitrogen Corp., Carlsbad, CA, U.S.A.). The mouse *c-mpl* cDNA constructed into the expression plasmids (pcDNA3.1-muMPL) was verified by DNA sequencing.

2.5. Construction of the human/mouse thrombopoietin receptor chimeras A, B, C, H499L-mutated human thrombopoietin receptor, and L490H-mutated mouse thrombopoietin receptor

All chimeras and mutants were prepared according to the PCR-based overlap extension method (Horton et al., 1990). The positions and nucleotide sequences of each primer used in these constructions are indicated in Fig. 2 and Table 1. To make chimera A, part of human c-*mpl* was amplified using primers H-Bgl II and HTMR, and part of mouse *c-mpl* was amplified using primers MCPF and C-Xba I (1st PCR). HTMR and MCPF contain partial sequences of the human transmembrane domain and the mouse cytoplasmic domain (shown in Table 1). These 1st PCR fragments were purified and mixed for use as a template for the next PCR using primers H-Bgl II and C-Xba I (2nd PCR). The 2nd PCR fragment was digested by Bgl II and Xba I, and the Bgl II-Xba I fragment of pGEM3-huMPL was replaced by the PCR product. For chimera B, the 1st PCR using primers H-Bgl II and HECR was done for human *c-mpl* cDNA and that using primers MTMF and C-Xba I for mouse *c-mpl* cDNA. HECR and MTMF contain partial sequences of the human extracellular domain and the mouse transmembrane domain. The purified 1st PCR fragments were combined and subjected to the 2nd PCR using primer set of H-Bgl II and C-Xba I. After digestion by Bgl II and Xba I, the Bgl II-Xba I fragment of pGEM3-huMPL was replaced by the 2nd PCR product. For chimera C, the 1st PCR was performed on mouse *c-mpl* with a primer set of M-Sac I and MTHR or another set of MTHS and C-Xba I. The primers MTHR and MTHS have sequences of the transmembrane domain of human *c-mpl* and a small portion of an extracellular domain or cytoplasmic domain of mouse *c-mpl* on the 3′ end, respectively. The purified 1st PCR products were mixed and subjected to the 2nd PCR using a primer set of M-Sac I, C-Xba I. After the digestion by Sac I and Xba I, the Sac I-Xba I fragment of pcDNA3.1-muMPL was replaced by the 2nd PCR product. To construct H499L-mutant human *c-mpl* (H499L-huMPL), the 1st PCR was performed on human *c-mpl* cDNA using primers of H-Bgl II and H499LA or using primers of H499LS and C-Xba I. The purified 1st PCR products were subjected to the 2nd PCR using primers H-Bgl II and C-Xba I. After the digestion by Bgl II and Xba I, the Bgl II-Xba I fragment of pGEM3-huMPL was replaced by the PCR product. To construct L490H-mutated mouse *c-mpl* (L490H-muMPL), PCR using primers M-Sac I and L490HA was performed on pcDNA3.1-muMPL. The PCR product was digested using Sac I and Bpu1102 I, and the Sac I-Bpu1102 I fragment of pcDNA3.1-muMPL was replaced by the PCR fragment. The L490H-muMPL was recloned into a retroviral vector pQCXIP (Clontech). These newly constructed expression plasmids were all verified by DNA sequencing.

Table 1
Nucleotide sequences of primers used for construction of chimeras and mutants of the thrombopoietin receptor

Primer	Nucleotide sequence (5′–3′)
H-Bgl II	AGG GAG ATC TCC AGT GGG CAT CTG
HTMR	CCA CTT CAG CAG CAG CAG GCC CAG GAC
MCPF	CTG CTG AAG TGG CAA TTT CCT GCG CAC TAC
C-Xba I	C TCT TCT AGA TCA GGG CTG CTG CCA ATA GCT TAG
HECR	GGT GAT CCA GGC GGT CTC GGT GGC TTG
MTMF	GCC TGG ATC ACC TTG GTG ACT GCT CTG CTC
M-Sac I	CCC CGA GCT CGC TAC AGC TTG
MTHR	GCC CAG CAC CAG ATG CAG AGC AGT CAC CAA GGA GAT CCA
MTHS	GTG CTG GGC CTC AGT GCC GTC CTG GGC CTA CTG CTG CTA
H499LA	CAC TAG **CAG** CAG AGC GGT CAC CAA GGA GAT
H499LS	GCT CTG **CTG** CTA GTG CTGGGC CTC AGC GCC
L490HA	GAG GCT CAG CAC CAG **ATG** CAG AGC AGT CAC CAA GGT GAT

Underlining; sequences corresponding to mouse *c-mpl*, Bold lettering; trinucleotides corresponding to the amino acid replaced at residue 499 of human thrombopoietin receptor or at residue 490 of mouse thrombopoietin receptor, Boxed sequences; TCTAGA, GAGCTC, and GCTCAGC are recognition sites of restriction enzymes Xba I, Sac I and Bpu1102 I, respectively.

2.6. Transfection of the thrombopoietin receptor expression plasmids

Parental Ba/F3 cells were washed twice and 1×10^7 cells were suspended in 0.6 ml PBS. Cells were mixed with 20 μg of each expression plasmid carrying the human or mouse thrombopoietin receptor gene, chimeric thrombopoietin receptor genes A, B, or C, or the H499L mutant thrombopoietin receptor gene, and transfected by electroporation at 220 V and 960 μFD. After 24-h incubation at 37 °C in RPMI medium supplemented with 10% fetal bovine serum (HyClone) and mouse IL-3, the cells were collected by centrifugation and resuspended in the same medium containing 2 mg/ml Geneticin (G418, Invitrogen-Gibco) as a selection marker. Ba/F3-huMPL, Ba/F3-muMPL, Ba/F3-chimeras A, B, C, and Ba/F3-huMPL(H499L) were established and maintained in RPMI1640 medium containing 10% fetal bovine serum, mouse IL-3 and 2 mg/ml Geneticin. A retroviral packaging cell line 293GP2 was cotransfected with pQCXIP-L490H-muMPL and pVSV-G plasmids using Fugene 6 (Roche). The culture supernatant was collected as a viral suspension and added to Ba/F3 cells in the presence of polybrene. After 24 h of infection, Ba/F3-muMPL(L490H)

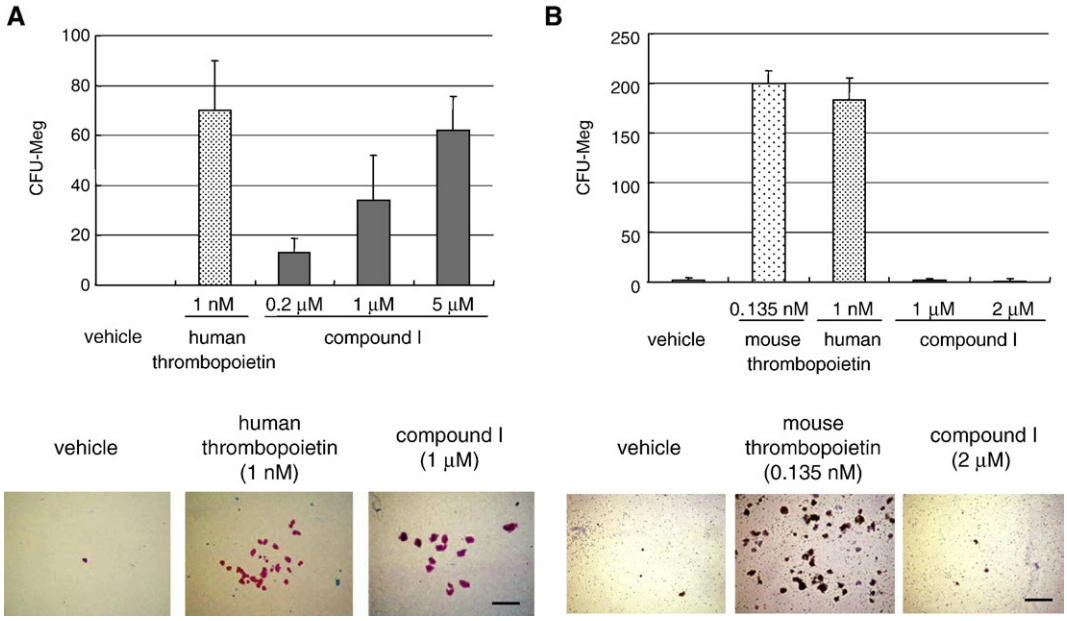

Fig. 3. Effects of compound I on human and mouse megakaryocyte colony formation. (A) Human $CD34^+$ cells were cultured with 1 nM recombinant human thrombopoietin or 0.2, 1, 5 μM compound I, respectively. After 12 days, megakaryocyte colonies were labeled with anti-CD41 antibody and the number of them was counted. (B) Mouse bone marrow cells were cultured with 0.135 nM (10 ng/ml) recombinant mouse thrombopoietin, 1 nM recombinant human thrombopoietin, or 1, 2 μM compound I, respectively. After 7 days, megakaryocyte colonies were stained for acetylcholinesterase activity and the number of them was counted. All results are shown as the mean±S.D. of four determinations. Lower panels of (A) and (B) show their typical images. Bar=250 μm.

cells were selected by 3-day incubation with 1.25 μg/mL puromycin.

2.7. Cell proliferation assay

Cells growing in log-phase were collected and washed once in RPMI1640 medium (without any supplements) to remove residual mouse IL-3 and resuspended at a density of 5×10^5 cells/ml in RPMI1640 medium containing 10% fetal bovine serum (but without IL-3). Fifty thousand cells were dispensed into each well of 96-well tissue culture plates containing recombinant human thrombopoietin or compound I at a variety of concentrations. At 20 h after incubation, WST-1 reagent (Takara Bio Inc.) was added to each well (10 μl/well), and the plates were incubated for a further 4 h at 37 °C. Finally the amount of viable cells was measured as the absorbance at 450 nm. Data was represented as a percentage of the maximum viability of each cell stimulated by recombinant human thrombopoietin.

2.8. Human megakaryocyte colony formation assay

Human megakaryocyte colony formation assay was performed according to the method of MegaCult™-C (StemCell Technologies). Briefly, $CD34^+$ cells from human bone marrow were washed and resuspended at a concentration of 3.3×10^3 cells/ml in Iscoves modified Dulbecco medium (IMDM) supplemented with 10 μg/ml recombinant human insulin, 200 μg/ml human transferrin (iron-saturated), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 1.1 mg/ml collagen as serum-free medium (Mega-IMDM). An aliquot of 0.75 ml of cell suspension was dispensed into each well of double chamber slides (StemCell Technologies) in the presence of different concentrations of compound I, recombinant human thrombopoietin, or vehicle (0.1% DMSO). The slides were incubated at 37 °C in humidified air containing 5% CO_2 for 12 days. After being dehydrated, fixed, and blocked with 5% human serum in Tris-buffered saline, the cells on the slides were incubated with 10 μg/ml anti-human CD41a antibody, then biotin-conjugated goat anti-mouse IgG and avidin alkaline phosphatase conjugate and alkaline phosphatase substrate were applied in sequence. Evans blue counter-staining was done for each slide. $CD41a^+$ colonies were enumerated as megakaryocyte

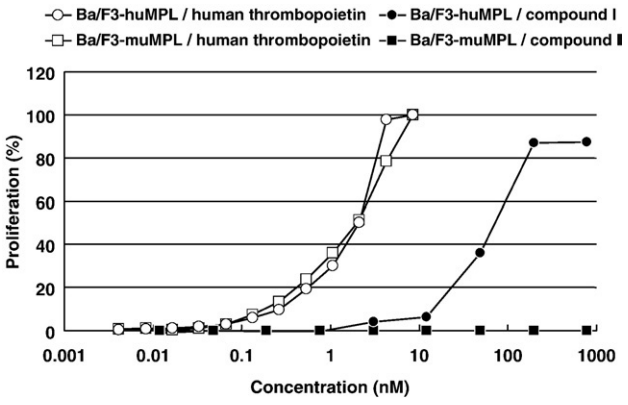

Fig. 4. Proliferative activity of compound I for each thrombopoietin-dependent cell line expressing human or mouse receptor. Proliferative responses of Ba/F3-huMPL stimulated by recombinant human thrombopoietin (open circles) or compound I (closed circles), and Ba/F3-muMPL stimulated by recombinant human thrombopoietin (open squares), or compound I (closed squares) are shown. The values are shown as the means of duplicate determinations.

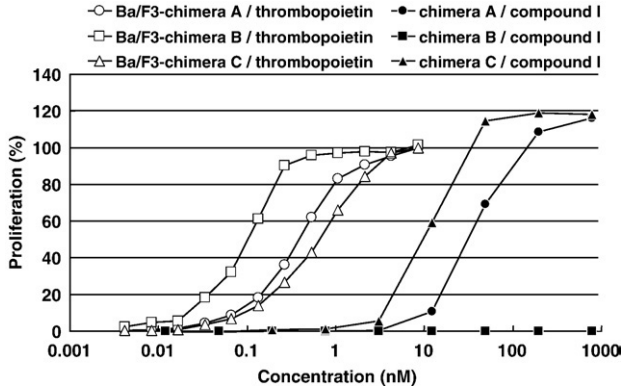

Fig. 5. Responses of three chimera receptors to compound I in proliferation assay. Three thrombopoietin-dependent cell lines Ba/F3-chimera A (circles), B (squares) or C (triangles) were stimulated by recombinant human thrombopoietin (open) or compound I (closed). The values are shown as the means of duplicate determinations.

colony-forming units (CFU-Meg) using a stereoscopic microscope (OPTIHOT-2, Nikon, Japan). The values are shown as the means±S.D. of 4 determinations. Simultaneously, photographs of the colonies were obtained with a stereoscopic microscope-mounted camera.

2.9. Mouse megakaryocyte colony formation assay

Mouse megakaryocyte colony formation assay was performed according to the method of MegaCult™-C (StemCell Technologies Inc.). Female Balb/c mice 7 to 8 weeks old were sacrificed and bone marrow cells were collected from their femurs. Next, the cells were cleared of erythrocytes by lysing buffer (Beckman Coulter, Tokyo, Japan) and washed with Mega-IMDM twice. They were then resuspended in Mega-IMDM at a concentration of 3.3×10^5 cells/ml and subjected to the same assay as that for human megakaryocyte colony formation. After being dehydrated and fixed in ice-cold acetone, megakaryocyte colonies were stained for acetylcholinesterase activity with 0.5 mg/ml acetylthiocholiniodide in 75 mM sodium phosphate buffer (pH 6.0) including 5 mM sodium citrate, 3 mM copper sulfate, and 0.5 mM potassium ferricyanide at room temperature for 3.5 h. Slides were refixed and counterstained with Harris' hematoxylin solution. Megakaryocyte colonies were defined as consisting of at least 3 megakaryocytes per colony and scored with a stereoscopic microscope. The values are shown as the means±S.D. of 4 determinations.

3. Results

3.1. Species specificity of the thrombopoietin mimetics

We had discovered novel thrombopoietin mimetics with a cell-based assay using the thrombopoietin-dependent Ba/F3-huMPL cells expressing the human thrombopoietin receptor on their surface (Yamane et al. in submission). Fig. 1A shows the chemical structure of compound I, a tool compound as a representative of the thrombopoietin mimetics examined in this study.

Compound I stimulated not only proliferation of Ba/F3-huMPL cells, but also the generation of $CD41^+$ megakaryocytic colonies from $CD34^+$ human bone marrow cells in a dose-dependent manner, as did recombinant human thrombopoietin (Fig. 3A). Prior to evaluation of the *in vivo* activity of compound I, megakaryocyte colony formation assay using mouse bone marrow cells was performed. In this assay, acetylcholinesterase activity (AchE) (Jackson, 1973) was measured to detect mouse megakaryocytes. Although 1 nM of recombinant

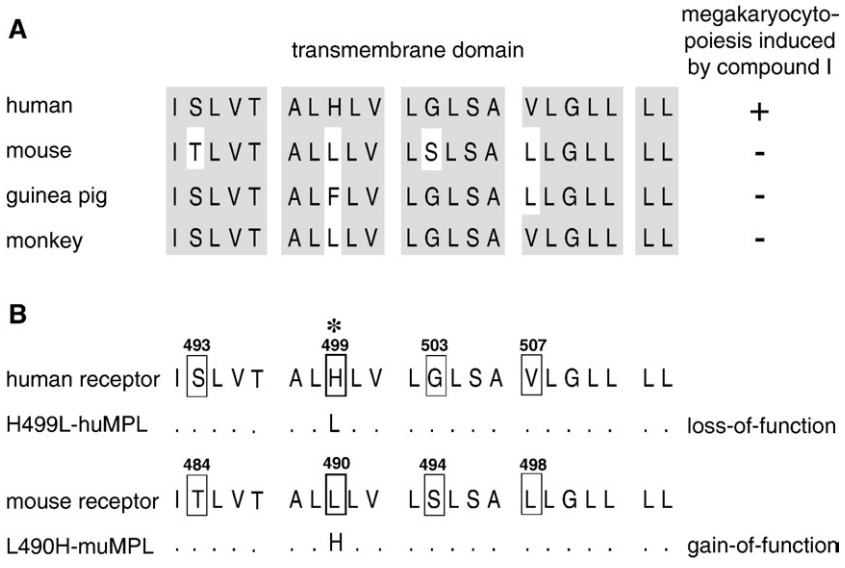

Fig. 6. The amino acid residue unique to human in the transmembrane domain. (A) Comparison of mouse, guinea pig, cynomolgus monkey and human thrombopoietin receptor transmembrane domains. Shaded boxes show the residues identical to human receptor. The results of megakaryocyte colony formation assay are shown by +/− on the right. (B) Construction of loss-of-function mutant and gain-of-function mutant. Open rectangles show mismatched amino acid residues between human and mouse receptors. Asterisk shows His at residue 499 is unique to the human receptor. Leu at residue 490 of mouse receptor corresponds to human His^{499}.

human thrombopoietin increased the equivalent number of mouse megakaryocyte colonies that were induced by 0.135 nM of recombinant mouse thrombopoietin, even 2 μM of compound I had no effect on the mouse bone marrow cells (Fig. 3B).

To confirm the species-specificity of compound I, the proliferation assay was conducted using thrombopoietin-dependent cells expressing the human thrombopoietin receptor (Ba/F3-huMPL) or the mouse thrombopoietin receptor (Ba/F3-muMPL). While recombinant human thrombopoietin stimulated proliferation of both Ba/F3-huMPL and Ba/F3-muMPL cells, compound I stimulated proliferation of only Ba/F3-huMPL and not Ba/F3-muMPL cells (Fig. 4).

3.2. Responses of chimera thrombopoietin receptors to the thrombopoietin mimetics

We tried to identify the pivotal site for the activation of the thrombopoietin receptor by exploiting the species-specificity of compound I. We established three thrombopoietin-dependent Ba/F3 cell lines expressing different types of chimera receptors (Ba/F3-chimeras A, B and C). As shown in Fig. 2, chimera A has the human extracellular and human transmembrane domains. Chimera B has the human extracellular and mouse transmembrane domains. Chimera C has the mouse extracellular and human transmembrane domains. All chimera receptors have the mouse cytoplasmic domain. A cell proliferation assay was done using the cell lines expressing these chimera receptors (Fig. 5). All three chimera receptors were confirmed as being functional because recombinant human thrombopoietin could react with them. Compound I led to the proliferation of Ba/F3-chimeras A and C, but not Ba/F3-chimera B. The peculiar domain to chimera B was the mouse transmembrane domain. These results suggested that the human-type transmembrane domain was pivotal for the receptor activation by compound I. Interestingly, Ba/F3-chimera C proliferated at a lower concentration of the compound than Ba/F3-chimera A or Ba/F3-huMPL shown in Fig. 4.

3.3. Comparison of amino acid sequences from humans and other animals

Fig. 6A shows the comparison of the amino acid sequences of the transmembrane domains from human and three other animals. The human sequence differs from mouse, guinea pig, and cynomolgus monkey sequences by four, two and one amino acid, respectively. We examined the reactivity of bone marrow cells from these animals to compound I by the megakaryocyte colony formation assay, but found no response to it. These results strongly suggested that the unique histidine at residue 499 of the human thrombopoietin receptor was the pivotal site of the thrombopoietin receptor activation by compound I.

3.4. Responses of point-mutated thrombopoietin receptors to thrombopoietin mimetics

To confirm that His at residue 499 in the transmembrane domain is required for activity of compound I, we established two cell lines expressing mutant receptors: the human thrombopoietin receptor in which His^{499} is replaced with Leu (Ba/F3-huMPL(H499L)) for a loss of the function and the mouse thrombopoietin receptor in which Leu^{490} is replaced with His (Ba/F3-muMPL(L490H)) for a gain of the function (Fig. 6B). Although the two mutant receptors were functionally active as both reacted to recombinant human thrombopoietin, the replacement of the residue of H^{499} or L^{490} led Ba/F3-muMPL(L490H) to gain the reactivity to compound I and Ba/F3-huMPL(H499L) to lose it (Fig. 7).

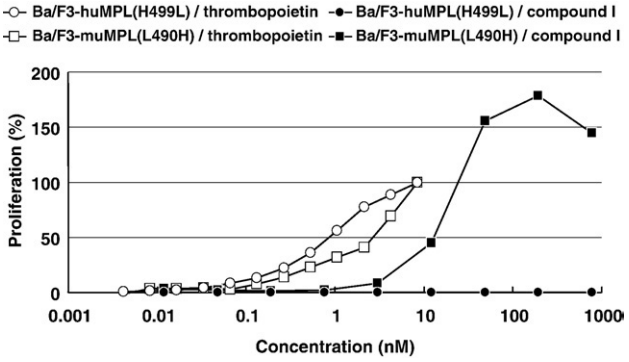

Fig. 7. Responses of the human H499L mutant receptor and the mouse L490H mutant receptor to compound I in proliferation assay. Two thrombopoietin-dependent cell lines expressing mutants, Ba/F3-huMPL(H499L) (circles) and Ba/F3-muMPL(L490H) (squares), were stimulated by recombinant human thrombopoietin (open) or compound I (closed). The values are shown as the means of duplicate determinations.

4. Discussion

We discovered a series of novel non-peptide small molecules with proliferation activities on the human thrombopoietin-dependent cells (Ba/F3-huMPL) (Yamane et al., in submission). These compounds activated signal molecules in the thrombopoietin signal transduction pathway that have been revealed by previous studies (Drachman et al., 1995, 1997; Kaushansky, 2005; Miyakawa et al., 1995, 1997; Rojnuckarin et al., 1999; Sattler et al., 1995). Our thrombopoietin mimetics induced megakaryocytopoiesis from human hematopoietic progenitor cells, but did not act on mouse bone marrow cells. Further investigation using bone marrow cells of guinea pig and cynomolgus monkey revealed that the compound could stimulate only the human thrombopoietin receptor. These results suggested that it would be difficult to estimate the thrombopoietin-like activity of our compounds in an *in vivo* system using laboratory animals. Analysis of human/mouse chimera receptors demonstrated that the transmembrane domain was essential for the exhibition of the activity of the compound. Comparison of the transmembrane domains among mouse, guinea pig, cynomolgus monkey and human revealed that His^{499} is a residue unique to the human thrombopoietin receptor. To confirm the hypothesis that His^{499} in the transmembrane domain of the human thrombopoietin receptor is pivotal for the receptor activation by our compound, we conducted a cell proliferation assay using two thrombopoietin-dependent cell lines expressing

the mutant receptor: Ba/F3-huMPL(H499L) or Ba/F3-muMPL (L490H). Ba/F3-huMPL(H499L) cells showed that the human thrombopoietin receptor lost the reactivity to compound I by the replacement of His^{499} with Leu. Conversely, Ba/F3-muMPL(L490H) cells showed that the mouse thrombopoietin receptor gained reactivity to compound I by the replacement of Leu^{490} with His. These results confirmed that His^{499} in the transmembrane domain of the human thrombopoietin receptor is essential for the thrombopoietin-like activity of the compound.

Among the non-peptidyl thrombopoietin receptor agonists reported to date, SB394725, NIP-004 and YM77 showed high specificity to the human receptor, and SKF-57626, SB-497115 and NIP-004 were reported to need the transmembrane region surrounding His^{499} of the human thrombopoietin receptor (Erickson-Miller et al., 2004, 2005; Inagaki et al., 2004; Kimura et al., 1998; Nakamura et al., 2006; Sakai et al., 2005; Suzuki et al., 2005). These findings suggest that several compounds having different chemical structures activate the human thrombopoietin receptor by a common mechanism concerned with its transmembrane domain. An earlier report stated that the point mutation of Ser^{505} to Asn in the transmembrane domain made the thrombopoietin receptor constitutively active (Onishi et al., 1996). This mutation was found in patients with familial essential thrombocythemia (Ding et al., 2004). It is likely that the transmembrane domain plays an important role in controlling the activation of the thrombopoietin receptor. An amphipathic motif which keeps the unliganded receptor inactive has been found at the transmembrane-cytoplasmic junction of the thrombopoietin receptor (Staerk et al., 2006). Considering hydrophobicity as a structural peculiarity of our thrombopoietin mimetics, the compound might be inclined to interact with a region abundant in hydrophobic amino acids like the transmembrane domain. A plausible explanation is that compound I binds at some other position on the external part of the receptor and needs the transmembrane His 499 to affect the conformational change in the receptor.

The *in vivo* activity of our thrombopoietin mimetics cannot be evaluated using common experimental animals. In the case of SB-497115, the chimpanzee was used for *in vivo* study because the chimpanzee thrombopoietin receptor has a human-type transmembrane domain containing His^{499} (Erickson-Miller et al., 2004; Sellers et al., 2004). For other *in vivo* assay systems, the transplantation of human bone marrow cells into experimental animals (Nakamura et al., 2006) or the transgenic mouse expressing human *c-mpl* should be useful. While His^{499} in its transmembrane domain should be essential for the activation by compound I, there might be a region which regulates the sensitivity to the compound in the extracellular domain of the receptor. Figs. 4, 5 and 7 show that Ba/F3-chimera C and Ba/F3-muMPL(L490H) containing mouse type extracellular domains were more sensitive to compound I than Ba/F3-chimera A and Ba/F3-huMPL. Further investigation using chimeras or mutants of the thrombopoietin receptor should help us understand the mechanism of the receptor activation by these compounds and lead to the development of more active thrombopoietin mimetics.

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