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A non-peptide compound which can mimic the effect of thrombopoietin via c-Mpl

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Abstract Thrombopoietin (TPO) is a cytokine which plays a central role in megakaryopoiesis and platelet production by binding to its cell surface receptor, termed c-Mpl. In the present study, two benzodiazepinones that compete with the binding of TPO to the extracellular region of c-Mpl were identified, and one of them stimulated the proliferation of a human TPO-dependent megakaryocytic cell line, UT-7/TPO. It stimulated the activation of signal transducer and activator of transcription 5 in UT-7/TPO cells. These results suggest that a non-peptide compound can mimic the effect of TPO via c-Mpl.

Key words: Thrombopoietin; c-Mpl; Benzodiazepinone; Signal transducer and activator of transcription

1. Introduction

Thrombopoietin (TPO) is a cytokine which has biological effects as a major mediator of megakaryocyte growth and platelet production [1–8]. TPO supports the proliferation of megakaryocyte progenitors and induces their differentiation into mature megakaryocytes. TPO exerts its action by binding to a specific cell surface receptor encoded by *c-mpl* [1–5]. c-Mpl is a member of the cytokine receptor superfamily [9].

The binding of a cytokine to its specific receptor on the cell surface induces tyrosine phosphorylations and activation of several proteins, including a receptor itself, receptor-associated tyrosine kinases such as Janus kinases (JAKs) and signal transducers and activators of transcriptions (STATs) [10–15]. Activated STATs then form dimers, translocate into the nucleus, and bind to a specific DNA sequence and modulate transcription [10–15]. TPO induces the tyrosine phosphorylation of JAK2 and TYK2 and the activation of STAT1, STAT3 and STAT5 in several cell lines, including mouse megakaryocytes and human platelets [16–27].

For several years, random phage peptide libraries have been used to identify novel agonist peptides for erythropoietin (EPO) receptor [28]. Recently we and Cwirla et al. independently detected novel agonist peptides for c-Mpl [29,30]. These reports were the first proof that small peptides could exhibit bioactivity of a large polypeptide hormone through its receptor. There is no report that a non-peptide compound can do so, to our knowledge.

The human leukemia cell line UT-7 was established from the bone marrow cells of a patient with acute megakaryoblastic leukemia [31]. UT-7/GM, a subline of UT-7, can proliferate in response to granulocyte-macrophage colony stimulating factor (GM-CSF) or TPO [32,33]. The subline UT-7TPO was established from UT-7/GM by culture at lower concentrations of TPO and had morphologically mature megakaryocytic characteristics [33].

In the present study, we identified two benzodiazepinones that compete with the binding of TPO to human immunoglobulin fusion protein containing the extracellular region of human c-Mpl (MPL-IgG). One of these benzodiazepinones, TM41, stimulated the proliferation of UT-7/TPO cells. In addition, it activated STAT5 in a manner similar to that of TPO. These results show that TM41 mimics the effect of TPO via c-Mpl.

2. Materials and methods

2.1. Hematopoietic growth factor and reagents

Benzodiazepinones which we termed TM compounds were synthesized by Hokuriku Seiyaku Co. (Fukui, Japan). Recombinant human TPO was obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human GM-CSF was obtained from Genzyme (Cambridge, MA, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Wako (Tokyo, Japan).

2.2. Competition binding assay

MPL-IgG is the soluble form of human c-Mpl containing the extracellular ligand binding domain of c-Mpl and the human immunoglobulin Fc region. It was prepared as described previously [29]. Briefly, 96-well plates were coated with MPL-IgG at 10 ng/well in phosphate-buffered saline (PBS), and incubated at 4°C overnight. The wells were then blocked with PBS containing 1% bovine serum albumin (BSA). Mixtures of TM compounds at concentrations ranging from 3 μM to 1 mM and the constant concentration of 2 ng/ml TPO were added and incubated in MPL-IgG immobilized wells. After incubation for 1 h, the wells were washed and the remaining bound TPO was detected by goat anti-human TPO antibody (R&D Systems) and then donkey anti-goat IgG conjugated to horseradish peroxidase (HRP; Chemicon International, Temecula, CA, USA). The absorbance was measured at a wavelength of 450 nm using a microplate reader (model M-Vmax; Molecular Device, Menlo Oaks, CA, USA).

2.3. Cells and culture

The human leukemia cell lines UT-7 and UT-7/TPO were kindly provided by Dr. N. Komatsu (Jichi Medical School, Tochigi, Japan) [31,33]. UT-7 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco, Rockville, MD, USA) containing 10% fetal calf serum (FCS; Intergen, Purchase, NY, USA) and 0.5 ng/ml GM-CSF. UT-7/TPO cells were maintained in IMDM containing 10% FCS and 1 ng/ml TPO.

2.4. WST-1 assay for cell proliferation

UT-7 cells and UT-7/TPO cells were starved of growth factor for 24 h. These cells were resuspended at a density of 2×10^4 cells/0.1 ml IMDM containing 10% FCS and incubated in 96-well plates in the absence or presence of various concentrations of TM compounds,

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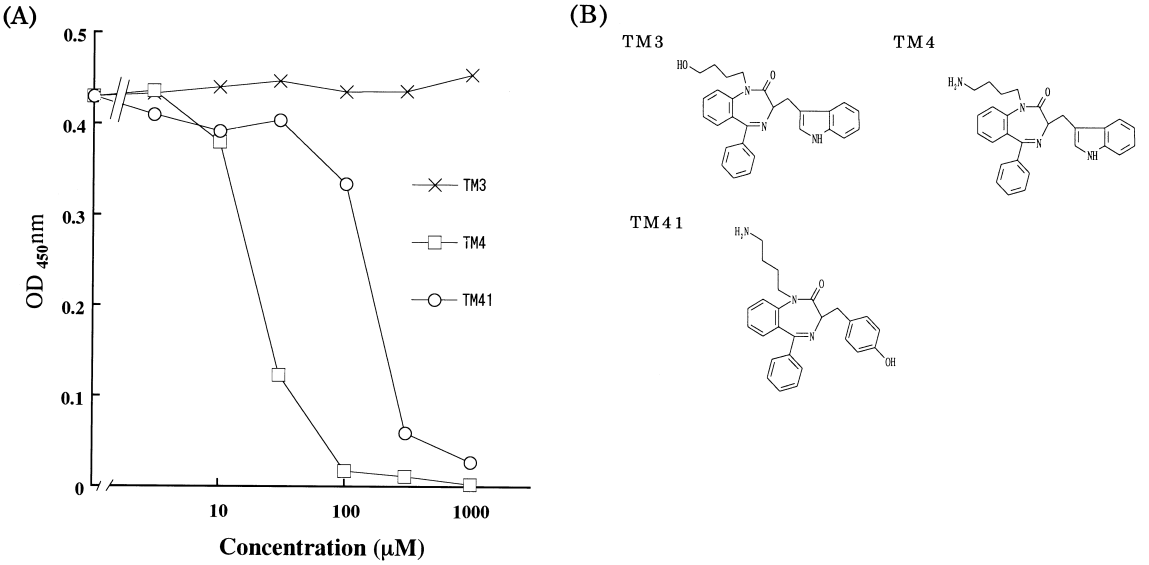

Fig. 1. Competition binding assays and chemical structure of TM compounds. A: 96-well plates were coated with MPL-IgG. TM compounds (3 μM–1 mM) and 2 ng/ml TPO were added and incubated in MPL-IgG immobilized wells. The wells were then washed and the remaining bound TPO was detected by goat anti-TPO antibody and then donkey anti-goat IgG conjugated to HRP. The absorbance was measured. The values shown are the means of duplicate determination. B: Structures of TM compounds. TM3, (±)-1,3-dihydro-1-(4-hydroxybutyl)-3-(1*H*-indol-3-ylmethyl)-5-phenyl-2*H*-1,4-benzodiazepin-2-one; TM4, (±)-1-(4-aminobutyl)-1,3-dihydro-3-(1*H*-indol-3-ylmethyl)-5-phenyl-2*H*-1,4-benzodiazepin-2-one; TM41, (±)-1-(4-aminobutyl)-1,3-dihydro-3-[(4-hydroxyphenyl)methyl)-5-phenyl-2*H*-1,4-benzodiazepin-2-one.

TPO or GM-CSF for 3 days. 10 μl of 1 mM WST-1 and 0.2 mM 1-methoxy PMS were added to each well, and the plates were incubated for 4 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader.

2.5. Electrophoretic mobility shift assay (EMSA)

UT-7 cells and UT-7/TPO cells were starved of growth factor for 24 h. These cells were incubated with TM compounds, TPO or GM-CSF for 15 min at 37°C. Nuclear extracts were prepared as described by Sadowski et al. [34]. The STAT5 binding site of the bovine β-casein promoter (5′-AGATTTCTAGGAATTCAATCC-3′) was used as a probe [35]. The probe was end-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase and used about 1 ng (about 1×10^5 cpm) per reaction. Extracts (from 2.5×10^5 cells/point) were incubated in 20 μl of reaction mixture containing 10 mM HEPES, pH 7.9, 5% glycerol, 30 mM NaCl, 0.1% NP-40, 1 mg/ml BSA, 1 mM EDTA and 0.5 mg/ml of salmon sperm DNA at room temperature for 30 min. For the competition experiment, 100-fold excess of unlabeled probe was added to the reaction mixture. For supershift experiment, 2 μg of anti-STAT5 antibody which reacted with STAT5a and STAT5b (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the reaction mixture. Samples were loaded onto 5% polyacrylamide gels and then analyzed with a bioimaging analyzer BAS2000 (Fujix, Tokyo, Japan).

3. Results

3.1. TM compounds inhibit the binding of TPO to the MPL-IgG

MPL-IgG was utilized in a competition binding assay to screen many compounds that inhibited the binding of TPO to c-Mpl. Most compounds, represented by TM3, did not inhibit the TPO binding to MPL-IgG (Fig. 1A), but several compounds such as TM4 and TM41 were identified as dose-dependent inhibitors of the binding of TPO to MPL-IgG (Fig. 1B). These compounds, with a benzodiazepinone structure with an amino residue at position 1, are shown in Fig. 1B.

3.2. TM41 stimulates the proliferation of UT-7/TPO cells

To determine whether TM4 and TM41 have the same biological activity as TPO, we tested these compounds in a cell proliferation bioassay. UT-7/TPO is a useful cell line for the study of the action of TPO, because this cell line has a morphologically mature megakaryocytic character and grows in response to TPO [33]. TM4 did not stimulate the proliferation

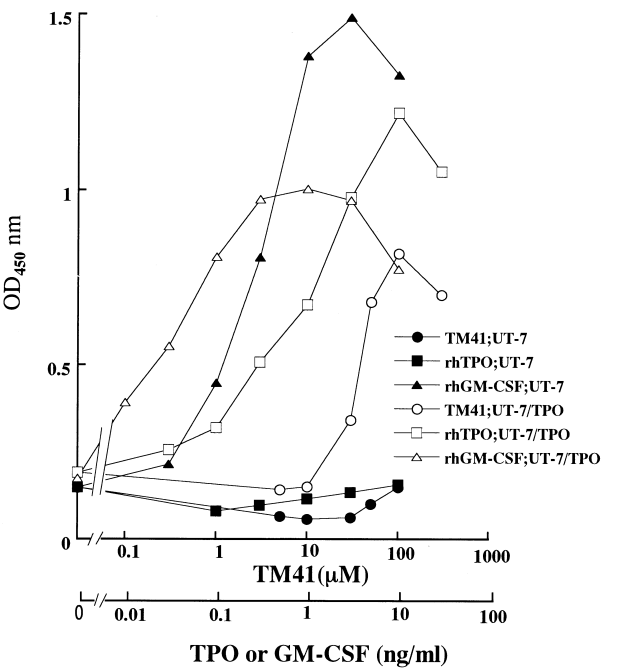

Fig. 2. Proliferative response of UT-7 cells and UT-7/TPO cells to TM41. UT-7 cells and UT-7/TPO cells were deprived of growth factor for 24 h and then plated at a density of 2×10^4 cells/well in IMDM containing 10% FCS and incubated in the absence or presence of TM41, TPO or GM-CSF. After 3 days, the absorbance was measured by the WST-1 assay described in Section 2. The values shown are the means of triplicate determinations.

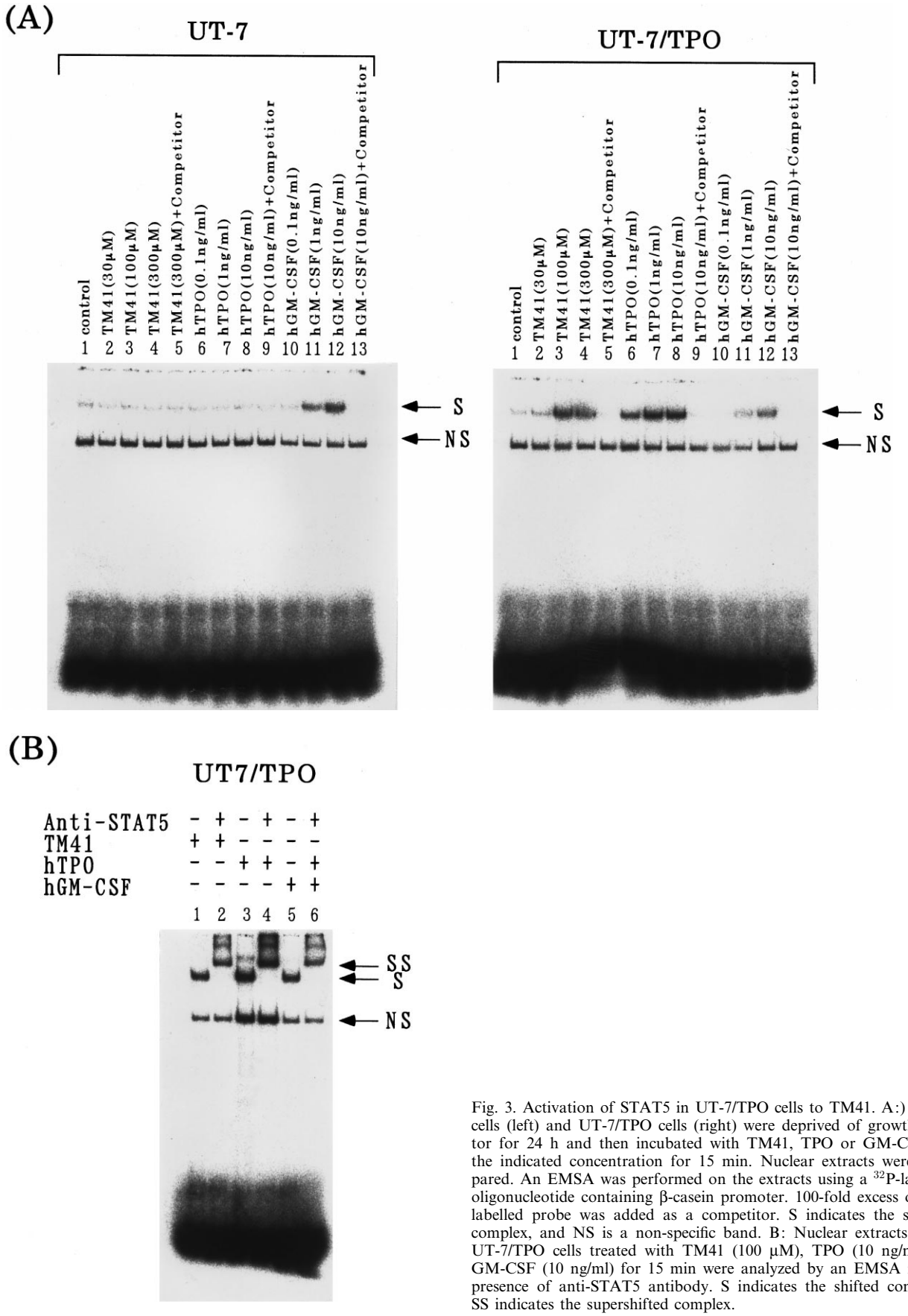

Fig. 3. Activation of STAT5 in UT-7/TPO cells to TM41. A:) UT-7 cells (left) and UT-7/TPO cells (right) were deprived of growth factor for 24 h and then incubated with TM41, TPO or GM-CSF at the indicated concentration for 15 min. Nuclear extracts were prepared. An EMSA was performed on the extracts using a ^{32}P-labeled oligonucleotide containing β-casein promoter. 100-fold excess of unlabelled probe was added as a competitor. S indicates the shifted complex, and NS is a non-specific band. B: Nuclear extracts from UT-7/TPO cells treated with TM41 (100 μM), TPO (10 ng/ml) or GM-CSF (10 ng/ml) for 15 min were analyzed by an EMSA in the presence of anti-STAT5 antibody. S indicates the shifted complex. SS indicates the supershifted complex.

of UT-7/TPO cells and UT-7 cells (data not shown). TM41 stimulated the proliferation of UT-7/TPO cells, although it did not stimulate the proliferation of parental UT-7 cells, which do not respond to TPO (Fig. 2). This indicates that although the biological potential of TM41 is weaker than that of rhTPO, it may act as an agonist for c-Mpl.

3.3. TM41 stimulates the activation of STAT5 in TPO-dependent cells

TPO induces the tyrosine phosphorylation of several proteins, including c-Mpl, Shc, JAK2, STAT5 and so on [16–27]. GM-CSF, EPO and IL-3 also activate JAK2 and STAT5 [10,14]. Because STAT5 is an early and important substrate of activated JAK2 via c-Mpl, we investigated whether TM41 can induce the activation of STAT5 in UT-7/TPO cells by EMSA. UT-7 cells and UT-7/TPO cells express JAK2 and STAT5 [24,33,38]. The EMSA was performed using bovine β-casein promoter as a probe for STAT5 [35]. Nuclear extracts prepared from TM41-, TPO- or GM-CSF-treated UT-7/TPO cells dose-dependently possessed DNA binding activity (Fig. 3A). The density of the shifted complex derived from TM41-, hTPO- or hGM-CSF-treated UT-7/TPO cells paralleled the growth stimulation activity against UT-7/TPO cells. The formation of these complexes was completely inhibited by a 100-fold excess of unlabeled probe, indicating that the proteins bound specifically to the probe. Although the nuclear extracts prepared from GM-CSF-treated UT-7 cells also possessed the same activity, those of TM41- or TPO-treated UT-7 cells did not possess the activity (Fig. 3A).

To further examine these results, a supershift experiment was performed with an anti-STAT5 antibody. The supershift experiment showed that the presence of anti-STAT5 antibody apparently altered the mobility of the DNA-protein binding complex, indicating the presence of an immunologic STAT5 in TM41-, TPO- or GM-CSF- treated extracts of UT-7/TPO cells (Fig. 3B).

4. Discussion

TPO will be useful for the treatment of thrombocytopenia, but it has significant drawbacks including oral unavailability and potential immunogenicity. The function of TPO and EPO via receptors can be mimicked by smaller peptides which were isolated from random peptide libraries on filamentous phage [28–30]. However, such mimetic peptides may not have the same effects when they are orally administered. Non-peptide agonistic ligands for the cholecystokinin and somatostatin receptors have been obtained [36,37]. The most prominent bioactive forms of these hormones are 8 and 14 amino acids, respectively. There has been no report, to our knowledge, that a non-peptide compound mimics a cytokine or growth factor which is a large polypeptide hormone. In this study, we tried to find a non-peptide compound which has physiological activities similar to those of TPO.

TPO and TPO-mimetic peptides bind to MPL-IgG [1,29]. We used MPL-IgG as a source to search for a non-peptide compound which can mimic an activity of TPO via c-Mpl. Several synthetic compounds were identified that inhibited the binding of TPO to MPL-IgG in a concentration-dependent manner. Two compounds, TM4 and TM41, were investigated. They exhibit a close structural relationship (Fig. 1B); they each have benzodiazepinone with amino residues at position 1. We next checked the effect of these compounds on the proliferation of UT-7/TPO cells. TM41, but not TM4, stimulated the proliferation of UT-7/TPO cells. When UT-7/TPO cells were cultured with TM4 at 10–100 μM in the presence of 1 ng/ml hTPO, the proliferation of the cells was dose-dependently inhibited by TM4 (data not shown). TM4 may be an antagonist for c-Mpl.

TPO induces the tyrosine phosphorylation of JAK2 and TYK2 and the activation of STAT1, STAT3 and STAT5 in several cell lines, including mouse megakaryocytes and human platelets [16–27]. Activation of STAT1 and STAT3 was not detected in UT-7/TPO cells by TPO [33]. We thus examined whether TM41 also induced the activation of STAT5. TM41 induced the activation of STAT5 in UT7/TPO cells. These results indicate that a low molecular weight compound can attain the affinity of c-Mpl binding and potency of c-Mpl activation possessed by TPO.

TPO shows homology to the primary amino acid sequences of EPO [1,2]. The amino acid sequence of c-Mpl is highly conserved to the common βc receptor of GM-CSF, IL-3 and IL-5, and EPO receptors [9]. The question remains whether TM41 activates other cytokine receptors. UT-7 cells grow dependent not only on GM-CSF but also on EPO and IL-3, and their maximal proliferation activity is the same as that of GM-CSF [31]. Since TM41 did not stimulate the proliferation and the activation of STAT5 in UT-7 cells, the target of TM41 seems to be specific for c-Mpl.

c-Mpl seems to be activated as a homodimer, similar to the receptors for growth hormone, GM-CSF and EPO [39–42]. The activation of c-Mpl by TM41 could be explained in the following two ways: (i) TM41 causes conformational changes of receptor which induce receptor-receptor interactions; or (ii) TM41 binds to two receptor molecules in a bivalent manner, and this mimics receptor homodimerization. The actual mode of action may be resolved by three-dimensional nuclear magnetic resonance or X-ray crystal analysis of the compound-receptor complex.

In conclusion, TM41 stimulates the proliferation of human megakaryocytic cell lines. To our knowledge, this is the first demonstration of the activation of cytokine receptor by a low molecular weight compound. TM41 is useful in vitro as a unique tool for investigating the biological role of TPO via c-Mpl. Moreover, the use of TM41 could lead to the design and discovery of more potent low molecular weight drugs that mimic the therapeutically important protein TPO.

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