LETTER

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Zinc Is Not Required for Activity of TPO Agonists Acting at the c-Mpl Receptor Transmembrane Domain

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ABSTRACT Molecules that mimic the cytokine thrombopoietin that act by an atypical mechanism of binding to a receptor transmembrane (TM) domain are widely understood to require zinc for their biological activity. We investigated potent thrombopoietin mimetics from three chemical classes including the recently registered drug Eltrombopag, which operate *via* this novel mechanism, to determine whether zinc is essential for inducing cell proliferation. Using addition of zinc and a potent metal chelator, we show that the existing paradigm is incorrect and the compounds exhibit excellent thrombopoietin-mimetic activity even in the presence of high concentrations of EDTA. The implications of these findings for the mechanism of action are discussed.

Human thrombopoietin (TPO) is a circulatory cytokine that is the primary regulator of megakarocytopoiesis and platelet production. In addition, TPO is an early acting synergistic cytokine regulating quiescence and proliferation of hematopoietic stem cells (*1, 2*). TPO binds to its receptor, human myeloproliferative leukemia virus oncogene (c-Mpl), and initiates an intracellular signaling cascade (*1*), resulting in production of platelets essential in wound healing (*3, 4*). Although TPO alone has been used clinically, concerns about its immunogenicity and, to some extent, cost of the native cytokine and conjugates have limited widespread clinical application (*5, 6*). The development of small, synthetic, orally active agonist compounds of TPO would be very beneficial to treat thrombocytopenia and to drive hematopoietic stem cells to desired cellular fates (*7*).

Random screening has identified a relatively diverse range of synthetic compounds that act as c-Mpl agonists, including pyrazol-4-ylidenehydrazines (*8*), salicylaldehyde thiosemicarbazones (*9*), naphtho(1,2-*d*) imidazoles (*10*), xanthocillins (*11*), benzimidazoles (*12*), hydrazinothiophenes (*13*), arylthiazoles (*14*), benzamides (*15–18*), benzocarbazoles (*19, 20*), and butyzamides (*21*). One of these classes has yielded a new drug, Eltrombopag, which has recently received registration from the FDA for the treatment of thrombocytopenia (*20*).

Unusually, these compounds interact with the transmembrane helix of the receptor. In the human and chimpanzee receptor, the transmembrane helix contains a unique histidine residue that, *via* single point knock out and knock in mutation experiments, has been shown to be essential for the activity of this class of TPO agonist (*15*). No other animal species carry this histidine mutation, and their c-Mpl receptors are unaffected by these agonists (*13, 15, 22*).

Early work on this class of mimetic (*10*) identified a molecular feature that was hypothesized to chelate zinc ions and carry them into the c-Mpl transmembrane domain, where they interact with H499 to activate the receptor (see Figure 1).

Subsequent work attributed this "zinc-chelating" role to the 15 or so chemotypes that exhibit TPO agonist activity *via* this unusual mechanism (*19, 23*). Evidence for this hypothesis was published in a patent by Delorme *et al.* (*24*). They reported experiments in which two pyrazol-4-ylidenehydrazines exhibited activity against mutant C610H and double mutant G607T murine GCSF receptor constructs in the presence of added zinc ions. Chelation of zinc by EDTA abolished receptor activity. This single experiment using a receptor construct was extrapolated to

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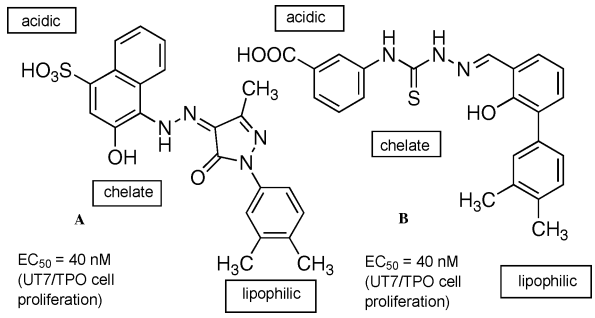

Figure 1. Comparison of pyrazol-4-ylidenehydrazine TPO mimic A and thiosemicarbazone TPO mimic B, illustrating the three key features for potent thrombopoietic activity: central metal chelate, lipophilic end portion, and a distal acidic functionality (from ref 9, used with permission).

conclude that all compounds of this type required zinc to function as TPO agonists. No primary, peer-reviewed publication has formally demonstrated this general prerequisite for zinc, although subsequent NMR and mutation experiments on chimeric constructs of c-Mpl (*25*) cast doubt on the hypothesis. Our preliminary work caused us also to question the role of zinc in compounds acting at the TM domain of c-Mpl. In particular, the high TPO agonist activity of the benzocarbazoles (*19, 20*) that are covalently constrained and lack a strong chelating group is inconsistent with a requirement for zinc.

We screened four mimetics of TPO using a human transfected factor-dependent murine hematopoietic cell line (FD-Mpl) in combination with a high concentration of zinc sulfate or EDTA. We wished to determine whether high or enhanced levels of zinc could improve the binding of these mimetics to the c-Mpl receptor and enhance cell proliferation and whether high levels of EDTA eliminate mimetic activity.

We assessed the effect of zinc and EDTA across a range of concentrations on the growth and viability of FD-Mpl cells growing in media supplemented with rhTPO (30 ng mL^{-1}) to ensure that the metal ion and the chelating agent did not affect cell proliferation on their own. We saw no statistically significant difference between the numbers of cells cultured with rhTPO only, rhTPO plus 100 μM zinc, and rhTPO plus 100 μM EDTA.

The TPO− and TPO+ controls all gave the expected results (see Methods), with either no viable cells or strong growth at 30 ng mL^{-1} of rhTPO respectively. Figure 2 shows cell proliferation results for compounds **1**, **2a**, **2b**, and **3** with and without added Zn^{2+} and with added EDTA. Cell proliferation was normalized using the cell numbers obtained from culture in media supplemented with 30 ng mL^{-1} (0.84 nM) rhTPO expressed as a percentage. Asterisks indicate where the responses were different at the 95% confidence limit.

The effect of added high dose zinc and chelator on factor-dependent cells stimulated by Eltrombopag (**1**) is summarized in Figure 2, panel a. Added $ZnSO_4$ had negligible effect on cell growth (cell numbers comparable to those with 10 and 30 ng mL^{-1} rhTPO). Addition of EDTA to **1** gave slightly enhanced cell numbers and more variable results, but the difference was not statistically significant. The addition of zinc and EDTA appear to have no effect, giving cell numbers virtually identical to **1** alone at 1 μM. The ED_{50} values for the control and both treatment groups were essentially the same.

Figure 2, panels b and c summarize the results of the experiments adding Zn or EDTA to the pyrazolohydrazinonaphthalene (PHN) compounds **2a,b**. The compounds gave a good dose−response curve and high cell numbers when used alone, although marginally less than **1**. The addition of $ZnSO_4$ to **2a** gave cell responses identical to those with **2a** alone (Figure 2, panel b). The addition of EDTA did not significantly change cell growth compared that of the control (**2a** alone), generating a dose−response curve very similar to that of **2a** alone, and **2a** with added Zn. The graph shows that the ED_{50} values for all three samples were essentially the same within experimental error, as was observed for **1**.

The addition of $ZnSO_4$ to **2b** marginally decreased cell numbers at 1 and 10 μM compared to the control of compound **2b** alone and also produced an EC_{50} value slightly higher than that of **2b** alone. The addition of EDTA marginally decreased cell growth below that of the control but exhibited an EC_{50} similar to that of the control (Figure 2, panel c). Dose response curves for the three conditions were similar, but the maximum proliferation for the treated groups was lower than the control.

The benzocarbazole, **3**, was an important compound because it has a conformationally constrained structure and no clear metal chelating group, suggesting it does interact with zinc strongly. However, it clearly exhibited a different response compared to the other agonists (Figure 2, panel d), although the differences were still small. Both treated groups showed reduced maximum proliferation compared to the control, as was seen with other agonists. EDTA-treated cells had a dose−response curve and EC_{50} value similar to those of the control. Interestingly, the zinc-treated cells exhibited a EC_{50} value lower than those of the control and EDTA-treated cells, and this difference was statistically significant. At the highest dose tested, there were crystals in all cultures, indicating precipitation. Paradoxically, the class of compounds least likely to chelate zinc showed a small decrease in EC_{50} on addition of zinc ions.

Thrombopoietin is a critical cytokine for generating megakaryocytes and platelets from hematopoietic stem and progenitor cells cultured *ex vivo*. Small molecule mimics that interact with the TM domain of c-Mpl may be replacements for native TPO. It is im-

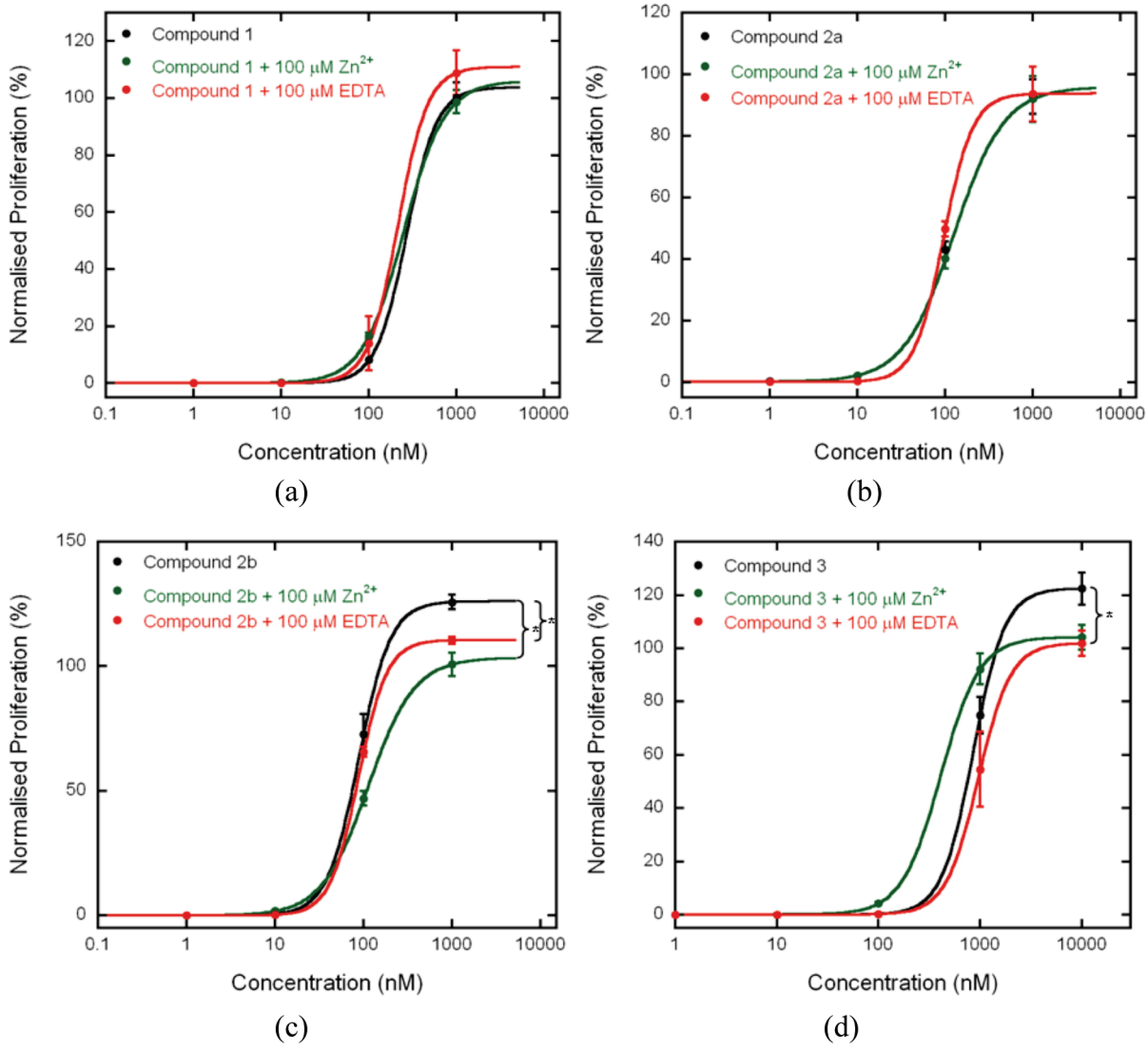

Figure 2. a) Growth-response curves of factor-dependent cells with Eltrombopag 1 **alone, with Eltrombopag** 1 **and 100 μM $ZnSO_4$, and with Eltrombopag** 1 **and 100 μM EDTA. b) Growth-response curves of factor-dependent cells with PHN** 2a **alone, with** 2a **and 100 μM $ZnSO_4$, and with** 2a **and 100 μM EDTA (note that the black curve lies directly under the green curve). c) Growth-response curves of factor-dependent cells with PHN** 2b **alone, with** 2b **and 100 μM $ZnSO_4$, and with** 2b **and 100 μM EDTA. d) Growth-response curves of factor-dependent cells with benzocarbazole** 3 **alone, with** 3 **and 100 μM $ZnSO_4$, and with** 3 **and 100 μM EDTA.**

portant to understand how these molecules work so that culture conditions be deployed that ensure their maximal bioactivity. As previous reports had suggested that zinc was essential for bioactivity (*10, 19, 23*), it is also important to consider the baseline levels of zinc normally present in most culture media like DMEM, supplemented with 10% FCS. This has been estimated to be 4 μM (*26*). *In vivo*, zinc is bound to albumin in the circulation, where it is maintained at approximately 1 μg mL^{-1} in mammals. The total intracellular zinc content of a typical fibroblast, in cell culture, is 0.25 fmol or ~200 μM (*29*). These levels are similar to the concentrations of the TPO agonists used in our experiments and would be sufficient to activate them if zinc binding was necessary for their activities.

EDTA binds zinc extremely strongly with a K_d of $\sim 10^{-16}$, compared to a K_d for magnesium of 10^{-9} and K_d for calcium of 10^{-11}, and will selectively deplete a solution of free zinc ions in the presence of other free diva-

lent ions, even if they are present at high concentration (*28*). The kinetic behavior of zinc is summarized by Nyborg (*29*). Intraventricular injection of EDTA has been reported to deplete intracellular zinc levels so that the levels of EDTA used for our experiments should remove any incidental zinc in the culture medium and in the cells (*30*). Accordingly, our data strongly suggest that TM acting mimics do not require zinc for their activity.

We also conducted a computational study of structure−activity relationships in ~400 members of this class of TPO agonists (*31*). The model was very successful in explaining the molecular features required for agonist activity and did not require zinc chelation. Our study suggested that the putative metal binding moiety in most agonists has a different role than binding zinc. The heteroatoms in the linker region contribute to stabilization of a pseudo planar conformation of the agonists *via* intramolecular hydrogen bonds, amide planarity, tautomeric effects, and π conjugation, suggesting this conformation is essential for activity.

Dimerization of c-Mpl and signaling by agonists will be dependent on intramolecular distances between ligands and receptors and the fit of the two surfaces which, from our results, does not require the presence of a zinc ion. The exact molecular mechanism by which the small molecule agonist acting at or near the key histidine residue in the transmembrane helix is far from clear.

It is also not clear why the highest levels of zinc used in this study sometimes enhance the cell proliferation activity of the small molecule agonists. The levels used are much higher than physiological, so the observations may not be biologically relevant. These high levels of zinc did not significantly alter the activity of TPO, and not all classes of small molecule TPO agonists showed substantial effects. We speculate that the enhancement may be due to additional stabilization of the required binding conformation of agonist by zinc chelation in the linker region (*31*). Our QSAR studies suggest that agonists wrap around the transmembrane helix and that the linker region heteroatoms interact with transmembrane histidines. This could lock them into a position where the Lewis base region of agonists interact with the extracellular juxtamembrane domain of c-Mpl and cause receptor dimerization.

In conclusion, our experiments with a TPO-dependent cell line show that zinc is not required for agonist activity of four compounds in three chemical classes, including the recently registered drug Eltrombopag, that act *via* the atypical transmembrane mechanism. While the linker region of TPO agonist compounds is still able to chelate zinc in some cases, this is not essential for activity. The previous mechanistic paradigm needs revising, as the addition of EDTA does not abolish activity.

METHODS

Compounds **1**, **2a**, and **2b** (Supplementary Figure 1) were prepared by published methods (*8, 24*). The benzocarbazole **3** was prepared by the method of Marsilje *et al.* (*19, 32*) and references cited therein. The identities of the four synthesized compounds were confirmed by comparison of their ^{1}H NMR spectra with those reported in the literature (see Supporting Information).

Biological Screening. A TPO-dependent cell line was created by retroviral transduction of the murine hematopoietic cell line FDCP-1 (*33*) with the ecotropic Psi2 vector containing full-length human c-MPL. These cells are absolutely dependent on human TPO for survival and were used throughout the study for bioassay of all c-Mpl agonists. The FD-Mpl line was validated with native ligand TPO and Eltrombopag, a potent small molecule mimetic of TPO acting *via* the transmembrane mechanism. The dose−response rhTPO curve is shown in Supplementary Figure 2.

Proliferation of 5000 FD-Mpl cells/96 well in the absence of added Zn^{2+}, 100 mM Zn^{2+} as $ZnSO_4{\cdot}7H_20$ (BDH, Poole, England), and 100 mM EDTA (BDH, Poole, England) was carried out for 48 h at 37 °C (humidified air, 5% CO_2) in DMEM with 10% FCS. After 48 h cell proliferation was measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, no. G7570) and expressed as a percentage of cell proliferation obtained in media supplemented with either 30 ng mL^{-1} of rhTPO, 30 ng mL^{-1} of rhTPO and 100 μM $ZnSO4$, or 30 ng mL^{-1} of rhTPO and 100 μM EDTA. Importantly, in the absence of activation on the c-Mpl receptor, the FD-Mpl cells die within 48 h (Supplementary Figure 3).

We also varied the sequence of addition of reagents, but this produced no change to the outcomes reported here. Statistical analysis was carried out using a two-way ANOVA with interactions comparing factor 1 (control, no added Zn^{2+}) with factor 2 (100 μM added Zn^{2+} or 100 μM added EDTA).

Supporting Information Available: Structures and characterization of TPO agonist compounds, response of factor-dependent (fd-Mpl) cells to TPO, fd-Mpl cell viability in response to TPO starvation. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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