Identification of a Pharmacophore for Thrombopoietic Activity of Small, Non-Peptidyl Molecules. 1. Discovery and Optimization of Salicylaldehyde Thiosemicarbazone Thrombopoietin Mimics

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Abstract: High-throughput screening has resulted in the discovery of thiosemicarbazone thrombopoietin mimics. A shared pharmacophore hypothesis between this series and a previously identified class, the pyrazol-4-ylidenehydrazines, led to the rapid optimization of both potency and efficacy of the thiosemicarbazones. The application of high-throughput chemistry and purification techniques allowed for the rapid elucidation of structure−activity relationships.

The 332 amino acid cytokine thrombopoietin (TPO) plays a critical role in the regulation of megakaryocytopoiesis and is the primary regulator of platelet production.[1] TPO elicits its biological effect through binding and activation of the cell-surface receptor, c-mpl (the TPO receptor). Binding of TPO to c-mpl initiates a cascade involving several cell-signaling pathways leading to the irrevocable commitment of the progenitor cell along the megakaryocytic lineage.[2−5] Mature megakaryocytes then undergo cell fragmentation to produce the platelet bodies essential for the eventual formation of a protective thrombus in compromised blood vessels. Severely low platelet counts, thrombocytopenia, is a serious complication in patients receiving intensive chemotherapy. Currently, management of thrombocytopenia is primarily based on platelet transfusion, which is becoming increasingly costly.[6−9] The only approved platelet growth factor available at this time is the protein therapeutic rhuIL-11 (Neumega).[10] rhuTPO[11] and pegylated megakaryocyte differentiation factor[12] (peg-MGDF, a pegylated, truncated N-terminal region of TPO) are two protein agents currently undergoing clinical trials for the treatment of thrombocytopenia. Recently we have reported the discovery of the pyrazol-4-ylidenehydrazines, represented by compound **1**, as small-molecule TPO mimetic agents.[13] These compounds were shown to stimulate the proliferation and differentiation of TPO-responsive cell lines and human bone marrow cultures with full TPO efficacy at EC_{50} values as low as 1−100 nM. In this report, we describe the discovery of a second class of small-molecule TPO mimics, the salicylaldehyde thiosemicarbazones.

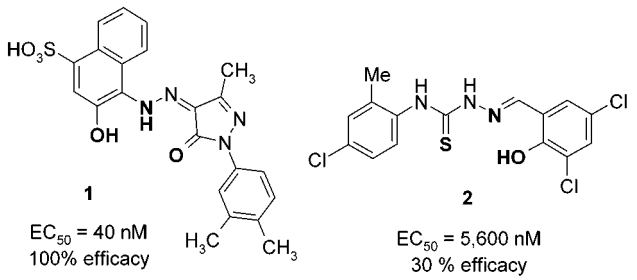

Figure 1. Comparison of salicylaldehyde thiosemicarbazone screening-hit **2** and potent pyrazol-4-ylidenehydrazine TPO agonist **1**. Activity is measured by expression of luciferase in the BAF3/TPO-Rluc cell line.[15]

High-throughput screening for the induction of a luciferase reporter gene in a TPO-responsive cell line[13,14] resulted in the identification of salicylaldehyde thiosemicarbazone **2** (Figure 1) as a weak TPO agonist. We recognized that this compound shared some structural similarities with the pyrazol-4-ylidenehydrazine class of TPO mimics; both the pyrazol-4-ylidenehydrazine **1** and the salicylaldehyde thiosemicarbazone **2** contain an arrangement of heteroatoms that can potentially engage a metal ion in a bicyclic, tridentate chelate. At the time, SAR trends for the pyrazol-4-ylidenehydrazines had already been elucidated, with acidic functionality being optimal on the naphthalene ring and lipophilic aryl groups being preferred as N substituents on the pyrazole ring. Because we were intrigued by the possibility that pyrazol-4-ylidenehydrazines and salicylaldehyde thiosemicarbazones may share the same pharmacophore for thrombopoietic activity, studies to improve the potency in the luciferase assay of salicylaldehyde thiosemicarbazone screening hit **2** were initiated.

Incorporation of acidic functionality was found to be important for potent TPO agonist activity in the pyrazol-4-ylidenehydrazine series.[13] As an initial test of our common pharmacophore hypothesis, both the sulfonic and carboxylic acid analogues **5a** and **5b** were prepared as shown in Scheme 1. This did indeed result in an over 10-fold improvement in potency for both **5a** and **5b**; however, maximum efficacies remained around 30% of that induced by saturating concentrations of TPO.

To further improve the potency and efficacy of the salicylaldehyde thiosemicarbazone TPO mimics, a molecular model was constructed in which the tridentate chelation atoms of salicylaldehyde thiosemicarbazone **5b** and pyrazol-4-ylidenehydrazine **1** were overlayed (Figure 2). As can be seen, these heteroatoms superimpose very well. This model also suggests that the incorporation of bulky aryl substituents at the 3-position of the hydroxyphenyl ring will allow access of the putative lipophilic pocket previously recognized to greatly enhance potency in the pyrazol-4-ylidenehydrazine series.[13] To test this hypothesis, a series of nine *o*-methoxy biaryl aldehydes **7** and the corresponding nine *o*-hydroxy

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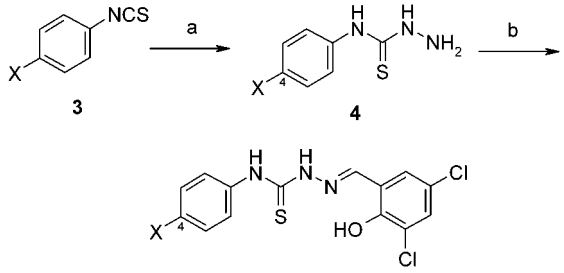
Scheme 1[a]



[a] (a) NH_2NH_2, THF; (b) 3,5-dichlorosalicylaldehyde, DMF.

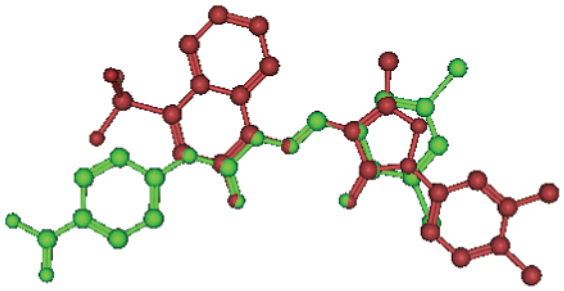


Figure 2. Model[16] of salicylaldehyde thiosemicarbazone **5b** (green) and pyrazol-4-ylidenehydrazine **1** (red) with the tridentate heteroatom motif overlayed.

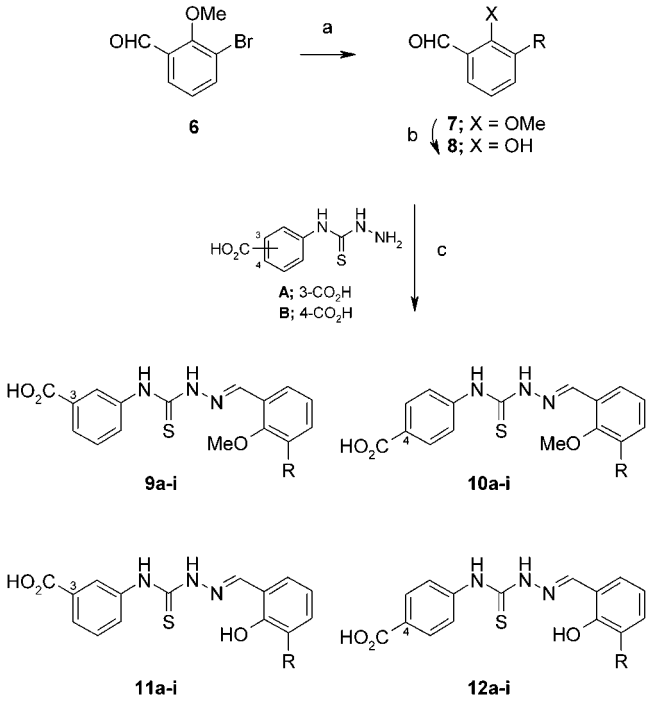
biaryl aldehydes **8** were prepared as shown in Scheme 2.[17] These 18 aldehydes were condensed with both the 3-carboxythiosemicarbazide **A** and the 4-carboxythiosemicarbazide **B** to afford 36 thiosemicarbazones, **9a**−**i**, **10a**−**i**, **11a**−**i**, and **12a**−**i**.

The TPO mimetic activity of the 36 salicylaldehyde thiosemicarbazones was tested by their ability to induce the proliferation of the TPO-responsive UT7/TPO-R cell lines in vitro.[18] Efficacies at the concentration of maximal effect[19] for all compounds are illustrated in Figure 3 as a percentage relative to the maximal effect of saturating TPO. As was predicted from the model in Figure 2, introduction of bulky, lipophilic aryl groups did indeed result in substantial increases in both potency and efficacy. The *o*-hydroxyl group was critical for high activity, as attested by the weak or lack of activity for the 18 compounds **9** and **10** containing a methoxy group in this position. 3-Carboxy and 4-carboxy analogues were roughly equally efficacious, suggesting a less stringent requirement for the optimal position of the acidic functionality.

For ease of representation, the array data in Figure 3 relates only to the maximal efficacy of the salicylaldehyde thiosemicarbazone TPO mimics. Potencies also improved by incorporating the lipophilic biaryl functionality as shown for selected agonists in Table 1. The most potent analogues **11b** and **12c** have potencies of 20 and 30 nM, respectively, with efficacies similar to those induced by maximal TPO concentrations.

It is important to note that all active salicylaldehyde thiosemicarbazones showed complete selectivity for TPO-responsive cell lines (UT7/TPO-R, BAF3/TPO-R) versus other cytokine-responsive cell lines tested [e.g., BAF-3 parental (IL-3), UT7/EPO-R, Hep-G2/G-CSF-R]. Further, the agonists induced a pattern of kinase activation and protein phosphorylation similar to that induced by TPO itself (data not shown).

Scheme 2[a]



R			
a	2-methylphenyl	**f**	1-dibenzofuranyl
b	3,4-dimethylphenyl	**g**	1-naphthyl
c	3-trifluoromethylphenyl	**h**	3-nitrophenyl
d	4-fluorophenyl	**i**	3-chlorophenyl
e	4-chlorophenyl		

[a] (a) R-$B(OH)_2$, $Pd(PPh_3)_4$, 1,4-dioxane, aqueous Na_2CO_3; (b) BBr_3, CH_2Cl_2; (c) **a** or **b**, DMF, 4 Å molecular sieves.

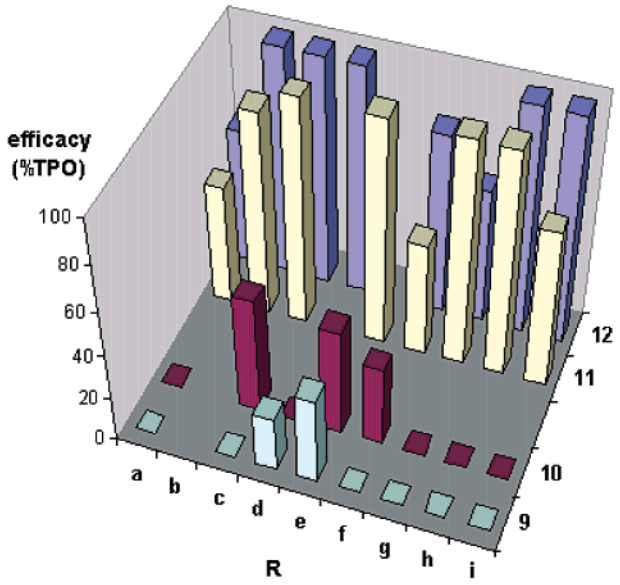
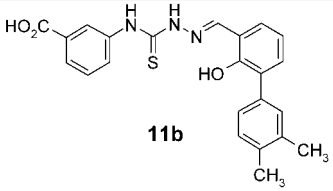
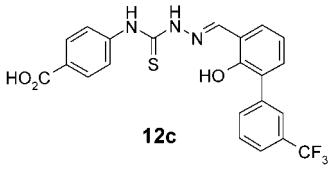


Figure 3. Maximal proliferative effect in UT7/TPO-R cell lines for salicylaldehydes **9a**−**i**, **10a**−**i**, **11a**−**i**, and **12a**−**i**. Efficacies are expressed as a percentage of the maximal proliferation induced by saturating TPO concentrations.

In conclusion, high-throughput screening has resulted in the discovery of a new series of TPO mimics, the salicylaldehyde thiosemicarbazones. A shared pharma-

Table 1. UT7/TPO-R Proliferation Data for Selected Salicylaldehyde Thiosemicarbazones

Compound	EC_{50} (nM)	efficacy ($\%TPO_{max}$)
 **11b**	30	90
 **12c**	20	100

cophore hypothesis between this series and a previously identified class, the pyrazol-4-ylidenehydrazines, in conjunction with molecular modeling led to the rapid optimization of both potency and efficacy of the salicylaldehyde thiosemicarbazones. The application of high-throughput chemistry and purification techniques facilitated the simultaneous synthesis of arrays of salicylaldehyde thiosemicarbazones and allowed for the rapid elucidation of structure−activity relationships. This pharmacophore hypothesis also made possible the invention of a completely novel class of TPO mimics.[20]

Supporting Information Available: UT7TPO-R proliferation assay potencies and efficacies for all thiosemicarbazones **9**−**12**, experimental details for the synthesis, and characterization of representative salicylaldehyde thiosemicarbazone TPO mimics. This material is available free of charge via the Internet at http://pubs.acs.org.

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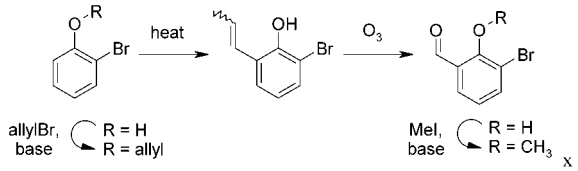
(15) Luciferase assay: BAF3/TPO-Rluc cells [(1×10^5)/mL] (starved of IL-3 overnight) in growth medium containing fetal bovine serum (FBS) (0.5% v/v) and $ZnCl_2$ (30 μM) were incubated with compounds (0.32% DMSO final concentration) or rhuTPO at 37 °C (5% CO_2, 95% relative humidity) for 3 h. Luciferase activity was recorded using a Dynatech model 1000 luminometer. Each data point is the average of triplicate assays.

(16) *MOE*, version 2001.01; Chemical Computing Group Inc.: Quebec, Canada, 1997−2001.

(17) Bromo-2-methoxybenzaldehyde was prepared from 2-bromophenol as show below. Full experimental details are given in the Supporting Information.



(18) UT7/TPO proliferation assay: UT7/TPO cells (0.2×10^6 cells/mL) were suspended in IMDM supplemented with FBS (10% v/v) and glutamine and then incubated with compounds (0.32% DMSO final concentration) or rhTPO at 37 °C (5% CO_2, 95% relative humidity) for 72 h. After centrifugation, cell pellet total DNA content is measured using a BrdU proliferation kit (Boehringer Mannheim, catalog no. 1647229). Each data point is the average of duplicate assays.

(19) For ease of representation, potencies are not considered in this analysis.

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