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Discovery and biological evaluation of benzo[*a*]carbazole-based small molecule agonists of the thrombopoietin (Tpo) receptor

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ABSTRACT

A novel series of benzo[*a*]carbazole-based small molecule agonists of the thrombopoietin (Tpo) receptor is reported. Starting from a 3.4 μM high throughput screen hit, members of this series have been identified which are full agonists with functional potency <50 nM and oral bioavailability in mice.

The condition of low platelet count is referred to as thrombocytopenia. A patient is considered to be at risk of spontaneous hemorrhaging if their platelet count falls below 20,000/μL. The causes of thrombocytopenia are manifold, including e.g., idiopathic thrombocytopenic purpura (ITP), chronic liver disease, HCV infection, certain varieties of cancer and as a side effect of certain forms of cancer chemotherapy. Thrombopoietin (Tpo) is a hematopoietic growth factor whose agonism of the Tpo receptor (TpoR, also called cMpl) has been shown to be the principal regulator of megakaryocyte growth and differentiation into platelets. TpoR is a cell surface receptor that initiates an intracellular signaling cascade that is mediated by the JAK2 kinase and the transcription factor Stat5. The administration of PEG modified, truncated Tpo was tested clinically but this therapeutic strategy has been abandoned due to immunogenicity issues. Novel methods of agonism of the TpoR thus represents an alternative strategy to address thrombocytopenia in patients, currently an area of unmet medical need.[1,2]

Despite the ostensibly difficult task of replicating the effect of a protein–protein interaction with a small molecule ligand, TpoR agonism has recently received increasing attention from the pharmaceutical industry. In addition to the injectable peptidic agent Romiplostim™[3] which recently obtained regulatory approval, a number of nonpeptidyl small molecule agonists have been disclosed (Fig. 1). Eltrombopag™ (**1**)[4–7] was recently the first small molecule TpoR agonist to be submitted for regulatory approval. Several additional reports of small molecule drug discovery efforts have been published.[8–23]

We screened for TpoR agonists by measuring activation of a Stat5 response element-driven reporter gene in a human TpoR responsive Ba/F3 cell line (Ba/F3-hTpoR RGA).[12,16] A benzo[*a*]carbazole (**7**, Fig. 2) was identified as a novel agonist of hTpoR with 3.4 μM potency and 100% efficacy relative to 30 ng/mL Tpo. The structure of **7** is a novel chemotype in comparison to the small molecule TpoR ligands which had been previously described in the literature,[4–23] (Fig. 1). A search of the literature indicated that the only previously described biological activity of **7** is weak antibacterial and antifungal activity.[24] Based upon the unique chemotype, low micromolar potency and full efficacy of **7**, a preliminary SAR analoging effort was undertaken. An initial series of benzo[*a*]carbazoles was synthesized using known methodology[25] and evaluated in vitro in order to map out the key pharmacophore features necessary for TpoR activation. The functional activities of this initial series of compounds (**7**–**14**) against TpoR are shown in Table 1.

Reduction of the carboxylic acid in **7** generated the benzyl alcohol **8** which is inactive. Likewise, methylation of either the carbazole nitrogen (**9**) or the phenol (**10**) is not tolerated. Converting the carboxylic acid in **7** to the corresponding methyl ester (**11**) generated a compound with retained potency (1.3 μM vs 3.4 μM) but

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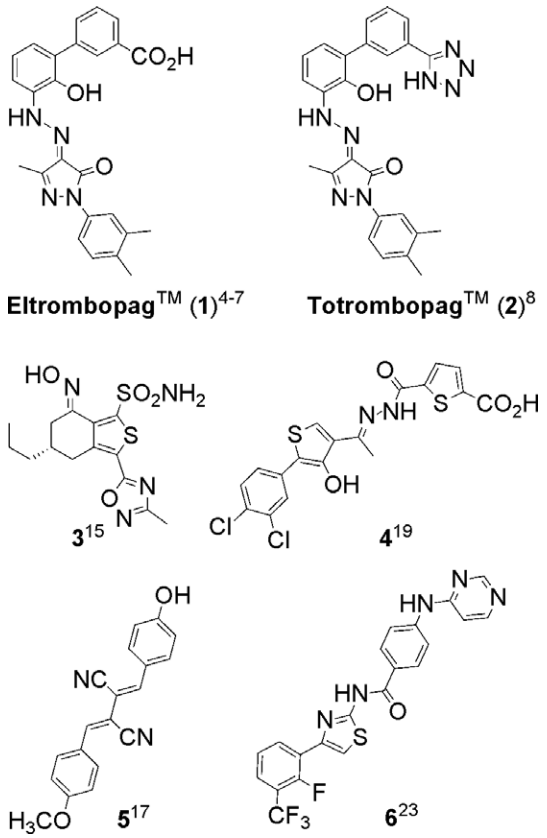


Figure 1. Representative structures of reported small molecule TpoR agonists.

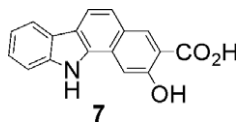
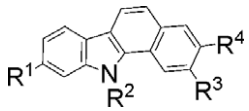


Figure 2. HTS hit molecule.

Table 1
In vitro human TpoR functional activity of benzo[*a*]carbazole derivatives



Compound[a]	R^1	R^2	R^3	R^4	EC_{50}[b] (μM)	% Efficacy[c]
7	H	H	OH	CO_2H	3.4	100
8	H	H	OH	CH_2OH	>30	NA
9	H	CH_3	OH	CO_2H	>30	NA
10	H	H	OCH_3	CO_2H	>30	NA
11	H	H	OH	CO_2CH_3	1.3	11
12	CH_3	H	OH	CO_2H	0.87	76
13	CH_3	H	OH	CH_2CO_2H	9.2	70
14	CF_3	H	OH	CO_2H	0.143	128
Eltrombopag™ (**1**)					0.038	127

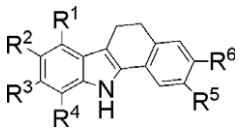
[a] All compounds demonstrated satisfactory LC–MS and ^1H NMR characterization.
[b] Ba/F3-hTpoR RGA as previously described.[10,14]
[c] Relative to 30 ng/mL Tpo. NA, not applicable.

dramatically reduced efficacy (11% vs 100%). This indicates that the salicylic acid portion of the molecule is very important for both potency and efficacy. Initial SAR exploration on the opposite side of the scaffold generated compound **12** which incorporates a methyl group in the 9-position of the benzo[*a*]carbazole ring system. Compound **12** was the first sub-micromolar compound identified in the scaffold with a potency of 870 nM (76% efficacy). A methylene homologation of the benzoic acid in **12** to an acetic acid (**13**)[26] results in a ca. 10× less potent compound (0.87 μM vs 9.2 μM) but interestingly, efficacy is retained (70% vs 76%). Encouraged by the sub-micromolar activity of **12**, we replaced the methyl group in **12** with a trifluoromethyl group (**14**) which significantly increases both potency and efficacy (143 nM, 128%). This level of potency and efficacy led us to consider **14** as a lead molecule and a starting point for the further optimization of the benzo[*a*]carbazole scaffold. At this point, we desired to modify the 5,6-olefin in this series of molecules for two reasons: (a) the possibility of oxidation of the 5,6-double bond in vivo potentially generating a reactive species and (b) the flat and fully aromatic nature of this molecule making intercalation into DNA an undesired possibility. We decided to attempt to switch from the fully aromatic benzo[*a*]carbazole to a partially reduced dihydro-benzo[*a*]carbazole ring system[27] and the resulting compounds are shown in Table 2.

Compound **15** is the partially reduced direct analog of the lead compound **14**. We were pleased to see that partially reducing the aromaticity of the lead compound **14** generates a compound which retains much of the potency (473 nM) and efficacy (96%) of the fully aromatic system. Compound **15** was thus able to serve as a lead molecule in the optimization of a partially reduced dihydro-benzo[*a*]carbazole scaffold. The CF_3 group in **15** was systematically moved to other regiopositions on the scaffold (**16**–**18**) in order to determine if its effect on potency is primarily due to its electronic effects on the ring system or if it demonstrates a potency effect in a regiospecific fashion. Compound **16** incorporates a fluorine in the R^4 position for synthetic reasons but we have demonstrated that the hydrogen to fluorine switch in position R^4 reproducibly causes only a ca. 1–2× effect on potency in the dihydro-benzo[*a*]carbazole scaffold (data not shown). Placing the CF_3 group in the R^1 position (**16**) or R^2 position (**17**) results in compounds with similar activities to **15** but it is clear that a CF_3 group is not tolerated at the R^4 position (**18**). This indicated to us that the positive effects of the CF_3 group on potency may be due to its electron withdrawing electronic effect with perhaps an unfavorable steric clash in the R^4 position. In order to test this hypothesis, we incorporated two alternative electron withdrawing groups: a nitrile at the R^2 position (**19**) or a carboxylic acid at the R^3 position (**20**).[28] Both compounds have significantly reduced activity, demonstrating that the CF_3 group's effect on potency is due to factors other than ring electronics.

We next systematically affixed a phenyl group to various regiopositions on the scaffold in order to gauge the steric limitations afforded by the binding site of the TpoR. A phenyl ring was affixed in the R^1 (**21**), R^2 (**22**) or R^3 (**23**) positions. Compound **21** is inactive, showing potential steric intolerance at the R^1 position. The R^2 position (**22**) partially tolerates the phenyl ring (569 nM, 68%) but the R^3 position (**23**) shows complete tolerance of a phenyl group as well as similar potency and efficacy compared to the lead molecule **15**. Even though **23** and **15** have similar potencies, we were encouraged by the fact that a tolerated aromatic ring in the R^3 position would afford a wider range of potential follow-up compounds to synthesize in our attempt to increase potency. Interestingly, a cyclohexyl group is not tolerated in the R^3 position (**24**) indicating a possible preference for an aromatic system in the R^3 position. The activity of **23** and synthetic accessibility led us to focus our efforts on aromatic analogs in the R^3 position. Based upon SAR reported in the literature[13] and the possibility of a similar binding mode in the Tpo receptor, we reasoned that hydrophobic substitution on the R^3 phenyl ring may increase activity and synthesized the derivatives **25**–**37**. In general, the SAR indicates that increased potency correlates with an increase in lipophilicity in

Table 2
In vitro human TpoR functional activity of dihydro-benzo[*a*]carbazole derivatives



Compound[a]	R^1	R^2	R^3	R^4	R^5	R^6	EC_{50}[b] (μM)	% Efficacy[c]
15	H	H	CF_3	H	OH	CO_2H	0.473	96
16	CF_3	H	H	F	OH	CO_2H	0.799	101
17	H	CF_3	H	H	OH	CO_2H	0.333	115
18	H	H	H	CF_3	OH	CO_2H	7.83	37
19	H	CN	H	H	OH	CO_2H	>30	NA
20	H	H	CO_2H	H	OH	CO_2H	7.79	74
21	Phenyl	H	H	H	OH	CO_2H	>30	NA
22	H	Phenyl	H	H	OH	CO_2H	0.569	68
23	H	H	Phenyl	H	OH	CO_2H	0.373	136
24	H	H	*c*-Hexyl	H	OH	CO_2H	>30	NA
25	H	H	3′-Methyl-phenyl	H	OH	CO_2H	0.194	128
26	H	H	4′-Methyl-phenyl	H	OH	CO_2H	0.109	154
28	H	H	2′,6′-Dimethyl-phenyl	H	OH	CO_2H	0.232	30
29	H	H	3′,4′-Dimethyl-phenyl	H	OH	CO_2H	0.065	127
30	H	H	3′,5′-Dimethyl-phenyl	H	OH	CO_2H	0.067	128
31	H	H	4′-Fluoro-3′-methyl-phenyl	H	OH	CO_2H	0.035	126
32	H	H	3′-CF_3-phenyl	H	OH	CO_2H	0.148	127
33	H	H	4′-$N(CH_3)_2$-phenyl	H	OH	CO_2H	0.284	136
34	H	H	4′-*n*-Butyl-phenyl	H	OH	CO_2H	0.026	143
35	H	H	4′-Propoxy-phenyl	H	OH	CO_2H	0.041	129
36	H	H	4′-Methoxymethyl-phenyl	H	OH	CO_2H	0.452	132
37	H	H	3′-Propoxy-phenyl	H	OH	CO_2H	0.043	147
38	H	H	4′-*n*-Butyl-phenyl	H	H	CO_2H	0.111	141
39	H	H	4′-*n*-Butyl-phenyl	F	H	CO_2H	0.055	140
40	H	H	4′-*n*-Butyl-phenyl	H	H	1*H*-Tetrazole-5-yl	0.119	128
41	H	H	4′-*n*-Butyl-phenyl	H	H	$C(O)NH_2$	0.076	111
42	H	H	4′-*n*-Butyl-phenyl	H	H	$C(O)N(CH_3)_2$	0.127	132
43	H	H	4′-Fluoro-3′-methyl-phenyl	F	H	2-Imidazoline	>30	NA
Eltrombopag™ (**1**)							0.038	127

[a] All compounds demonstrated satisfactory LC–MS and ^1H NMR characterization.
[b] Ba/F3-hTpoR RGA as previously described.[10,14]
[c] Relative to 30 ng/mL Tpo. NA, not applicable.

this region of the ligand although individual heteroatoms are tolerated. This indicates that this portion of the scaffold binds to a highly hydrophobic pocket of the receptor. Tolerance for large substituents in either the 3′ or 4′-positons of the phenyl ring at R^3 indicates that the binding pocket in this region of the TpoR is sterically large. The 2′,6′-dimethyl phenyl analog **28** retains the potency but not the efficacy of the parent molecule **23**, which indicates the potential preference of a flat aromatic ligand geometry in the R^3 position for receptor activation. The most potent analogs identified from these efforts (**31**, **34**, **35**, and **37**) have potencies <50 nM and efficacies >100% relative to Tpo. Two additional compounds (**29** and **30**) are only slightly less potent with potencies of ca. 65 nM. The in vitro Ba/F3-hTpoR RGA potency and efficacy of these analogs are similar to that seen for the clinically efficacious compound Eltrombopag™ (**1**).

With a potent compound (**34**) in hand, we next investigated the possibility of modifying the salicylic moiety. Deleting the phenolic oxygen of **34** to generate **38** results in a 4× loss of potency. This loss in potency is partially addressed by introducing a fluorine in the R^4 position generating compound **39** which has 55 nM potency with 140% efficacy. Substituting the benzoic acid in **38** with a tetrazole (**40**), amide (**41**) or *N*,*N*-dimethylamide (**42**) generates compounds which all show similar potencies and efficacies as **38,** indicating that a negatively charged moiety is not required for receptor binding and activation. The 2-imidazolone (**43**) however does not retain activity, indicating the intolerance of the receptor to a positively charged moiety in that portion of the ligand.

The pharmacokinetic profile of compound **30** was determined in BALB/c mice. It has 10% bioavailability with a C_{max} of ca. 2 μM and a half-life of 4.1 h following 20 mg/kg oral dosing.[29] This was considered a reasonable starting point for the final optimization of the in vivo properties of this series of compounds. However, at this time we noted that both in vitro and in vivo, the dihydro-benzo[*a*]carbazole series of compounds exemplified by **30** is chemically unstable and reverts to the fully aromatic benzo[*a*]carbazole ring system over time via oxidation of the 5,6-ethylene bridge.

In summary, we have described a structurally novel class of small molecule agonists of the human Tpo receptor. Beginning with a 3.4 μM hit (**7**), we were able to increase potency 130× generating compounds with <50 nM potency and full efficacy in activating the human Tpo receptor. A representative compound from this series has oral bioavailability in mice. We were successful in dearomatizing the original hit molecule but noted that the resulting compounds are chemically unstable with rearomatization to the benzo[*a*]carbazole ring system being facile. Our efforts to address this chemical instability pharmaceutical development issue are discussed in the following communication in this journal.

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26. Prepared from **12** by the following sequence: (a) BnBr (3.3 equiv), NaH (3.3 equiv), DMF, 60 °C, 2 h; (b) LAH (2 equiv), THF (74% over two steps); (c) $SOCl_2$ (1.9 equiv), pyridine (0.4 equiv), chloroform; (d) KCN (4 equiv), 160 °C, 10 min (50% over two steps); (e) LiOH, dioxane, H_2O (160 °C, 10 min), Na, NH_3, THF, −78 °C (15% over two steps).
27. The dihydro-benzo[*a*]carbazole ring system (**15**–**43**) is prepared by reacting a 0.1 M solution of 1 equiv of the appropriate hydrazine hydrochloride, 1 equiv of the appropriate tetralone, and 2.5 equiv of $ZnCl_2$ in acetic acid at 105 °C overnight. The final compounds are generated using standard synthetic methodology as described in Alper, P., et al. WO 2007/009120.
28. Prepared by treatment of **15** with LiOH at 150 °C for 10 min in a mixture of water and dioxane.
29. Formulated as a 2.5 mg/mL in PEG300/D5W (3:1) solution.