Modulation of Peripheral Serotonin Levels by Novel Tryptophan Hydroxylase Inhibitors for the Potential Treatment of Functional Gastrointestinal Disorders

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Abstract: The discovery of a novel class of peripheral tryptophan hydroxylase (TPH) inhibitors is described. This class of TPH inhibitors exhibits excellent potency in in vitro biochemical and cell-based assays, and it selectively reduces serotonin levels in the murine intestine after oral administration without affecting levels in the brain. These TPH1 inhibitors may provide novel treatments for gastrointestinal disorders associated with dysregulation of the serotonergic system, such as chemotherapy-induced emesis and irritable bowel syndrome.

Serotonin (5-hydroxytryptamine, 5-HT[a]) is a neurotransmitter that modulates central and peripheral functions through action on neurons, smooth muscle, and other cell types. The rate-limiting step in serotonin biosynthesis is the hydroxylation of tryptophan, which is catalyzed by tryptophan hydroxylase (TPH). The resulting 5-hydroxytryptophan is then decarboxylated by an amino acid decarboxylase to 5-HT.[1–4] Together with phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH), TPH belongs to the pterin-dependent aromatic amino acid hydroxylase family.[1–4] Two vertebrate isoforms of TPH, TPH1 and TPH2, have been described.[5–7] TPH1 is primarily expressed in the pineal gland and non-neuronal tissues, such as enterochromaffin (EC) cells of the gastrointestinal (GI) tract.[5,8,9] TPH2 (the dominant form in the brain) is expressed exclusively in neuronal cells, such as dorsal raphe or myenteric plexus cells.[5,8,9]

5-HT is involved in the control and modulation of multiple physiological and psychological processes. In the central nervous system (CNS), 5-HT regulates mood, appetite, and other behavioral functions. In the GI system, where approximately 90% of the body's 5-HT is synthesized and stored, 5-HT plays a general prokinetic role and is an important mediator of sensation (e.g., nausea and satiety) between the GI tract and the brain.[10–12] Dysregulation of the peripheral 5-HT signaling system is involved in the etiology of several conditions such as functional GI disorders, chemotherapy-induced emesis, and heart valve damage.[13–16] The large number of pharmaceutical agents that block or stimulate 5-HT receptors is indicative of the wide range of medical disorders that have been associated with 5-HT dysregulation.[17] One example, *p*-chlorophenylalanine (pCPA), a weak inhibitor of TPH, has proven effective in treating chemotherapy-induced emesis, as well as diarrhea, in carcinoid tumor patients. Unfortunately, administration of pCPA has been linked to the onset of depression and other alterations of CNS function in patients.[18–20]

In contrast to known serotonin reduction treatments, disruption of the mouse TPH1 gene results in loss of most peripheral 5-HT while leaving neuronal levels unaffected.[8,21] We therefore hypothesized that TPH inhibitors could be developed to selectively modulate only peripheral serotonin levels, thereby avoiding CNS-related side effects. Such selectivity can be achieved by developing (1) a TPH1 inhibitor that is selective against TPH2, (2) a TPH inhibitor that does not penetrate the blood−brain barrier, (3) a TPH inhibitor that acts locally in the GI tract, or (4) a combination of these approaches.

Herein, we report the discovery of a novel class of tryptophan hydroxylase inhibitors that selectively reduce peripheral serotonin levels in animal models without affecting central serotonin levels. Compounds within that class were optimized to act locally in the intestine with minimal systemic exposure after oral administration and do not penetrate the blood−brain barrier. When administered orally to mice, these compounds were able to reduce 5-HT levels in the GI tract without affecting brain 5-HT levels.

Several biological assays were developed to determine the inhibitory effect of compounds against TPH in vitro and in vivo. In vitro biochemical assays for human, mouse, and rat TPH1 and human TPH2, PAH, and TH were used to measure inhibition of enzyme activity and the selectivity among TPH1, TPH2, PAH, and TH. Cell based assays using rat mastocytoma cells (RBL) or human carcinoid cells (BON) were developed to measure inhibition of serotonin biosynthesis in whole cells. In addition, an in vivo tissue 5-HT assay was developed to determine the effect of compounds on 5-HT levels in various tissues after oral administration of TPH inhibitors.[21]

Discovery of the compounds began with high-throughput screening of a library of approximately 200,000 compounds using a radiometric enzyme assay. Several classes of hits were identified. Among them, phenylalanine derivative **1** was confirmed as a TPH1 inhibitor with an IC_{50} of 0.67 μM. The *S*-isomer **1a** was found to be the active form, with an IC_{50} of 0.24 μM against TPH1. *p*-Ethynylphenylalanine (pEPA) and pCPA were tested in the same in vitro assays, side by side with **1a**, and have an IC_{50} of 30 and 92 μM, respectively (Scheme 1). Compared to pCPA and pEPA, **1a** shares the same phenylalanine moiety but is about 380-fold more potent than pCPA and 120-fold more potent than pEPA. It was envisioned that **1a** could be further optimized not only to improve in vitro and in vivo potency but also to improve physicochemical properties to achieve local action in the GI tract and to ensure that no penetration of the blood−brain barrier occurred.

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[a] Abbreviations: 5-HT, 5-hydroxytryptamine; TPH, tryptophan hydroxylase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; EC, enterochromaffin; pCPA, *p*-chlorophenylalanine; GI, gastrointestinal; CNS, central nervous system; RBL, rat mastocytoma cell; BON, human carcinoid cell; pEPA, *p*-ethynylphenylalanine.

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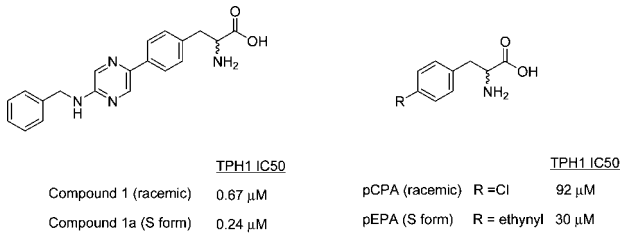
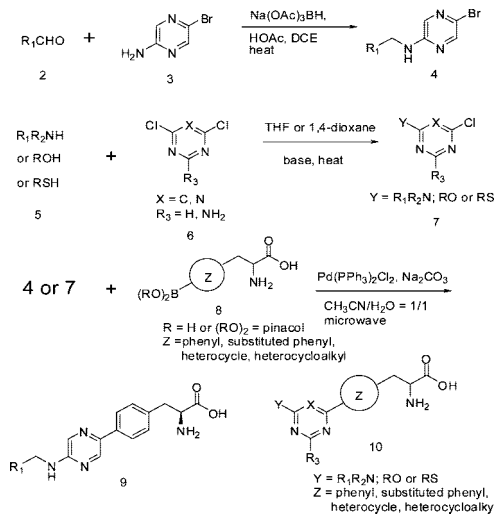
Scheme 1. Chemical Structures of **1a**, pCPA, and pEPA



Scheme 2. Synthesis of TPH1 Inhibitors



Analogues were designed and synthesized to develop structure−activity relationships of the lead molecules and to improve potency and physicochemical properties. Scheme 2 outlines the general synthetic route for the preparation of these analogues.

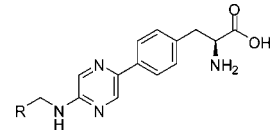
Intermediates **4** and **7** were made through reductive amination of 2-amino-5-bromopyrazine **3** with various aldehydes **2** or nucleophilic displacement of pyrimidine or triazine dichloride **6** with nucleophiles such as amines, thiols, or alcohols. Halides **4** and **7** were then coupled with **8** using microwave-assisted Suzuki coupling to give **9** and **10**. Compounds **9** and **10** were purified by preparative HPLC using a MeOH/H_2O/TFA system.

The initial SAR of the 2,5-disubstituted pyrazine class revealed that a lipophilic substitutent at the 2 position of 2,5-pyrazine was preferred. Arylmethylamino substituted analogues generally had good in vitro potencies against TPH1. Introduction of polarity at the 2 position decreased in vitro activity (Table 1).

The SAR also suggested that a 4′-phenylalanine moiety is essential to the in vitro potency against TPH and cannot be substituted by common acid isosteres. Replacement of phenylalanine with phenylglycine, tetrahydroisoquinoline carboxylic acid, or *N*-methyl-L-phenylalanine, as well as its corresponding amino alcohol or α-aminotetrazole, all resulted in significant loss of activity (**11**−**15**; chemical structures and data are in Supporting Information).

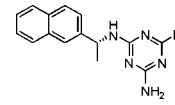
Compared to **10a**, analogues with various changes on the L-phenylalanine moiety (**10b**, **10c**, **16**, and **17**, Table 2) all showed significantly decreased in vitro activity against TPH1. Furthermore, replacing the phenyl group of the phenylalanine moiety with pyridine, piperidine, or pyrazole (**10d**−**f**) significantly reduces in vitro activity against TPH1. These data suggest that the 4′-phenylalanine moiety is essential to the potency.

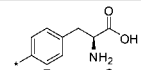
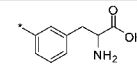
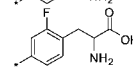
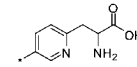
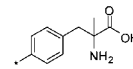
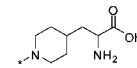
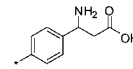
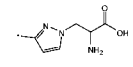
Table 1. In Vitro Potency of Compounds **9a–l**



compd	R	TPH1 IC_{50} (μM)
9a	cyclohexyl	0.24
9b	3-furanyl	0.96
9c	4-cyanophenyl	0.11
9d	2-pyridyl	6.15
9e	3-quinolinyl	0.19
9f	2-tetrahydronaphthyl	0.046
9g	2-naphthyl	0.013
9h	3-cyclopentyloxy-4-methoxyphenyl	0.045
9i	3-(9-ethyl-9H-carbazolyl)	0.031
9j	3,4-dimethoxyphenyl	0.069
9k	4′-biphenyl	0.044
9l	2′-methyl-ortho-biphenyl	0.040

Table 2. In Vitro Potency of L-Phe Analogues



cmpd	R	TPH1 IC50 (μM)	cmpd	R	TPH1 IC50 (μM)
10a		0.026	**10c**		> 11
10b		0.20	**10d**		0.89
16		11.1	**10e**		0.77
17		3.77	**10f**		12.6

This finding was further confirmed by a solid-state crystal structure of TPH1 protein with inhibitor **21** (LP-533401)[21] (Figure 1A and Figure 2). As observed upon the binding of ligands to the phenylalanine hydroxylases,[22] this protein undergoes substantial conformational change, with the mobile loops "closing" on the ligand relative to the "partially closed" form seen for the apo structure of tryptophan hydroxylase.[3] Also, Arg 287 moves to form a salt bridge with the ligands' carboxylate groups. A web of hydrogen bonds ties the amino portion of the amino acid to residues of the binding pocket directly and via a bridging water molecule.

Once the SARs at the 2 and 5 positions of the pyrazine were developed, our efforts turned to the modification of the pyrazine core. A variety of heterocycles were studied. Several examples are given in Table 3. The results suggest that the pyrazine core is sensitive to alteration but can be replaced with a 2-amino-4,6-disubstitued triazine (**10g**) or a 2-amino-4,6-disubstitued pyrimidine (**10h**). The SAR that led to compounds with activity in cells and in vivo were derived from the 2-amino-4,6-disubstituted pyrimidine class.

During the development of SAR and optimization of lead compounds, potent TPH1 inhibitors in biochemical assays were tested in cell-based assays. One significant finding based on the cell potency of these TPH1 inhibitors was that introduction of an α-trifluoromethylalkoxy linker at the 4 position of the 2-amino-4,6-disubstituted pyrimidine analogues greatly improved the TPH inhibitory activity in cells. Table 4 summarizes the comparison of the α-methylamino linker (**10i**), α-methyl-

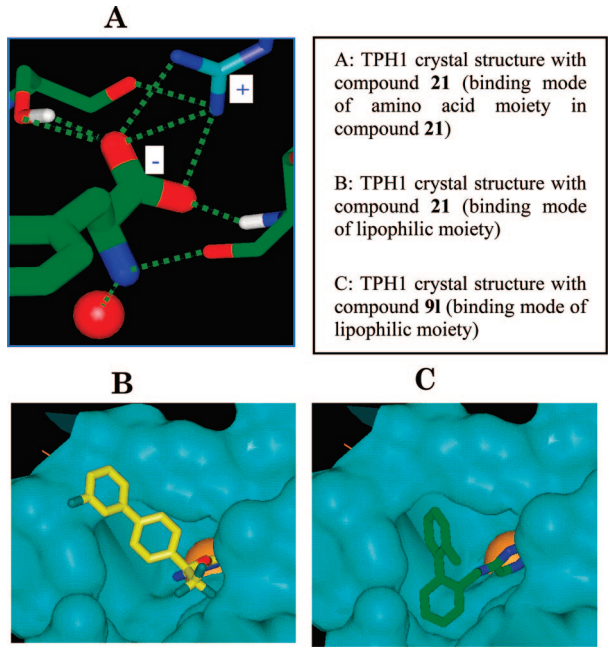


Figure 1. TPH1 crystal structures.

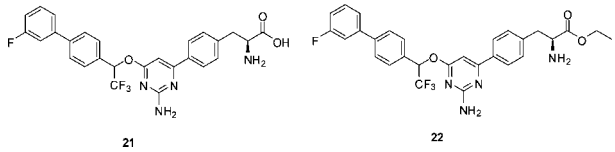
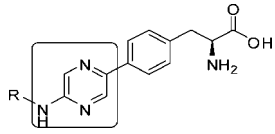


Figure 2. Chemical structures of **21** and **22**.

alkoxy linker (**10j**), and α-trifluoromethylalkoxy linker (**10k**). The data showed that the α-CF_3 alkoxy analogue (**10k**) has decreased activity in the in vitro enzyme assay but has improved activity in the cell-based assay. The ratio EC_{50}/IC_{50} is about 3, which is significantly lower than that for the α-methylalkoxy analogue (ratio of 29).

Although the exact reason for the improved cell potency of these α-CF_3 alkoxy analogues is unknown, this finding is consistent across a wide variety of structural variations in the pendent aryl substituent and sets the stage for the in vivo optimization of these compounds.

Table 3. Replacement of Pyrazine Core



compd	R	"core"	TPH1 IC_{50} (μM)
9g	A	E	0.013
10g	A	F	0.024
9h	B	E	0.045
18	B	G	>10
19	B	H	0.97
9i	C	E	0.031
20	C	I	>10
10h	D	J	0.024

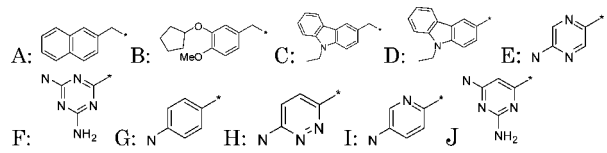
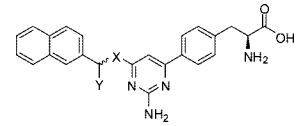
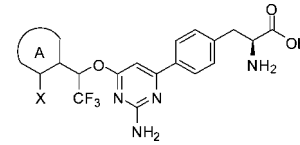


Table 4. α-CF_3 Alkoxy Linker Improves Cell Potency



compd	X/Y	TPH1 IC_{50} (μM)	RBL EC_{50} (μM)
10i	NH/CH_3 (R)	0.032	1.7
10j	O/CH_3 (R)	0.38	11
10k	O/CF_3 (RS)	0.18	0.51

Table 5. In Vitro Potency of TPH1 Inhibitors



compd	A/X	TPH1 IC_{50} (μM)	RBL EC_{50} (μM)	BON EC_{50} (μM)
10l	Ph/3′-Me-Ph	0.070	0.056	0.56
10m	Ph/2′-furanyl	0.024	0.048	0.80
10n	Ph/3′-CF3-pyrazole	0.029	0.070	0.75
10o	Thiophene/2′-furanyl	0.054	0.063	1.19

The crystal structure of TPH1 with another inhibitor **9l** further revealed the binding modes of the lipophilic moiety of the TPH1 inhibitors within the protein's active site (Figure 1B,C). The pendent 2′-substituent on the pyrazine core is generally tolerant of substitution, with lipophilic substituents being preferred. However, the solid-state structures of **21** and **9l** showed that the orientations of the aryl substituent differ significantly.

For both ligands, this lipophilic moiety occupies the outermost regions of the pterin cofactor binding site. In the case of **21**, it stretches across this site to make nonspecific interactions with the outside of the protein. However, **9l** binds with one ring nestled into a shallow sub-binding pocket. It has enhanced binding to TPH1 compared to the para-biaryl substitution pattern (**21**). This is also in agreement with the in vitro TPH1 potency of these two compounds, where the ortho-biaryl analogue **9l** is about 10 times more potent than the para-biaryl analogue (**21**). This information was further applied to our optimization strategy and the design of new analogues.

With the understanding of SAR, the binding mode in the TPH1 active site, and the contribution of the α-trifluoromethylalkoxy linker to the improved cell potency, we further optimized the lead compounds by introducing a variety of ortho-biaryl α-trifluoromethylalkoxy substituents at the 4 position of 2-amino-4,6-disubstitued pyrimidine (Table 5). These compounds showed excellent potencies in in vitro biochemical assays and good cellular potencies.

Compounds with good in vitro potencies were evaluated in in vitro ADME assays and in vivo pharmacokinetic assays. In general, these compounds have poor systemic exposure and do not penetrate the blood−brain barrier in mouse pharmacokinetic studies. For example, **21** showed a low plasma clearance (3.33 ± 0.56 (mL/min)/kg) and a small volume of distribution (0.73 ± 0.07 L/kg) following intravenous injection (1 mpk). After oral administration, **21** was poorly absorbed with a low oral bioavailability (7.6 ± 1.6%). The compound level in the brain is negligible following oral administration, indicating no penetration of the blood−brain barrier (see detailed **21** PK results in Supporting Information).

To test the in vivo efficacy of the compound, **21** was given to mice orally at 30 or 90 mg/kg, twice a day, for a total of six

doses. Compared to the vehicle control group, 5-HT levels in the small intestine (jejunum and ileum) were significantly lower in the **21**-dosed animals, averaging a 55% and 70% reduction at 30 and 90 mg/kg, respectively. 5-HT levels in the colon were also reduced, averaging a 24% and 36% reduction at 30 and 90 mg/kg, respectively. In contrast, no significant change in brain 5-HT levels was observed at either dose.[21]

Compound **22** (LP-615819),[21] the ethyl ester prodrug of **21**, was also tested in the mouse side by side with a known TPH inhibitor (pCPA) to compare their in vivo effects. Compound **22** was given orally at 45 mg/kg, while pCPA was given at 90 mg/kg, twice daily. After 3 days of dosing, both compounds reduced 5-HT levels in the intestine, with **22** showing greater efficacy in the jejunum. More importantly, pCPA, as expected, significantly lowered 5-HT levels in the brain while **22** showed no effect. In a follow-up study, **22** was given to mice orally at three different dose levels (20, 45, 90 mg/kg) for 3 days. Treatment with **22** caused robust reduction of 5-HT along the intestinal tract in a dose-dependent fashion with a 88% reduction in the jejunum at 90 mg/kg. Most importantly, the compound did not alter brain 5-HT levels significantly, even at the highest dose tested.[21]

In summary, we describe here a novel class of potent TPH inhibitors. We show that these TPH inhibitors, such as **21** and **22**, can selectively inhibit peripheral 5-HT synthesis without affecting CNS 5-HT synthesis, because of the very poor systemic exposure and their inability to cross the blood−brain barrier. This novel class of peripheral TPH inhibitors may provide potential treatment for a variety of gastrointestinal diseases caused by dysregulation of the serotonergic pathway.

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Supporting Information Available: Experimental details, analytical data of the compounds, in vitro potency data of **11**−**15**, and PK of LP-533401. This material is available free of charge via the Internet at http://pubs.acs.org.

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