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Discovery and Characterization of Novel Tryptophan Hydroxylase Inhibitors That Selectively Inhibit Serotonin Synthesis in the Gastrointestinal Tract

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Abbreviations: GI, gastrointestinal; 5-HT, 5-hydroxytryptamine; pCPA, para-chlorophenylalanine; TPH, tryptophan hydroxylase

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Abstract

Serotonin (5-HT) is a neurotransmitter with both central and peripheral functions, including the modulation of mood, appetite, hemodynamics; and gastrointestinal (GI) sensation, secretion, and motility. Its synthesis is initiated by the enzyme tryptophan hydroxylase (TPH). Two isoforms of TPH have been discovered: TPH1, primarily expressed in the enterochromaffin cells of the gastrointestinal tract, and TPH2, expressed exclusively in neuronal cells. Mice lacking *Tph1* contain little to no 5-HT in the blood and GI tract while maintaining normal levels in the brain. Since GI 5-HT is known to play important roles in normal and patho physiology, we set out to discover and characterize novel compounds that selectively inhibit biosynthesis of GI 5-HT. We describe here two of a series of these inhibitors that are potent for TPH activity both in biochemical and cell-based assays. This class of compounds has unique properties with respect to their pharmacokinetic and pharmacodynamic effects on GI serotonin production. Similar to the *Tph1* knockout results, these TPH inhibitors have the ability to selectively reduce 5-HT levels in the murine GI tract without affecting brain 5-HT levels. In addition, administration of these compounds in a ferret model of chemotherapy-induced emesis caused modest reductions of intestinal serotonin levels as well as a decreased emetic response. These findings suggest that GI-specific TPH inhibitors may provide novel treatments for various gastrointestinal disorders associated with dysregulation of the GI serotonergic system, such as chemotherapy-induced emesis and irritable bowel syndrome (IBS).

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter with both synaptic and paracrine activities that modulates central and peripheral functions through action on neurons, smooth muscle, and other cell types. It is synthesized from tryptophan by the sequential actions of tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase. Only one form of TPH was known to exist until Walther *et al.* published a second distinct TPH-encoding gene (Walther et al., 2003a). The originally identified isoform was renamed TPH1 and the newly discovered isoform was designated TPH2. The two enzymes share an overall identity of ~70%, and are both ~50% identical to the other two members of the aromatic amino acid hydroxylase family, phenylalanine and tyrosine hydroxylases. *Tph1* is primarily expressed in the pineal gland and non-neuronal tissues, such as enterochromaffin (EC) cells of the gastrointestinal (GI) tract (Cote et al., 2003; Patel et al., 2004). On the other hand, *Tph2* is expressed exclusively in neuronal cells, such as the dorsal raphe and myenteric plexus (Cote et al., 2003; Walther et al., 2003a; Patel et al., 2004).

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 close to 90% of the body's 5-HT is synthesized and stored, 5-HT plays a prokinetic role in general and is an important mediator of sensation (e.g., nausea and satiety) between the GI tract and the brain (Gershon and Tack, 2007). 5-HT is released when EC cells are activated by

stimuli, such as distension or chemical signals; the resultant release of 5-HT increases motility of the gut. On the other hand, dysregulation of the GI 5-HT signaling system is involved in the etiology of several conditions such as functional gastrointestinal disorders, chemotherapy-induced emesis, and heart valve damage (Andrews et al., 1990; Kulke and Mayer, 1999; Gershon, 2003; Gershon, 2005; Gershon et al., 2005). 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 (De Ponti, 2004). Direct blockade of 5-HT synthesis through inhibition of TPH was also evaluated with a compound called para-chlorophenylalanine (pCPA) in man (Koe and Weissman, 1966; Engelman et al., 1967; Alfieri and Cubeddu, 1995). The compound proved effective in treating diarrhea in patients with carcinoid syndrome and emesis induced by chemotherapy (Engelman et al., 1967; Alfieri and Cubeddu, 1995). However, pCPA treatments have also been linked to depression and other alterations in CNS function (Engelman et al., 1967; Sjoerdsma et al., 1970), precluding the development of this agent for therapeutic use.

Based on the above observations, we wanted to determine if the specific loss of 5-HT from GI tissues would have an effect on emesis. We hypothesized that selective reduction of GI 5-HT synthesis could be achieved with inhibitors exhibiting either isoform selectivity or restricted tissue distribution; and that selectively targeted inhibition of TPH could potentially exert a therapeutic effect on emesis or other functional gastrointestinal disorders like irritable bowel syndrome (IBS) without exerting undesired CNS effects. We show here that mice deficient for *Tph1* lack 5-HT in the GI tract but have normal 5-HT levels in the brain. We also describe novel, potent TPH inhibitors that

deplete 5-HT in the GI tract while not affecting brain 5-HT levels. When administered to animals in a chemotherapy-induced emesis model, one of these TPH inhibitors was able to decrease the emetic response.

Methods

Generation of *Tph1 homozygous null (-/-)* mice: A *Tph1* targeting vector was constructed using the λ knockout shuttle (KOS) system (Wattler et al., 1999). A λ KOS clone (pKOS5) and target vector were isolated essentially as described previously (Salojin et al., 2006). This clone spanned ~9.2kb of the *Tph1* gene including exons 2-4. In the pKOS5-derived vector, exon 3 (~184bp) and surrounding DNA was replaced by the IRES/LacZ/Neo selection cassette (supplemental data Fig. 1A); such vector was linearized and electroporated into Lex-1 embryonic stem (ES) cells which were derived from 129S5/SvEvBrd mice (formerly designated as 129/SvEvBrd). G418/FIAU-resistant clones were selected and targeted clones were identified and confirmed by Southern analysis (supplemental data Fig. 1B). The sequence of primers used to generate 5' external probes (79/47) and 3' external probes (55/80) were: TPH1-79 (5'-GCTCTTCTAAAACGTCCAGTAG-3'), TPH1-47 (5'-GTCTGAGTAAGATTAAACAATCCG-3'), TPH1-55 (5'-GCATCTTCCCTTTATTCAACC-3'), TPH1-80 (5'-CTTCATGTGAACTGTACTCTTGG-3'). ES cells from two targeted clones were microinjected into (albino) C57BL/6J- $Tyr^{c\text{-}Brd}$ (Zheng et al., 1999) blastocysts to generate chimeric mice, which were then bred to C57BL/6J-$Tyr^{c\text{-}Brd}$ (albino) to allow germline transmission. Heterozygous mice were interbred to produce *Tph1*-deficient animals ($Tph1^{tm1Lex}$). Genotype analysis was performed by conducting quantitative PCR for the Neo gene on mouse tail DNA. *Tph1* wild-type (+/+), heterozygous (+/-), and homozygous null (-/-) mice contain zero, one, and two copies of the Neo gene,

respectively. Homozygous *Tph1* homozygous null mice were born with the expected Mendelian frequency, and exhibited normal growth with no obvious abnormalities.

Analysis of 5-HT and 5-HIAA production in mice: Wild-type, heterozygous, and homozygous null mice were generated in a 129S5/SvEvBrd and C57BL/6 hybrid background. For tissue collection, one-year-old female mice were anesthetized using isoflurane. Blood was obtained via cardiac puncture, after which animals were immediately euthanized. Blood was collected into a Capiject tube with dipotassium EDTA (Terumo Medical Corp, Elkton, MD). Whole brain, stomach antrum, proximal duodenum, mid jejunum, distal ileum, and proximal and distal halves of the colon were collected. With intestinal tissues, mesenteric fat was removed, the gut lumen was opened, the contents were removed, and the tissue was blotted dry. Tissue wet weight was recorded and samples were snap-frozen immediately thereafter in liquid nitrogen, and stored at -80^{o}C for subsequent 5-HT analysis.

For 24-hour urinary 5-HIAA and creatinine analysis, 14-month-old male mice were housed singly and acclimatized to metabolic cages (Nalgene Nunc International Corporation, Rochester, NY) for one week prior to urine collection. To avoid contamination of spilled food into urine, mice were fed on daily prepared chow paste (1 g regular chow powder: 1 ml H_2O) in a feeding cup. Urine samples were collected every 24 h for 4 consecutive days; urine volumes were recorded and the samples were analyzed for 5-HIAA and creatinine.

Quantification of 5-HT, 5-HIAA, and creatinine: Fresh blood was mixed with 9 volumes of lysis solution containing 0.5 M trichloroacetic acid and 0.05 M sodium

ascorbate. After rigorous mixing, the samples were filtered through a GF/B 96-well filter plate (Whatman Inc., New Jersey, USA) by centrifugation at 650 x g for 5 minutes at 4°C. The filtrates were analyzed for 5-HT by an HPLC fluorometric method under the following conditions: 1) column, Water Symmetry C18 (4.6 x 50 mm); 2) mobile phase, 97% 100 mM sodium acetate, 3% acetonitrile, pH 3.5; 3) detection, excitation wavelength = 280 nm, emission wavelength = 330 nm. For intestinal tissues, frozen samples were homogenized in 4 volumes of homogenization buffer consisting of 0.3 M trichloroacetic acid, 0.1 M sodium acetate, 10 mM EDTA, 20 mM sodium bisulfate. Brain tissues were homogenized in 1.75 volumes of homogenization buffer; homogenate was centrifuged at 20,000 x g for 20 minutes at 4°C, and supernatant was then filtered and 5-HT levels were determined as described above. For urinary 5-HIAA analysis, each sample was diluted 5-fold with a buffer containing 100 mM sodium acetate, pH 3.5, 1 M sodium ascorbate, and 0.5 M EDTA. The diluted samples were centrifuged at 13,000 x g for 5 minutes at room temperature and the supernatant was then filtered as described above and 5-HIAA levels were determined using the HPLC fluorometric method described above. Creatinine levels were determined using a commercial assay (Creatinine Plus ver.2, Roche Diagnostics, Indianapolis, IN, USA).

In vitro TPH1 activity assays: Human full-length TPH1 and TPH2 were cloned into the expression vector pET24 (Novagen), expressed in *Escherichia coli*, and the TPH1 and TPH2 proteins were purified to apparent homogeneity using pterin-based affinity chromatography. Activities of both enzymes were measured using the fluorescence assay as previously described (Moran and Fitzpatrick, 1999). The rat mastoctyoma cell

line RBL-2H3, which expresses *Tph1* endogenously was obtained from The American Type Culture Collection (ATCC). The cells were seeded at 7,000 cells/well in a 96-well plate in complete medium. Cells were treated with TPH1 inhibitors at various concentrations for 3 days. Following treatment, media was removed and the cells were lysed with 0.1 N NaOH; lysates were then filtered and analyzed for 5-HT as described above.

Treatment of mice with compounds: All mouse experimental procedures were reviewed and approved by the Lexicon Institutional Animal Care and Use Committee (IACUC). Generally, 14-week-old C57BL/6J- $Tyr^{c\text{-}Brd}$ (albino) male mice were used for all pharmacological experiments. Animals were housed in microisolator cages in a temperature andlight/dark cycle-controlled environment with access to standard chow diet and water *ad libitum*. In general, compounds were given at 5 ml/kg by oral gavage twice daily for a period of 3 days. Vehicle controls consisted of PEG400 + 5% dextrose (40:60 ratio) for LP-533401 treatments and 0.1% Tween® 20 for all other treatments. Animals were sacrificed 5 h following the last dose and tissues were collected for 5-HT analyses as described above.

Immunohistochemistry: Mice were euthanized by CO_2 and tissues were fixed by immersion in 10% neutral buffered formalin for 48 h. Tissues were embedded in paraffin, sectioned at 4 µm, mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), stained with hematoxylin and eosin (H&E), and mounted with Permount (Fisher Scientific, Pittsburgh, PA) for histopathologic

examination. For immunohistochemistry, sections were incubated with primary antibody (Rabbit anti-Serotonin at 1:4,000; Sigma-Aldrich #S5545, St. Louis, MO) for 2 h at room temperature followed by a rinse in phosphate buffered saline (PBS) before blocking of endogenous peroxidase with hydrogen peroxide in methanol. Sections were rinsed in PBS and then incubated with biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:400 in PBS. Afterwards, sections were incubated for 1 h with an avidin-biotin horseradish peroxidase complex (Vector Elite ABC, Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the chromogenic substrate (Vector DAB Substrate Kit, Vector Laboratories, Burlingame, CA) and sections were counterstained with Meyer's hematoxylin. Control sections were incubated with non-specific primary antibodies (affinity-purified rabbit IgG).

Emesis studies in ferret: The cisplatin-induced emesis study was conducted in the ferret as described previously (Gardner et al., 1995). The in-life portion of the study was performed by Porsolt & Partners Pharmacology (Z.A. des Suhards - B.P. 9, 53940 Le Genest-Saint-Isle, France). Male *Mustela putorius furo* ferrets were divided into five groups (n = 8 per group). Three groups of animals were treated with LP-615819 by oral gavage for 5 days, twice a day at 10, 30, and 90 mg/kg (20, 60, 180 mg/kg/day). The remaining two groups were treated with vehicle (2 ml/kg of 5% dextrose) in the same way. On Day 5, 60 min before administration of cisplatin, ferrets were placed in individual inox cages (40 x 50 x 34 cm) with a grid floor. LP-615819 was administered to the respective dosage groups. Within the two control groups, one group was given vehicle, and the other group was given ondansetron (16 mg/kg) orally. Animals were

then given cisplatin 60 min later by intraperitoneal administration at 10 mg/kg, and observed over a 3 hr period for emetic behavior as previously described (Gardner et al., 1995).

Data analysis: Data are presented as mean ± SEM. Differences between vehicle and compound-treated groups were analyzed by the unpaired Student's *t* test, or one-way ANOVA followed by Dunnett's multiple comparison test. Unless indicated otherwise, *P* values less than 0.05 were considered significant.

Results

Analysis of 5-HT and 5-HIAA levels in *Tph1* homozygous null mice

We first examined 5-HT levels in various tissues from *Tph1*-deficient female mice. In such mice, 5-HT levels are reduced by 94% in blood and approximately 99% throughout the gastrointestinal tract (Fig. 1A-B and Supplemental Data Table 1), demonstrating that TPH1 is responsible for essentially all 5-HT synthesis there. These observations are consistent with earlier findings (Cote et al., 2003; Walther et al., 2003a). Interestingly, in *Tph1* heterozygous mice, 5-HT levels are 32% lower in blood and 28-47% lower in small and large intestines (Fig. 1A-B and Supplemental Data Table 1), indicating that TPH1 is truly the rate-limiting enzyme in the synthesis of GI 5-HT. Furthermore, the loss of 5-HT in whole blood is nearly equivalent to that of the GI tract in both the homozygous and the heterozygous animals, consistent with the well-established observation that platelets, without expressing *Tph1* or *Tph2*, take up 5-HT synthesized by the GI tract.

In contrast, brain 5-HT and 5-HIAA levels from *Tph1* heterozygous and homozygous null mice showed no statistically significant difference from their wild-type littermates (Fig. 1C and Supplemental Data Table 1), which is also consistent with previously published data (Walther et al., 2003a). Results obtained from male mice are similar to those from the female mice (data not shown). The normal brain 5-HT and 5-HIAA levels, together with the preserved general health and gastrointestinal function of the

Tph1 knockout animals indicates that inhibition of non-neuronal TPH activity represents a potentially safe mechanism of action for drug discovery and development.

Since 5-HIAA is the major metabolite of 5-HT, we compared 24-hour urinary output of 5-HIAA in *Tph1* wild-type and homozygous null mice (Supplemental Data Table 2). Total daily 5-HIAA excretion are ~80% lower in *Tph1* homozygous null mice compared to that of wild-type mice in each of the study days (Fig. 1D and Supplemental Data Table 2); whereas daily total urinary creatinine excretion was similar between the two groups of animals (Fig. 1E and Supplemental Data Table 2). After normalization to creatinine output, *Tph1*-deficient mice still showed ~ 80% reduction in daily 5-HIAA excretion when compared to their wild-type littermates (Fig.1F and Supplemental Data Table 2). These data demonstrate that approximately 80% of daily urinary 5-HIAA is derived from the metabolism of non-neuronal 5-HT sources. Brain 5-HT, only accounting for ~2% of the body's total amount in storage in the mouse, appears to be responsible for ~20% of the metabolism, implying a much higher turnover rate in the brain.

Identification of novel TPH inhibitors

In an attempt to discover and develop compounds that can inhibit 5-HT synthesis in the gut, we set out to identify novel TPH inhibitors. A high-throughput screen using purified TPH1 was conducted with a library of approximately 200,000 compounds using radiolabeled tryptophan. Several classes of inhibitors were identified and medicinal chemistry was applied to improve the potency of the compounds (manuscript in

preparation). One of the compounds, LP-533401 (Fig. 2A), was found to inhibit human TPH1 *in vitro* with an IC_{50} (concentration causing half of maximum inhibition) of 0.7 μM (Fig. 3A). It also inhibited purified, recombinant human TPH2 with similar potency (Fig. 3B). In comparison, para-chlorophenylalanine (pCPA) is a much less potent inhibitor with an IC_{50} of approximately 250 μM in the same assay (Fig. 3A). These compounds were also tested in a cell-based assay using the rat mastocytoma cell line RBL-2H3 which expresses *Tph1* endogenously. LP-533401 was found to completely inhibit 5-HT production in these cells with an IC_{50} of 0.4 μM (Fig. 3B). pCPA, as expected, showed a significantly less potent IC_{50} of 43 μM (Fig. 3C). To improve the efficacy of the compound *in vivo*, a prodrug was synthesized as an ethyl ester of LP-533401 and designated LP-615819 (Fig. 2B). The prodrug did not inhibit TPH1 *in vitro* at concentrations up to 40 μM (Fig. 3A). However, in the rat cell line, LP-615819 had about the same potency as LP-533401 (Fig. 3B), indicating that the prodrug had undergone conversion to the active compound in either cells, or cell culture media, or both. Since they have much improved *in vitro* potency, these compounds were tested in the mouse for 5-HT lowering effects.

Depletion of gastrointestinal 5-HT by novel TPH inhibitors

We examined *in vivo* activities of LP-533401 and LP-615819 by administering the compounds into mice through oral gavage for three-four days and then measuring the levels of 5-HT in the GI and brain. The employment of multiple day dosing was based on previous reports of slow 5-HT turnover in the GI of the rat following pCPA administration (Koe and Weissman, 1966; Weber, 1969). In one of the studies, LP-

533401 was given to mice at either 30 or 90 mg/kg, twice daily, while another group was given vehicle only, also twice daily. After six consecutive doses, 5-HT levels in the small intestine (jejunum and ileum) were significantly lower in the LP-533401-treated animals, averaging ~55% and ~70% reductions at 30 and 90 mg/kg, respectively (Fig. 4A and Supplemental Data Table 3). 5-HT levels in the colon were also reduced, averaging ~24% and ~36% reductions at 30 and 90 mg/kg, respectively. In contrast, no significant changes in brain 5-HT levels were observed at either dosage.

Since a prior TPH1 inhibitor, pCPA, was shown to lower both intestinal and brain 5-HT with stronger efficacy in the latter, we compared LP-615819, the prodrug of LP-533401, and pCPA side-by-side in the mouse. Animals were treated twice daily with LP-615819 at 45 mg/kg or pCPA at 90 mg/kg. After three days, both compounds had caused reduction of 5-HT levels in the intestine, with LP-533401 showing greater efficacy in the jejunum (Fig. 4B and Supplemental Data Table 4). More importantly, pCPA, as expected, significantly lowered 5-HT levels in the brain while LP-615819 caused no such effect. In a follow-up study, LP-615819 was given to mice twice daily at three different dose levels (20, 45, 90 mg/kg) for three days. Treatment with LP-615819 caused robust reduction of 5-HT along the entire gastrointestinal tract in a dose-dependent fashion (Fig. 5 and Supplemental Data Table 5). The best potency and efficacy with respect to 5-HT reduction was observed in the small intestine, including duodenum, jejunum, and ileum, while the least was seen in the stomach antrum (Fig. 5). Importantly, the compound did not reduce brain 5-HT levels significantly, even at the highest dose tested.

To confirm the observed near complete depletion of 5-HT in the intestine by LP-615819, immunohistochemistry studies were performed with anti-serotonin antibodies. Mice were given LP-615819 at 300 mg/kg, twice daily for four days. Intestinal sections from control animals showed a significant number of cells containing strong serotonin-immunoreactivity (Fig. 6A, C, E, G). In contrast, sections from LP-615819-treated animals showed only weak serotonin immunoreactivity in a few or no cells (Fig. 6B, D, F, H). Taken together these data clearly demonstrate that LP-615819 is able to deplete intestinal 5-HT almost completely without affecting brain 5-HT levels significantly.

Alleviation of emesis by peripheral TPH inhibition

5-HT is known to play a critical role in chemotherapy-induced emesis and various serotonin receptor 3 (5-HT3) antagonists are being used successfully in the clinic to prevent chemotherapy-induced nausea and vomiting (Schwartzberg, 2007). Previously, pCPA has been shown to reduce cisplatin-induced emesis in a ferret model as well as in human (Engelman et al., 1967; Alfieri and Cubeddu, 1995; Rudd et al., 1998). However, pCPA also depletes brain 5-HT, making it difficult to determine if the antiemetic effects are centrally or peripherally mediated. Therefore, we tested if the specific loss of 5-HT from GI tissues would prevent cisplatin-induced emesis. The use of *Tph1* knockout mice was precluded for such studies because mice do not exhibit an emetic response when challenged with cisplatin or other chemotherapeutic agents. Consequently, we tested LP-615819 in the commonly used ferret model of cisplatin-induced emesis. Ferrets were treated for five days with LP-615819 followed by cisplatin challenge. Emetic responses were monitored and compared to animals treated with vehicle or ondansetron (positive

control). Treatment with LP-615819 led to about a 50% reduction in the number of retches, vomits, and emesis periods at all three dosages (Fig. 7A , C, and Supplemental Data Table 6), but the observed differences did not reach statistical significance, likely because of large variances. However, when the data from all three LP-615819-treated groups were combined, the reduction in the number of retches and vomits was found to be statistically significant when compared to animals receiving vehicle only (Fig. 7B and Supplemental Data Table 6). At the end of the study, 5-HT levels were measured in the duodenum, jejunum, and brain. As shown in Figure 7D-F, LP-615819 lowered 5-HT levels by about 30% in the duodenum and jejunum at all three dosages but did not change brain 5-HT levels noticeably. As expected, ondansetron treatment did not alter intestinal 5-HT levels significantly.

Discussion

Knockout of *Tph1* in the mouse demonstrates that the vast majority of 5-HT synthesis in the GI tract is carried out by TPH1 (Cote et al., 2003; Walther et al., 2003b). Even though enteric neurons are known to express *Tph2* (Cote et al., 2003), they apparently contribute to only approximately 1% of total 5-HT content in the GI tract. In the work described here, we compared 5-HT synthesis and 5-HIAA excretion among *Tph1* wild-type, heterozygous, and homozygous null mice. Our data are consistent with previous conclusions that deletion of *Tph1* leads to ~99% loss of 5-HT in the GI tract and 94% reduction in the blood. Furthermore, the significant reduction of 5-HT levels in both the blood and gut of *Tph1* heterozygotes indicates that the 5-HT synthesis in the GI tract is influenced by *Tph1* copy number.

We have discovered and characterized a series of novel compounds that inhibit both TPH1 and TPH2 in vitro but selectively lower 5-HT levels in the GI tract. TPH is an intracellular enzyme; therefore, effective inhibitors must cross the plasma membranes to reduce 5-HT synthesis *in vivo*. pCPA displayed an IC_{50} or Ki of roughly 250 μM against purified, recombinant human TPH1 (Ki is approximately equal to IC_{50} here, since the tryptophan concentration was significantly below its Km in the assay). In the cell-based assay, pCPA showed an IC_{50} of 43 μM, indicating that it was taken up by these cells quite efficiently. LP-533401 showed a Ki of 0.7 μM against purified TPH1 and an IC_{50} of 0.3 μM with RBL-2H3 cells. Both pCPA and LP-533401 (disclosed here) are phenylalanine-based zwitterions, neither of which is expected to be able to diffuse passively across the

plasma membrane. We speculate that the two compounds enter the cells through a cognate transporter. The most likely candidates are amino acid transporters located in the plasma membrane. The exact mechanism of how pCPA and LP-533401 cross the cellular membrane, however, remains to be determined.

In contrast to pCPA, LP-533401 and LP-615819 do not lower 5-HT levels in the brain when administered orally. Since LP-533401 inhibits TPH2 as potently as it does to TPH1 (Fig. 2B), the most likely explanation for its lack of effect on central 5-HT is its inability to cross the blood-brain barrier. Preliminary experiments found that the concentration of LP-533401 in the brain is approximately 1% of that in the plasma after dosing at 10 mg/kg by oral gavage (6.1 nM in the brain *versus* 485 nM in the plasma at 2 hr post administration, manuscript in preparation), indicating that LP-78902 is unable to cross the blood brain barrier. Furthermore, we examined the effect of LP-533401 on brain 5-HT following intraperitoneal administration of this compound for 5 days at 100 mg/kg, twice daily, and found that brain 5-HT levels were not altered (vehicle group = 0.66 ± 0.03 ug/g; LP-533401 group = 0.67 ± 0.03 ug/g; $P > 0.05$, T Test). pCPA, which can deplete brain 5-HT quickly and robustly, apparently enters the brain by an unidentified transporter. While the exact mechanism of the observed differential tissue activity remains to be elucidated, we speculate that the greater molecular weight of LP-533401, as compared to pCPA (526.5 D *vs*. 199.6 D), may have prevented its uptake by such a hypothetical transporter.

5-HT released by EC cells following treatment with chemotherapeutic agents is one of the major factors responsible for nausea and vomiting in patients receiving such medications (Andrews et al., 1990). Even though 5-HT3 antagonists are effective in controlling acute nausea and vomiting in the majority of patients, delayed-onset nausea and vomiting continues to be a significant issue for patients receiving certain types of chemotherapy (Gregory and Ettinger, 1998; Lindley and Blower, 2000; Schwartzberg, 2007). TPH1 inhibitors alone or in combination with 5-HT3 antagonists may further increase the effectiveness in the control of nausea and vomiting. Consistent with this, pCPA has been tested in a small number of patients for such a purpose and found to be equally effective as the 5-HT3 antagonist ondansetron (Alfieri and Cubeddu, 1995). We tested LP-615819 in the ferret emesis model and found that it was able to partially reduce emesis induced by cisplatin. The limited efficacy may be due to the relatively modest (~30%) reduction in 5-HT levels observed in the duodenum and jejunum at all three dosages. It was unclear why all three dosages gave nearly the same level of 5-HT reduction. One potential explanation is that 5-HT turnover in the ferret intestine is very slow and, therefore, the maximum effect was achieved at the lowest dosage. Another possibility is that LP-615819 had a short-lasting effect and 5-HT turnover in the ferret is fast enough so that 5-HT in all the groups have rebounded to approximately the same level. Nevertheless, the fact that a similar degree of anti-emetic effect was observed at all three dosages is consistent with the corresponding equivalent loss of 5-HT at all dosage levels. Taken together, these data imply that the modest reduction of 5-HT in the upper intestine can lead to alleviation of emesis in the ferret. It is still interesting, however, given the abundance of 5-HT in the small intestine, that a partial antiemetic effect was

observed with a limited (30%) reduction of 5-HT. One potential explanation is that the large initial abundance of 5-HT in the GI tract may be masking any observed reduction, caused by LP-615819, in the more physiologically important 5-HT efflux. Nonetheless, our finding that a modest reduction in intestinal 5-HT reduces emesis is cause for further investigations of GI-specific TPH inhibitors for the treatment of nausea and vomiting induced by chemotherapy. Improved efficacy in emesis reduction, however, will be required for clinical use.

In the periphery, 5-HT participates in several key processes, including vasoconstriction, thrombosis, GI motility, secretion, and nociception. The dysregulation of 5-HT is associated with various maladies, as manifested by the significant number of therapeutic agents targeting the serotonergic system. However, direct inhibition of TPH enzymes has never been seriously explored as a therapeutic approach, due in part to the importance of 5-HT in the brain and the challenges associated with selective reduction of 5-HT outside the brain. The discovery of two distinct genes, TPH1 and TPH2, responsible for the synthesis of non-neuronal and neuronal 5-HT, respectively, offers the possibility of developing drugs that specifically inhibit one enzyme or the other. The generation of such specific inhibitors will be challenging, however, because TPH1 and TPH2 are 71% identical in amino acid sequence, and about 90% similar in the catalytic domain. The design and synthesis of compounds that can not cross the blood brain barrier may offer the best approach in this case.

In summary, we have discovered and characterized two compounds (LP-533401 and LP-615819) that selectively inhibit gastrointestinal 5-HT biosynthesis in animals, leaving brain 5-HT levels unaffected. This tissue-selective profile is likely due to the inability of these compounds to cross the blood-brain barrier, resulting in a restricted tissue distribution *in vivo*. Moreover, these compounds reduce emesis in a model system. We propose that specific inhibition of gastrointestinal 5-HT will offer therapeutic avenues for a multitude of diseases linked to the dysregulation of peripheral serotonergic pathways including gastrointestinal diseases such as irritable bowel syndrome.

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Legends for Figures

Fig. 1. Comparison of 5-HT levels in various tissues, and daily 5-HIAA and creatinine excretion in the urine among *Tph1* wild-type (+/+), heterozygous (+/-), and homozygous null (-/-) female mice. Error bars are SEM A-C: 5-HT levels in whole blood (A), jejunum (B), and brain (C). The number of animals was: wild-type (+/+) (n=10), heterozygous (+/-) (n=11), homozygous null (-/-) (n=4). *P<0.01 vs. control (Dunnett's post test following one-way ANOVA analysis). D-F: 24-hour urinary excretion of 5-HIAA (D), creatinine (E), ratio of 5-HIAA over creatinine (F). ** P <0.001 vs. control (unpaired student t test analysis on each day).

Fig. 2. Structure of LP-533401 and LP-615819. A. LP-533401; B. LP-615819.

Fig. 3. Inhibition of TPH1 and TPH2 activity in enzyme and cell-based assays. A. Inhibition of purified human TPH1. Open circle (○), pCPA; solid circle (●), LP-533401; open square (□), LP-615819. B. Inhibition of purified TPH1 and TPH2 by LP-533401. Solid circle (●), TPH1; open circle (○), TPH2. C. Inhibition of 5-HT production in RBL-2H3 cells. Open circle (○), pCPA; solid circle (●), LP-533401; open square (□), LP-615819. All error bars are SEM.

Fig. 4. Reduction of 5-HT in the intestine and brain of mice following compound treatment. A. Effect of vehicle control (open bars) *vs* LP-533401 at 30 mg/kg (grey bars) and 90 mg/kg (solid bars). B. Effect of vehicle control (open bars) *vs* pCPA (grey bars) and LP-615819

(solid bars). Data are presented as the mean % normalized to vehicle control. Error bars are SEM (n = 5 per group).

Fig. 5. Dose-dependent 5-HT reduction in the gastrointestinal tract by LP-615819 in the mouse. Open square (□), brain; open up triangle(△), stomach antrum; open down triangle(▽), colon; open circle (○), ileum; solid down triangle(▼), duodenum; solid circle (●), jejunum. Data are presented as the mean % normalized to vehicle control. Error bars are SEM (n = 5 per group).

Fig. 6. Immunohistochemistry analysis of intestinal 5-HT from untreated and LP-615819-treated animals. Serotonin-immunoreactive cells are stained brown. Control duodenum (A), jejunum (C), cecum (E), and colon (F). LP-615819-dosed duodenum (B), jejunum (D), cecum (G), and colon (H).

Fig. 7. Effect of LP-615819 on cisplatin-induced emesis and intestinal 5-HT levels in the ferret. A. number of retches and vomits. B. Combined number of retches and vomits from all LP-615819-treated animals. C. number of emesis periods. D. 5-HT in duodenum. E. 5-HT in jejunum. F. 5-HT in brain. Data are presented as the mean % normalized to vehicle control. Error bars are SEM (n = 8 per dosage group). * $P < 0.05$, ** $P < 0.01$ vs vehicle control (Dunnett test following one-way ANOVA).

Figure 1

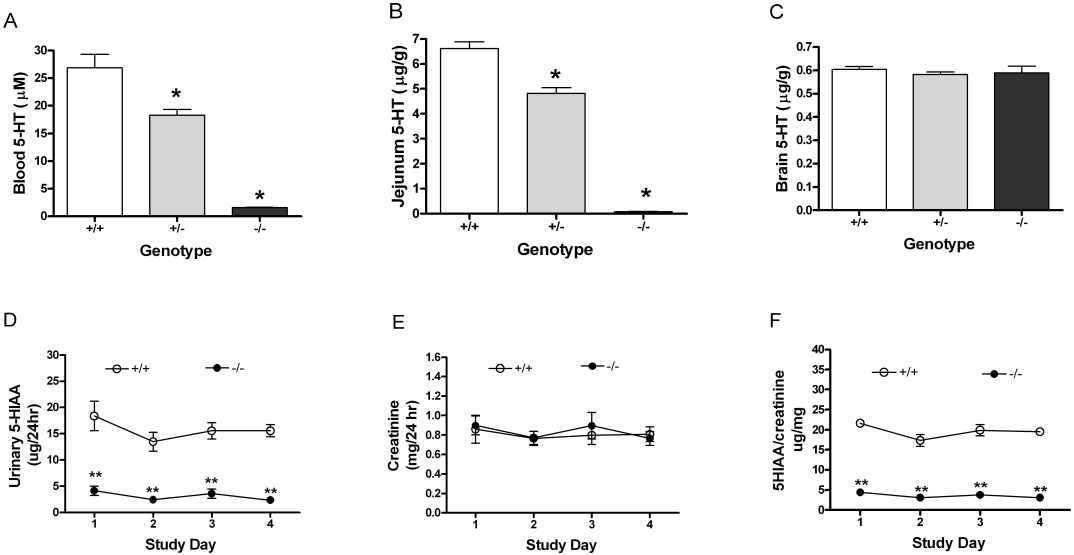
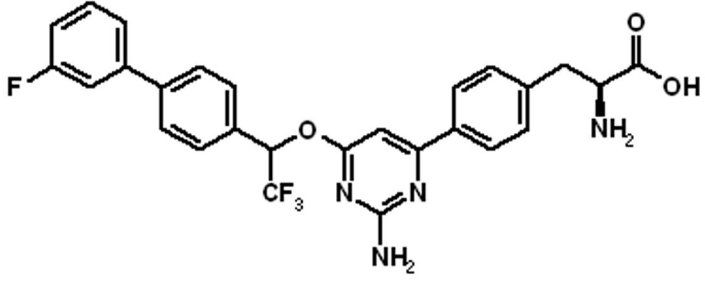

A
Blood 5-HT (μM)
30
25
20
15
10
5
0
*
*
+/+
+/-
-/-
Genotype
B
Jejunum 5-HT (μg/g)
7
6
5
4
3
2
1
0
*
*
+/+
+/-
-/-
Genotype
C
Brain 5-HT (μg/g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
+/+
+/-
-/-
Genotype
D
Urinary 5-HIAA (ug/24hr)
30
25
20
15
10
5
0
+/+
-/-
**
**
**
**
1
2
3
4
Study Day
E
Creatinine (mg/24 hr)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+/+
-/-
1
2
3
4
Study Day
F
5HIAA/creatinine ug/mg
40
35
30
25
20
15
10
5
0
+/+
-/-
**
**
**
**
1
2
3
4
Study Day

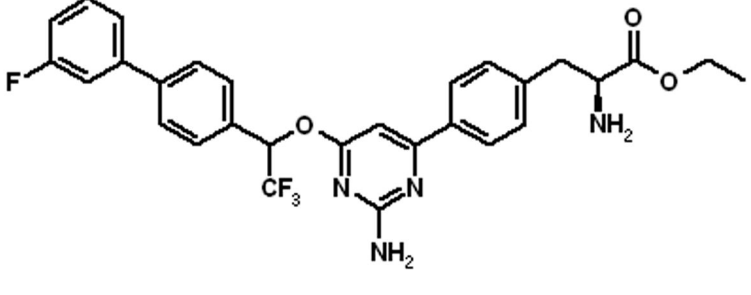
Figure 2

A.



LP-533401

B.



LP-615819

Figure 3

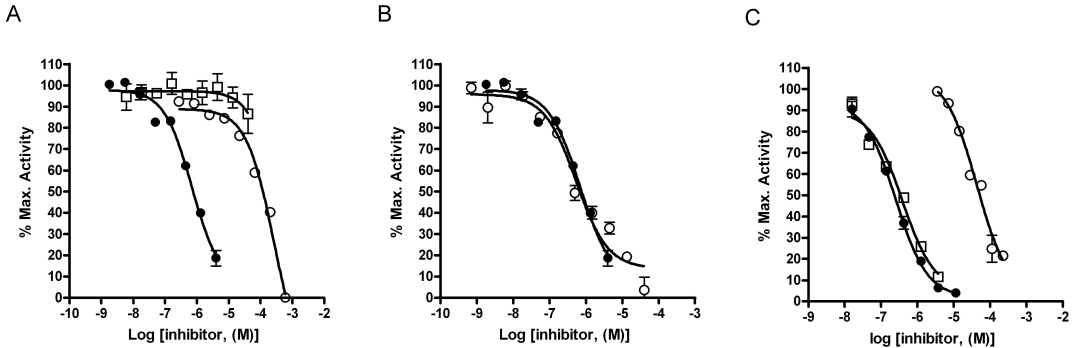
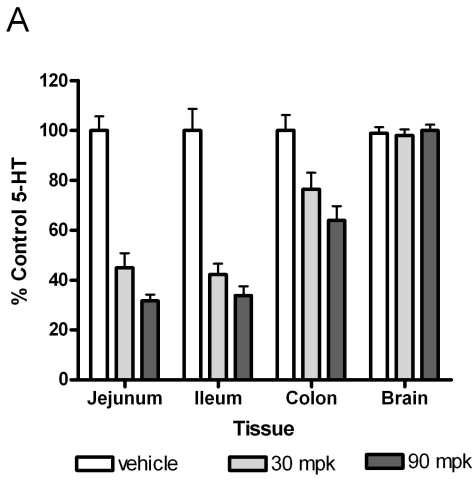


Figure 4


A
120
100
80
60
40
20
0
% Control 5-HT
Jejunum
Ileum
Colon
Brain
Tissue
vehicle
30 mpk
90 mpk

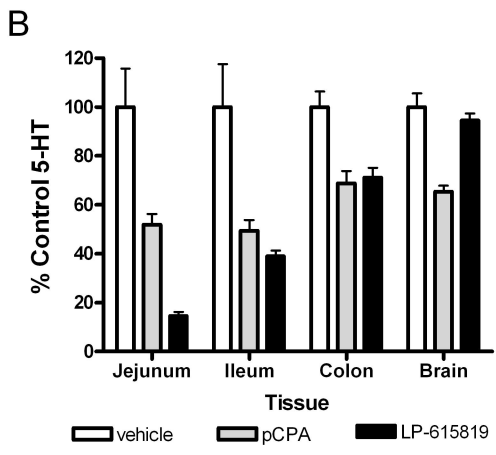

B
120
100
80
60
40
20
0
% Control 5-HT
Jejunum
Ileum
Colon
Brain
Tissue
vehicle
pCPA
LP-615819

Figure 5

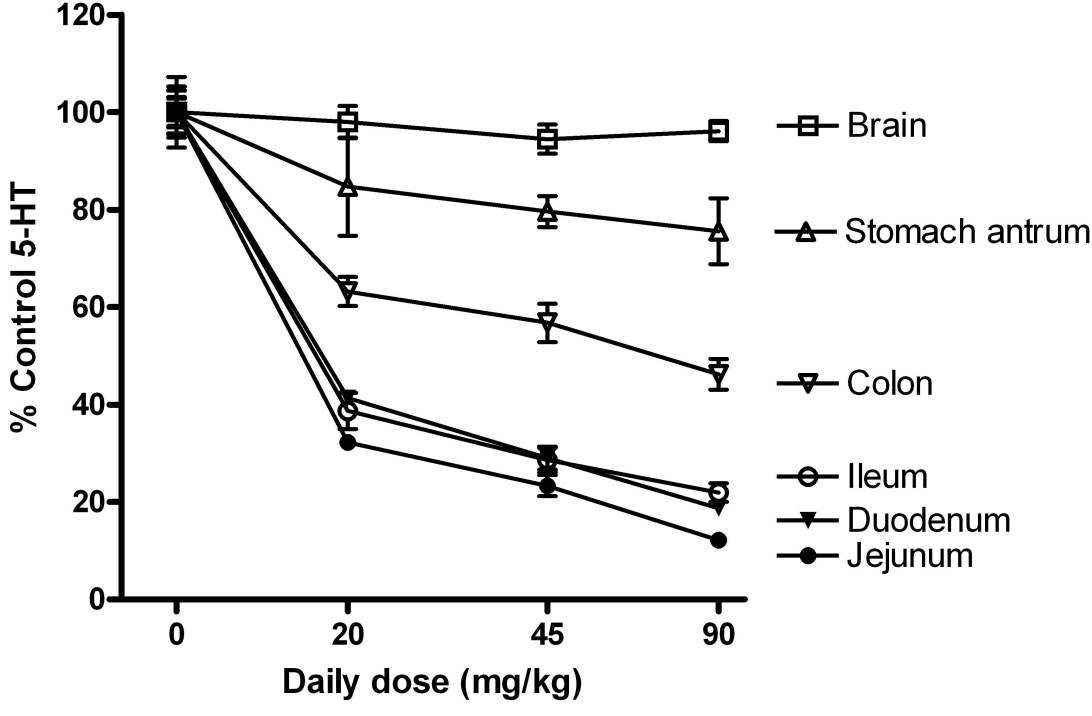

120
100
80
60
40
20
0
% Control 5-HT
0
20
45
90
Daily dose (mg/kg)
Brain
Stomach antrum
Colon
Ileum
Duodenum
Jejunum

Figure 6

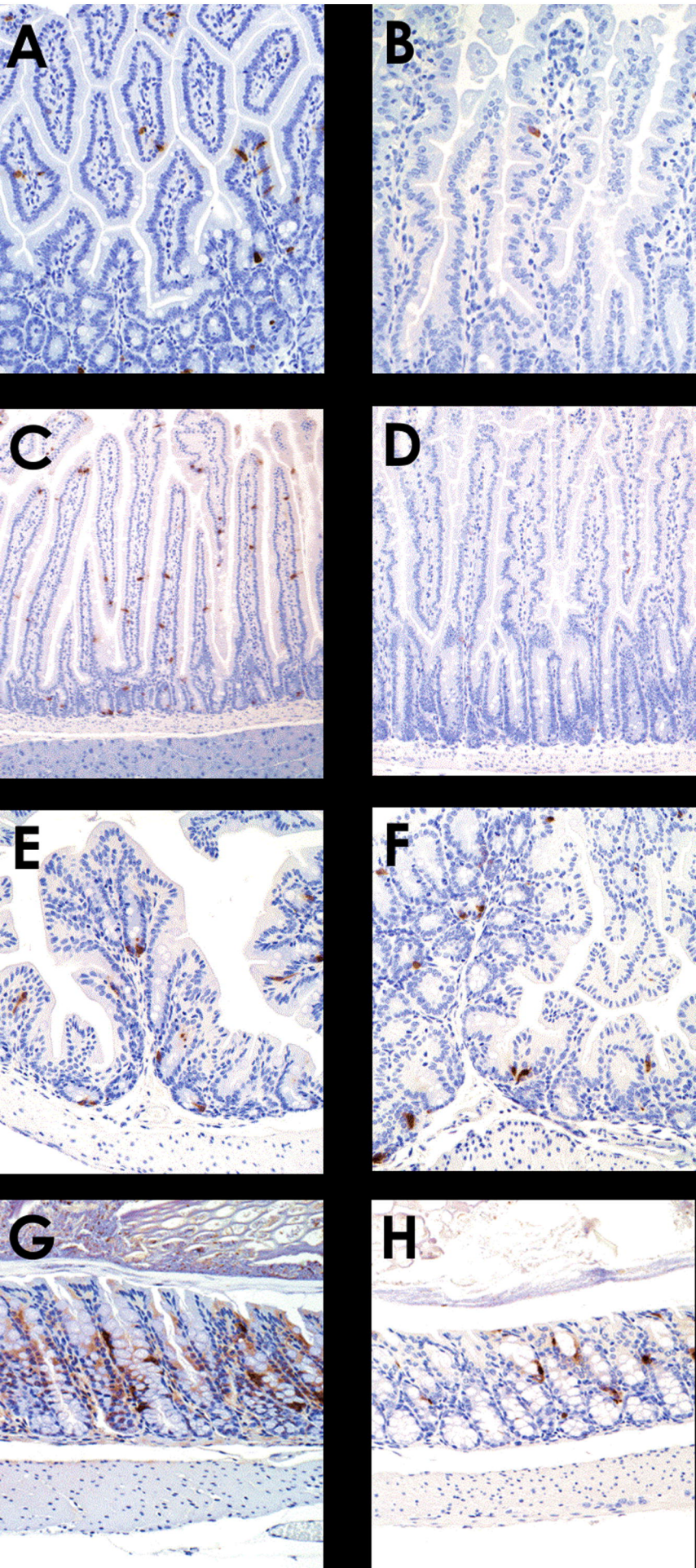
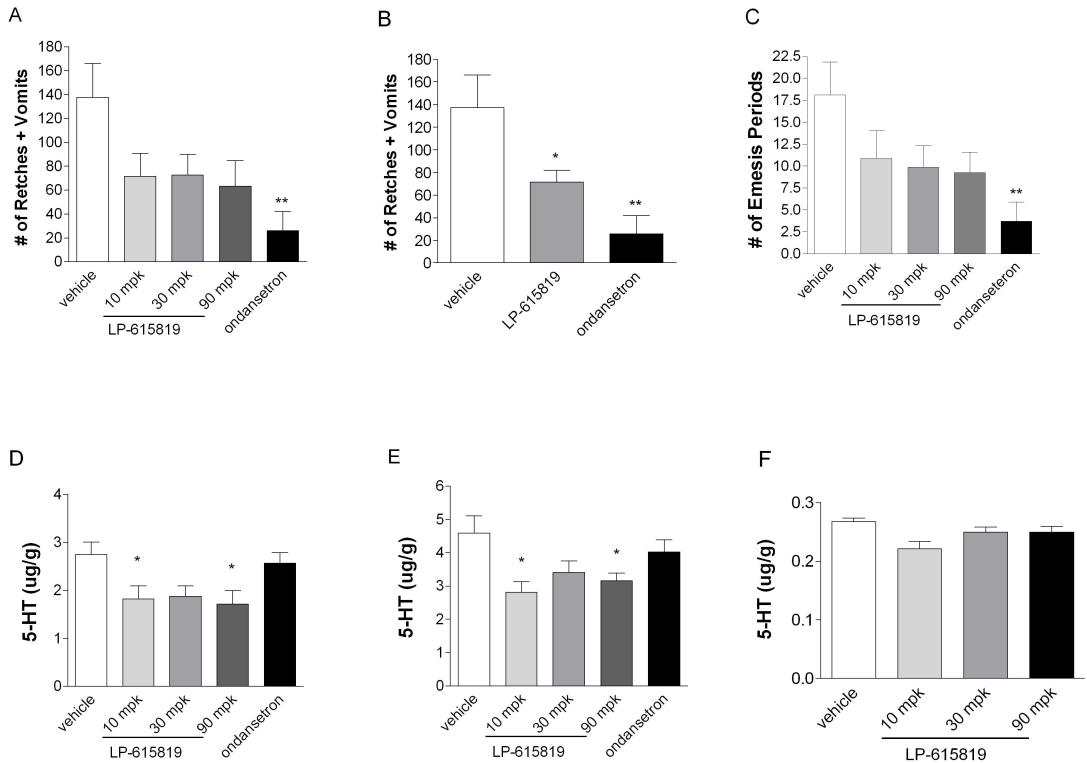


Figure 7


A
# of Retches + Vomits
vehicle
10 mpk
30 mpk
90 mpk
ondansetron
LP-615819
B
# of Retches + Vomits
vehicle
LP-615819
ondansetron
C
# of Emesis Periods
vehicle
10 mpk
30 mpk
90 mpk
ondanseteron
LP-615819
D
5-HT (ug/g)
vehicle
10 mpk
30 mpk
90 mpk
ondansetron
LP-615819
E
5-HT (ug/g)
vehicle
10 mpk
30 mpk
90 mpk
ondansetron
LP-615819
F
5-HT (ug/g)
vehicle
10 mpk
30 mpk
90 mpk
LP-615819