CSTI-300 (SMP-100); a Novel 5-HT₃ Receptor Partial Agonist with Potential to Treat Patients with Irritable Bowel Syndrome or Carcinoid Syndrome

Alexander Roberts, Gillian Grafton, Andrew D. Powell, Kristian Brock, Chunlin Chen, Dejian Xie, Jinkun Huang, Shuang Liu, Alison J. Cooper, Catherine A. Brady, Omar Qureshi, Zania Stamatakis, David D. Manning, Nicholas A. Moore, Bruce J. Sargent, Peter R. Guzzo, and Nicholas M. Barnes

Neuropharmacology Research Group, Institute of Clinical Sciences (A.R., G.G., A.J.C., C.A.B., O.Q., N.M.B.) and Institute of Immunology and Immunotherapy (Z.S.), College of Medical and Dental Sciences and DB – Diagnostics, Drugs, Devices and Biomarkers, Cancer Research UK Clinical Trials Unit (K.B.), University of Birmingham, Edgbaston, Birmingham, United Kingdom; Department of Life Science, School of Health Sciences, Birmingham City University, Birmingham, United Kingdom (A.D.P.); Shanghai Medicilon Inc., Shanghai, China (C.C.); Chengdu SciMount Pharmatech Co. Ltd., Chengdu, China (D.X., J.H.); ConSynance Therapeutics, Inc., Rensselaer, New York (S.L., P.R.G.); Albany Molecular Research, Inc., Albany, New York (D.D.M., N.A.M.); and Sargent Consulting, Hendersonville, North Carolina (B.J.S.)

ABSTRACT

The 5-hydroxytryptamine (5-HT) (serotonin) 5-HT₃ receptor represents a clinical target for antagonists to deliver symptomatic relief to patients with diarrhea-predominant irritable bowel syndrome (IBS-d) or carcinoid syndrome. Unfortunately, this pharmacological strategy can present side effects (e.g., severe constipation). The present study investigates the potential of a novel 5-HT₃ receptor partial agonist, CSTI-300, to treat patients with IBS-d and other conditions associated with discomfort from colonic distension, with a predicted reduced side-effect profile. The in vitro and in vivo preclinical pharmacology of the drug CSTI-300 was investigated to explore the potential to treat patients with IBS-d. CSTI-300 displayed selective high affinity for the human and rat 5-HT₃ receptor (Kᵢ approximately 2.0 nM) and acted as a partial agonist (approximately 30%–50% intrinsic efficacy) in vitro. In an in vivo model of IBS-d, the rat colon distension model, CSTI-300 displayed dose-dependent efficacy. In addition, oral administration of CSTI-300 to dogs that achieved plasma levels of the drug exceeding the Kᵢ value for the 5-HT₃ receptor failed to either evoke emesis or alter the state of feces. Pharmacokinetics for CSTI-300 in rat and dog identified high levels of oral availability with t½ range of 1.6–4.4 hours. The preclinical pharmacology of the lead candidate drug, CSTI-300, supports the potential of this novel drug to offer symptomatic relief to patients with irritable bowel syndrome and carcinoid syndrome with a rationale for a reduced “on-target” side-effect profile relative to 5-HT₃ receptor antagonists, such as alosetron.

SIGNIFICANCE STATEMENT

There is a lack of effective current treatment for diarrhea-predominant irritable bowel syndrome and carcinoid syndrome, and in both conditions, overactivity of the 5-hydroxytryptamine (5-HT) 5-HT₃ receptor is thought to be implicated in the pathophysiology. Because 5-HT₃ receptor blockade with antagonists results in significant side effects, we present evidence that treatment with a suitable 5-HT₃ receptor partial agonist will alleviate some symptoms associated with these conditions yet, without fully inhibiting the receptor, predict a less pronounced side-effect profile associated with this therapeutic strategy.

Introduction

Irritable bowel syndrome (IBS) is a defined functional bowel disorder (Rome IV criteria; e.g., Drossman, 2016; Drossman et al., 2014) that is often debilitating and sometimes presents severe abdominal pain (Lacy and Moreau, 2016) with colonic distension. Prevalence is around 10% to 11%, with a female:male ratio of 2:1 (Longstreth et al., 2006; Pleis and Lucas, 2009; Eck et al., 2016; Quigley et al., 2016). With comorbidities, including anxiety/depression (e.g., Fond et al., 2014), IBS contributes a considerable burden upon the healthcare system and loss of societal productivity (annual societal costs above $21 billion in the United States; https://www.aboutibs.org/facts-about-ibs/statistics.html; Ashburn and Gupta, 2006; Buono et al., 2017). IBS is subcategorized into four forms based on presenting clinical symptoms (Eck et al., 2016): diarrhea-predominant (IBS-d), constipation-predominant (IBS-c), alternating symptoms and Hasler, 2016) that is often debilitating and sometimes presents severe abdominal pain (Lacy and Moreau, 2016) with colonic distension. Prevalence is around 10% to 11%, with a female:male ratio of 2:1.
between diarrhea and constipation (IBS-mixed), and untyped IBS. IBS-d represents approximately one-third of the IBS patient population (Pleis and Lucas, 2009; Quigley et al., 2016).

Despite the substantial clinical need, the market for effective therapies for IBS is considered naïve and underpenetrated. There are no pharmaceuticals specifically indicated for IBS-mixed and just two products with IBS-c as an indication that often deliver insufficient benefit and/or are prone to side effects and are hence considered second-line treatments subsequent to failure of laxatives/diet adjustments, which themselves have very limited success (Camilleri and Boeckxstaens, 2017; Craig, 2018).

There is no established well-tolerated treatment of IBS-d, and common remedies (diet adjustments and fiber regimens) have very limited success. Within the United States, Allergan’s opiate receptor agonist, eluxadoline (Viberzi), was introduced, recently facilitated by Food and Drug Administration (FDA) “Fast Track” status, yet displays limited efficacy and is associated with common side effects, such as constipation and nausea as well as a report of ischemic colitis (Lacy and Moreau, 2016; Fragkos, 2017); moreover, some patients are at risk of developing pancreatitis (Camilleri, 2017), resulting in a warning from the FDA (https://www.fda.gov/Drugs/DrugSafety/ucm546154.htm). Another drug, the opioid receptor agonist ORP-101, also received “Fast Track” status (http://www.orphomed.com/newsroom/press-releases/2018-04-25/), demonstrating the continued recognition of the unmet therapeutic need.

It has been known for over 2 decades that 5-HT3 receptor antagonists, such as alosetron, deliver strong clinical efficacy to patients with IBS-d (e.g., Crowell, 2004), presumably by blocking elevated 5-HT function in these patients (Bearcroft et al., 1998; Dunlop et al., 2005; Enck et al., 2016; Fu et al., 2019; Gunn et al., 2019) that may arise from lower expression of the 5-HT transporter (Faure et al., 2010) to decrease gut motility and visceral sensation (e.g., Michel et al., 2005; Kapeller et al., 2011). Furthermore, increased expression of gut 5-HT3 receptor by patients with IBS-d (Gunn et al., 2019) and associated 5-HT3 receptor subunit polymorphisms (Kapeller et al., 2008, 2009; Kilpatrick et al., 2011; Gunn et al., 2019) support therapeutic targeting of this receptor.

However, patients with IBS-d receiving 5-HT3 receptor antagonists often report (potentially severe) constipation, and rare instances of ischemic colitis are evident (around 1:750 patients; see Zheng et al., 2017), which led to the withdrawal of the marketing authorization for alosetron by the FDA and may have contributed to the decision to stop clinical development of cilansetron (issues of severe constipation and ischemic colitis were also evident; Chey and Cash, 2005). Unusually, the FDA subsequently reinstated the marketing authorization for alosetron, albeit initially with a “black box” warning. Upon reflection, it would appear that high levels of 5-HT3 receptor inhibition are likely responsible for the severe constipation and possibly ischemic colitis. However, the latter side effect is likely a consequence of a multifactorial mechanism that includes the presence of IBS-d itself, since unmedicated patients can present this symptom and non-IBS-d patients receiving 5-HT3 receptor antagonists for other indications (e.g., emesis), including at relatively high dosage, do not present ischemic colitis (Tricco et al., 2016).

Knowledge of the pathophysiology of IBS-d forward an opportunity of a pharmacological mechanism predicted to deliver clinical efficacy with a reduced side-effect profile. Thus, a selective 5-HT3 receptor partial agonist would reduce receptor activity evoked by endogenous 5-HT yet would prevent complete receptor inhibition reducing the likelihood of constipation and perhaps ischemic colitis. A similar rationale is relevant to offer symptomatic relief to patients with carcinoid syndrome, which is associated with copious quantities of circulating 5-HT with arising chronic diarrhea (e.g., Davis et al., 1973; Kvlbs, 1994; Halperin et al., 2017) that can be controlled by the “off-label” use of 5-HT3 receptor antagonists (Anderson et al., 1987; Jacobsen, 1992; Platt et al., 1992; Schworer et al., 1995; Wymenga et al., 1998; Kiesewetter and Raderer, 2013; Halperin et al., 2017). The present report describes the preclinical pharmacology of the lead candidate novel 5-HT3 receptor partial agonist, CSTI-300 (Fig. 1), and the potential of the drug to treat some of the symptoms of IBS-d and carcinoid syndrome with a predicted reduced side-effect profile relative to 5-HT3 receptor antagonists like alosetron.

Materials and Methods

Nomenclature. The 5-HT3 receptor nomenclature conforms to international guidelines (Alexander et al., 2015).

Cell Culture

Human embryonic kidney 293 (HEK293) cells stably expressing the human functional 5-HT3A receptor (HEK293−h5-HT3A cells, Dubin et al., 1999; Brady et al., 2001) were cultured in Dulbecco’s modified Eagle’s medium [supplemented with 10% (v/v) FBS, 1.0% (v/v) penicillin/streptomycin (100 U/ml penicillin and 0.1 mg/ml streptomycin), and the selection antibiotic, G418 (250 µg/ml)] and maintained at 37°C, 5% CO2, and 95% air at 95% relative humidity. The same conditions were used for cells stably expressing the human 5-HT3AB receptor (HEK293−h5-HT3AB), except the media was also supplemented with Zeocin (80 µg/ml) as well as G418 (Brady et al., 2001). HEK293 cells stably expressing the rat functional 5-HT3A receptor (HEK293−r5-HT3A) were cultured the same as for HEK293−hour5-HT3A cells.

ABBREVIATIONS: AUC, area under the curve; CI-indole, 5-chloroindole; FDA, Food and Drug Administration; HEK293, human embryonic kidney 293; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; IBS, irritable bowel syndrome; IBS-c, constipation-predominant IBS; IBS-d, diarrhea-predominant IBS; p.o., per os; UPLC, ultra-performance liquid chromatography; Vd, volume of distribution.
Radioligand Binding. Radioligand binding assays were performed as described by us previously (e.g., Monk et al., 2004). Briefly, washed HEK293-h5-HT3A, HEK293-h5-HT3AB, or HEK293-r5-HT3A cells were homogenized (Polytron) in 25 mM Tris buffer (pH 7.4). For competition assays, individual binding assays were performed in triplicate, and binding tubes contained 100 μl of competing drug(s) or vehicle (Total binding; Tris buffer) and 100 μl of [3H]-granisetron (≈0.5 nM; ∼3.0 TBq/mmol; Perkin-Elmer, Seer Green, UK) before cell homogenate (100 μl) was added to initiate binding, which proceeded at room temperature for 60 minutes before termination by rapid filtration (Whatman GF/B filter) and subsequent washing (ice-cold Tris buffer) under vacuum through Whatman GF/B filters, followed by assay of the radioactivity remaining on the filters. For saturation binding assays, to demonstrate that CSTI-300 is a competitive ligand at the h5-HT3A and h5-HT3AB receptors, a range of [3H]-granisetron concentrations (0.1–30 nM) were used in the absence (vehicle) or presence of CSTI-300, with ondansetron (10 μM) used to define nonspecific binding, with each condition performed in duplicate. Cell homogenate samples were then incubated and harvested as for competition assays.

[Ca2+]i Assays. The day before performing intracellular calcium assays, HEK293-h5-HT3A, HEK293-h5-HT3AB, or HEK293-r5-HT3A cells were seeded at a density of 1 × 10^5 cells per well into black-sided, clear-bottomed 96-well plates (Sigma-Aldrich, Poole, UK). On the day of assay, the cells were washed twice with 1 × Hank's balanced salt solution (pH 7.4; Life technologies, Paisley, UK) and incubated with fluo-4 acetoxyethyl ester (5.0 μM; Life technologies, Paisley, UK) for 1 hour at room temperature. Cells were then washed twice with Hank's balanced salt solution and incubated for a further 30 minutes prior to assay (Newman et al., 2013). [Ca2+]i, was measured using a Flex station (Molecular Devices, Sunnyvale, CA) with fluorescence recorded every 3 seconds. At 80 seconds, 5-HT or CSTI-300 was applied rapidly to cells. At 320 seconds, ionomycin (750 μM) was added to demonstrate that the cells were still able to elicit an [Ca2+]i response at the end of the assay. For studies with the 5-HT3 receptor antagonist granisetron (500 nM), the drug was applied during the 30-minute incubation period before initiation of the [Ca2+]i measurements and remained present for the period of experimentation.

Single-Cell Electrophysiology. Approximately 18 hours prior to electrophysiology assays, HEK293-h5-HT3A cells were seeded directly onto 13-mm diameter glass coverslips coated with poly-L-lysine and fibronectin at a density of 2 × 10^5 HEK293-5-HT3A cells per coverslip. Macroscopic currents were recorded in the whole-cell recording mode of the patch-clamp technique. Currents were superfused at 2.0 ml/min with an extracellular solution composed of 140 mM NaCl, 2.8 mM KCl, 1.0 mM CaCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4, adjusted with NaOH. Patch electrodes were pulled from borosilicate glass (o.d. 1.2 mm, i.d. 0.69 mm; Harvard Apparatus, Edenbridge, UK) using a P-97 puller (Sutter, Novato, CA) and filled with intracellular solution consisting of 140 mM CaCl2, 2.0 mM MgCl2, 10 mM HEPES, 1.0 mM EGTA, 1.0 mM Mg-ATP, and 0.3 mM Na-GTP; pH was adjusted to 7.3 with CsOH (osmolarity ∼285 mOsm). Patch electrodes typically had open tip resistances of 4–7 MΩ. Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Wokingham, UK), low-pass Bessel-filtered at 1.0 kHz, and digitized at 10 kHz by a Digidata 1550B (Molecular Devices). Experiments were performed at room temperature with the cells voltage-clamped at −60 mV. Agonist-evoked currents were elicited by pressure ejection (1.3 bar; Picospritzer III; Parker Hannifin, Pine Brook, NJ) of agonist from patch pipettes placed ∼30 μm from the recorded cell.

Rat Colon Distension Model of IBS-d. A previously described IBS-d model (Banner and Sanger, 1995; Banner et al., 1995) was used to evaluate the activity of CSTI-300 versus alosetron. This in vivo model applies a noxious colorectal distension to conscious rats by acute balloon inflation and the effects observed as abdominal muscle contraction, with the threshold pressure noted as the readout. The protocol complied with and was approved by the Institutional Animal Care and Use Committee (Shanghai Medicilon Inc., Shanghai, China), and Shanghai Medicilon Inc. is accredited by the National Institutes of Health Office of Laboratory Animal Welfare (https://olaw.nih.gov/home.htm) and Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). Rats had access to food and water ad libitum in a temperature-controlled environment (12-hour light/dark cycle). Prior to the experiment, rats were fasted overnight.

Given the known ability of isolumine and other some volatile anesthetics to activate/sensitize transient receptor potential vanilloid 1 channels (Cornett et al., 2008) that would likely complicate the sensory readout from the IBS-d model, ether was selected as the anesthetic agent. Thus, under ether anesthesia, a 6- and 7-cm long latex balloon was carefully inserted intra-anally to a position ∼1 cm beyond the anorectal verge. The cannula from the balloon, which was taped to the tail to prevent expulsion of the balloon, was connected to a colored water-filled manometer and via a three-way tap to a syringe pump. Throughout the procedure, the singly housed animals (male Wistar rats; 180–250 g) were allowed unrestricted movement within a 20 × 20 × 14-cm clear acrylic box.

After recovery from the balloon insertion procedure, a ramp inflation of the colorectal balloon at a rate of 20 mm water per minute was performed until the visceromotor response (abdominal muscle contraction) was observed. At this point, the pressure was noted and released immediately by opening the three-way tap. The inflation procedure was repeated at 5-minute intervals until three stable responses were recorded.

Rats were then dosed with 5-hydroxytryptophan (5-HTP) (10 mg/kg) subcutaneously, and 5 minutes later a ramp inflation of the colorectal balloon was performed until the visceromotor response was observed. The inflation procedure was repeated at 5-minute intervals until three stable responses were recorded before subsequent treatment; either alosetron (0.01–1.0 mg/kg; 1.0 ml/kg s.c.), CSTI-300 (0.01–1.0 mg/kg; 1.0 ml/kg s.c.), or vehicle (0.9% NaCl; 1.0 ml/kg s.c.). The colonic distensions were performed at 5-minute intervals for a further 45 minutes with effective pressures monitored.

Dog Behavioral and Emesis Model. The protocol for the dog behavioral and emesis model complied with and was approved by the Institutional Animal Care and Use Committee (Shanghai Medicilon Inc.). Dogs were housed individually with access to food and water ad libitum in a temperature-controlled environment (18–29°C; relative humidity: 30%–70%; 12-hour light/dark cycle). Prior to the experiment, dosed for 30 minutes prior to CSTI-300 administration and for 30 minutes post-CSTI-300 administration.

A total of six naïve male Beagle dogs (10.15–11.45 kg) were administered CSTI-300 (1.0 mg/kg; 5.0 ml/kg p.o.) or vehicle (sterile water, 5.0 ml/kg p.o.). Each animal was monitored continuously (by an observer and a video recording) for 4 hours post-treatment (which would have been extended up to 6 hours if any remarkable observations had been evident during the first 4 hours postdose). During the observation period, each animal was observed primarily for signs of behavioral disturbances or emesis. Emesis was defined by retching and vomiting; “retch” was defined as the action, and “vomit” was defined as the delivery of gastric content through the retching action. Whether either or both of these emetic characteristics had occurred, they would have been recorded independently and recorded on a raw data sheet. Observations for other adverse events were also performed (e.g., abdominal contractions, excessive salivation, state of fecal matter [e.g., normal, loose, diarrhea], general distress, vocalizing [e.g., excessive barking or whimpering], atypical behavior, atypical feeding behavior, atypical drinking behavior, indication of pain, or mortality). With no emesis or adverse observations, the observation at the time point was noted as “normal.” Animals were also observed for 1 hour at 23 to 24 hours postdose, and the above observations were assessed. Whether any emesis or any adverse observation had been evident, the animals would have been monitored periodically until normal behavior was resumed (with the above observations monitored and recorded).

Six hours after treatment, a peripheral venous blood sample was taken (∼1 ml/sample into sodium heparin collection tubes) from each animal for bioanalysis of the plasma concentrations of CSTI-300 by high-performance liquid chromatography tandem mass spectrometry. Briefly, plasma samples (30 μl) were mixed with 150 μl 0.1% formic acid.
acid in water and transferred to a 96-well plate for injection. The liquid chromatography system was comprised of a Waters Ultra-Performance Liquid Chromatography (UPLC) (Waters Corporation) equipped with an ACQUITY UPLC binary solvent manager, ACQUITY UPLC Autosampler Module, ACQUITY UPLC sample organizer and ACQUITY UPLC column heater HT. Samples (3.0 μl) were injected onto an ACQUITY UPLC BEH C18 1.7-μm (50 × 2.10 mm) column, and the mobile phase was run at 600 μl/min. Postcolumn mass spectrometric analysis was performed using an API 4000 (triple-quadrupole) instrument from Applied Biosystems/MDS Sciex with an ESI Ionsource. The data acquisition and control system were created using Analyst 1.5.1 Software from Applied Biosystems/MDS Sciex. A calibration curve using CSTI-300 was created to enable quantification of CSTI-300 levels in the plasma.

Pharmacokinetic Studies. The pharmacokinetics of CSTI-300 was investigated in male Sprague-Dawley rats (288–322 g) and male Beagle dogs (8.0–12 kg).

Drug administration to rats was performed by NoAb BioDiscoveries (Mississauga, Ontario, Canada) after approval of procedures by the NoAb BioDiscoveries animal care committee and performed in accordance with the principles of the Canadian Council on Animal Care. Rats were housed two per cage with access to food and water ad libitum in a temperature-controlled environment (12-hour light/dark cycle). Prior to the experiment, rats were fasted overnight and for 2 hours post–CSTI-300 administration.

Drug administration to dogs was performed by Covance Laboratories Inc. (Madison, WI) and performed in compliance with the Animal Welfare Act Regulations (9 CFR 3). Dogs were housed individually with access to food and water ad libitum in a temperature-controlled environment (12-hour light/dark cycle). Prior to the experiment, dogs were fasted overnight and for 4 hours post–CSTI-300 administration.

CSTI-300 was administered either intravenously or orally (p.o) in sterile water as vehicle, with blood samples taken at predetermined times into K2 EDTA anticoagulant tubes. For oral studies, the dose volumes were 5.0 and 2.0 ml/kg for rat and dog, respectively. For intravenous studies, the dose volumes were 1.0 and 2.0 ml/kg for rat and dog, respectively. Plasma samples underwent protein precipitation with acetonitrile containing 0.1% formic acid. After sonication for 1 minute followed by vortex mixing for 2 minutes and refrigeration for 30 minutes, supernatants were transferred to a 96-well filter plate and filtered in a centrifuge. Filtered extracts (200 μl) were transferred into separate 96-well plates and blown dry under nitrogen at 40°C. The residue was reconstituted in 100 μl of mobile phase and submitted for analysis.

Table: CSTI-300 and 5-HTT Receptors

<table>
<thead>
<tr>
<th>Drug</th>
<th>h5-HT3A receptor</th>
<th>h5-HT3AB receptor</th>
<th>r5-HT3A receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ (nM)</td>
<td>Hill number</td>
<td>Kᵢ (nM)</td>
</tr>
<tr>
<td>CSTI-300</td>
<td>2.26±0.48</td>
<td>1.19±0.16</td>
<td>1.59±0.09</td>
</tr>
<tr>
<td>5-HT</td>
<td>327±62</td>
<td>1.49±0.19</td>
<td>563±89</td>
</tr>
</tbody>
</table>

Fig. 2. The affinity of CSTI-300 for h5-HT3A, h5-HT3AB, and r5-HT3A receptors. The ability of CSTI-300 or 5-HT to compete for [³H]-granisetron binding to h5-HT3A (top left), h5-HT3AB (top right), or r5-HT3A (bottom left) receptors stably expressed in HEK293 cells. Data represent mean ± S.E.M., n = 5. Table insert; arising quantitative pharmacological data for CSTI-300 or 5-HT for the h5-HT3A, h5-HT3AB, and r5-HT3A receptors determined by radioligand binding. Data represent mean ± S.E.M., n = 5.
liquid chromatography with tandem mass spectrometry analysis. CSTI-300 was quantified with Analyst 1.4.2 software using a calibration curve. Plasma concentration data were analyzed with WinNonlin 5.2 software using a noncompartmental model to calculate pharmacokinetic parameters.

**Data and Statistical Analysis.** Values are expressed as mean ± S.E.M. Curve-fitting data analysis was performed with KaleidaGraph (version 3.5). Two-way ANOVA with subsequent Tukey’s multiple comparisons test (with Bonferroni correction for multiple comparisons) were used to evaluate potential treatment differences in the rat IBS-d colon model; a Shapiro-Wilk procedure tested normality. A Kruskal-Wallis test with Dunn’s multiple comparisons was used to test any differences in 5-chloroindole (Cl-indole) affinity for the h5-HT3A receptor and any differences between CSTI-300 and vehicle treatment in rat IBS-d colon model. A Mann-Whitney U test was used to determine whether the impact of CSTI-300 upon [3H]-granisetron affinity for the h5-HT3 receptor was significant. Post hoc tests were only conducted if \( P < 0.05 \) and the variance of the data was homogenous. Significance criterion was \( P < 0.05 \).

Data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

**Results**

**Competition Radioligand Binding.** CSTI-300 and 5-HT competed for \([3H]\)-granisetron binding to the h5-HT3A, h5-HT3AB, or r5-HT3A receptors, with CSTI-300 demonstrating over 100-fold greater affinity compared with 5-HT (Fig. 2). The affinity of CSTI-300 was comparable at the h5-HT3A, h5-HT3AB, and r5-HT3A receptors. The Hill number for CSTI-300, like 5-HT, was above unity when competing for the h5-HT3A or r5-HT3A receptors (Fig. 2); however, this was not the case when either CSTI-300 or 5-HT were competing for the h5-HT3AB receptor (Fig. 2).

The endogenous agonist 5-HT, unlike 5-HT3 receptor antagonists, has been shown previously to reveal an impact of the cryptic orthosteric modulator Cl-indole, allowing Cl-indole to...
compete for [3H]-granisetron binding to the h5-HT3A receptor (Powell et al., 2016). CSTI-300, like 5-HT (see also Powell et al., 2016), revealed in a concentration-dependent manner (1.0–5.0 nM) the ability of Cl-indole to compete for [3H]-granisetron binding to the h5-HT3A receptor (Supplemental Fig. 1).

Fig. 4. CSTI-300 is a partial agonist at the h5-HT3A and r5-HT3A receptor. Ability of CSTI-300 to activate the h5-HT3A (upper four panels; h5-HT3A) or r5-HT3A (lower four panels; r5-HT3A) receptor assessed by an increase in [Ca2+]i, in HEK293 cells stably expressing the h5-HT3A or r5-HT3A receptor. Top left: concentration-dependent action of CSTI-300 or 5-HT (mean ± S.E.M., n = 4 to 5). Top right: representative traces displaying change in fluorescence over time prior to and subsequent to the administration of CSTI-300 (1.0 µM) or 5-HT (10 µM). Bottom panels: representative traces (n = 4 to 5) displaying change in fluorescence over time prior to and subsequent to the administration of 5-HT (3.0 µM) or CSTI-300 (0.3 µM) in the absence (vehicle) or presence of granisetron (500 nM; 30 minutes pretreatment). Table insert: arising quantitative pharmacological data. Data represent mean ± S.E.M., n = 4 to 5.
Saturation Radioligand Binding. To determine the nature of the interaction between CSTI-300 and the h5-HT3A or h5-HT3AB receptors, saturation radioligand binding studies using [3H]-granisetron were undertaken (Fig. 3). The presence of CSTI-300 (2.0–3.0 nM) reduced the affinity of [3H]-granisetron for both the h5-HT3A receptor and h5-HT3AB receptor without impacting the density of labeled receptors, indicating a competitive interaction with each receptor isoform.

Intracellular [Ca2+] Assays. CSTI-300 and 5-HT both activated the h5-HT3A, h5-HT3AB, and r5-HT3A receptors in a concentration-dependent manner to elicit an increase in intracellular Ca2+ concentration in populations of HEK293 cells stably expressing the h5-HT3A receptor and h5-HT3AB receptor without impacting the density of labeled receptors, indicating a competitive interaction with each receptor isoform.

Electrophysiology Studies. To further elucidate the effect of CSTI-300 at the h5-HT3A receptor, single-cell electrophysiology was performed with HEK293 cells stably expressing the h5-HT3A receptor. At a holding potential of -60 mV, a 60-second application of a maximal 5-HT concentration (10 μM) elicited an inward current of 1256 ± 231 pA (n = 5; Fig. 6). The time constant of the rising phase was 0.28 ± 0.07 seconds (n = 5), which decayed slowly back to baseline. The decay was best modeled by a two-exponential curve fit with time constants of 57.6 ± 26.5 and 245.8 ± 50.3 milliseconds (n = 5). In contrast, a 60-second application of a maximal concentration of CSTI-300 (100 nM) elicited a smaller inward current (248 ± 74 pA; n = 9; Fig. 6) with a slower rise time (3.64 ± 0.29 seconds; n = 9) and decay time (one-exponential fit; 260.9 ± 31.9 milliseconds; n = 9) when compared with 5-HT.

Rat IBS-d Model. To demonstrate the potential of CSTI-300 to reduce colonic sensitivity (a symptom associated with IBS-d), an in vivo rat model of IBS-d based on published work was used (Baker and Sanger, 1995; Banner et al., 1995). The sensitivity of the conscious rats to colon distension was increased by subcutaneous application of 5-HTP (10 mg/kg s.c.; Fig. 7), the biochemical precursor to serotonin. Consistent
with previous studies, this 5-HP—induced increased sensitivity of the colon was prevented by a selective 5-HT3 receptor antagonist (alosetron) and also by CSTI-300 in a dosedependent manner \((P < 0.01 \text{ to } <0.0001)\). In addition, a maximal effective dose of CSTI-300 displayed similar actions to a maximal effective dose of alosetron in this in vivo model (Fig. 7), and an \(ED_{50}\) of 0.55 mg/kg was calculated for CSTI-300.

**Dog Behavioral and Emesis Study.** CSTI-300 (1.0 mg/kg, p.o.) did not induce emesis in naive dogs (Table 1). In addition, CSTI-300 did not alter the state of the feces nor the general behavior of the dogs. Six hours after treatment of the dogs with CSTI-300 (1.0 mg/kg, p.o.), the concentration of CSTI-300 in plasma was found to be between 31 and 227 nM \((n = 6)\). The plasma protein binding in dog plasma was determined to be 30\% \pm 3\%. Therefore, the free drug concentrations achieved in this study were between 10 and 70 times the \(K_i\) for CSTI-300 at the h5-HT3 receptor, indicating that 5-HT3 receptor—saturating concentrations of CSTI-300 were achieved through the course of the investigation.

**Pharmacokinetic Studies.** The pharmacokinetics of CSTI-300 were evaluated in rat and dog (Fig. 8; Table 2). After intravenous administration of CSTI-300 (1.0 mg/kg), the \(t_{1/2}\) was comparable between rat \((1.6 \pm 0.2 \text{ hours} ; n = 3)\) and dog \((1.6 \pm 0.2 \text{ hours} ; n = 4)\). The volume of distribution values was moderate in the two species evaluated \((3.0–14.6 \text{ l/kg})\). The clearance values were also moderate with somewhat lower clearance values apparent in the larger species: dog \((1.3 \text{ l/h per kilogram})\) versus rat \((6.2 \text{ l/h per kilogram})\). The \(t_{1/2}\) for CSTI-300 was longer after oral administration and ranged from 2.3 to 4.4 hours in rat and 1.6 to 3.7 hours in dog (Fig. 8; Table 2). The oral bioavailability was good in rat \((24\%–41\%F)\) and excellent in dog \((89\%–100\%F)\). Increased doses of orally administered CSTI-300 trended toward supraproportional exposure [area under the curve (AUC)] within the species examined. After oral administration, the \(C_{max}\) and AUC are significantly increased for matched or similar doses in the larger animals (dog) versus the rodent species (rat).

**CSTI-300 Has Desirable Pharmaceutical Characteristics.** CSTI-300 displayed a variety of characteristics consistent with it being a suitable lead candidate drug for development (Table 3). Thus, CSTI-300 displayed a high level of pharmacological selectivity for the 5-HT3 receptor, with no significant off-target activity detected (affinity \(<<\) 1 \(\mu\)M for a range of neurotransmitter receptors and ion channels; Supplemental Table 1). The water solubility was excellent, and there was no need for any special formulation for in vivo work; sterile water or saline was used for all animal studies. The LogP of 1.95 is within the optimal historic range for orally active marketed drugs (Leeson and Young 2015). In a 7-day dose range finding toxicity study in rat, the no observed adverse effect level was determined to be 300 mg/kg per day, 300 times the effective dose observed in the rat colon distension model. CSTI-300 did not inhibit any of the common cytochrome 450—metabolizing enzymes, predicting a low probability for drug/drug interactions. Moreover, CSTI-300 was well-tolerated and demonstrated good oral pharmacokinetics in rodent and dog.

**Discussion**

This study presents the results of a detailed pharmacological investigation of the 5-HT3 receptor partial agonist CSTI-300, using both in vitro and in vivo models to demonstrate the potential therapeutic effectiveness of this drug as a treatment of symptoms associated with IBS-d and carcinoid syndrome (i.e., intermittent abdominal pain from bloating and gut distension).

The in vitro experiments demonstrated that CSTI-300 is a competitive and selective ligand for the h5-HT3 receptor with relatively high affinity for the h5-HT3A, h5-HT3AB, and r5-HT3A receptors in radioligand binding assays. CSTI-300 displayed partial agonist activity at the h5-HT3A, h5-HT3AB, and r5-HT3A receptors, with similar properties to the orthosteric endogenous agonist, 5-HT, albeit with a lower intrinsic efficacy. Relative to the marketed 5-HT3 receptor antagonists, ondansetron and alosetron, CSTI-300 displays a similar binding affinity to ondansetron [a current marketed therapeutic for nausea and vomiting (Zofran), used “off-label” to treat IBS-d] and \~10 times lower affinity than the very high-affinity 5-HT3 receptor antagonist, alosetron (Hirata et al., 2007).

The potential therapeutic activity of CSTI-300 was evaluated in a rodent model devised to mimic primarily the increased visceral sensitivity of patients with IBS-d, which can be demonstrated by intraluminal bowel distension (Houghton et al., 2002), although this symptom is also relevant to patients with carcinoid syndrome (https://www.cancer.org/cancer/gastrointestinal-carcinoid-tumor/detection-diagnosis-staging/signs-symptoms.html). This rat in vivo model had been shown previously to respond to 5-HT3 receptor antagonists predicting their efficacy (Banner and Sanger, 1995; Banner et al., 1995), which was subsequently established in patients with IBS-d and carcinoid syndrome. This preclinical model uses conscious rats in which a relevant noxious stimulus is delivered by the slow inflation of an intracolonic balloon; the pressure required to evoke an abdominal contraction being the quantitative readout (Banner and Sanger, 1995; Banner et al., 1995). Consistent with the previous studies (Banner and Sanger, 1995; Banner et al., 1995), peripheral administration of the precursor of 5-HT, 5-HP, increased the sensitivity of...
rats to the colonic distension such that the abdominal contraction was evident at lower balloon pressures. This was likely because of the conversion of 5-HTP to 5-HT, which would increase the signaling tone upon the 5-HT3 receptor; this also mimics the biochemical pathology reported in patients with IBS-d (Bearcroft et al., 1998; Dunlop et al., 2005; Enck et al., 2016; Fu et al., 2019; Gunn et al., 2019). This increased sensitivity to colon distension after 5-HTP administration was reversed by the clinically proven 5-HT3 receptor antagonist, alosetron; this finding supported the validation of the model. Since CSTI-300 is also a partial agonist at the r5-HT3A receptor, the ability of the lead candidate drug, CSTI-300, to display at least comparable activity to alosetron in this IBS-d model supports further development of this partial agonist as a treatment of patients with IBS-d and potentially for relief of gastrointestinal symptoms associated with carcinoid syndrome. As part of this development, the ability of CSTI-300 to attenuate diarrhea in preclinical models should be evaluated to see whether the compound has potential benefit to relieve additional symptoms associated with IBS-d and carcinoid syndrome.

Although 5-HT3 receptor antagonists from different chemical series display well-recognized efficacy in patients with IBS-d (e.g., alosetron, cilansetron, ondansetron, ramosetron; Andresen et al., 2008; Ford et al., 2009; Garsed et al., 2014; Fukudo et al., 2016) and relief from some symptoms for patients with carcinoid syndrome (Anderson et al., 1987; Jacobsen, 1992; Platt et al., 1992; Schwerer et al., 1995; Wymenga et al., 1998; Kiesewetter and Raderer, 2013; Halperin et al., 2017), their propensity to induce sometimes

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Emetic Episodes (Either Retching or Vomiting)</th>
<th>State of Feces</th>
<th>General Observations</th>
<th>Plasma (CSTI-300) 6 h Postdose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSTI-300 (1.0 mg/kg p.o.)</td>
<td>No emetic episodes for any of the six dogs</td>
<td>Normal for all six dogs</td>
<td>No abnormal observations</td>
<td>105 ± 87 nM (31–227 nM)</td>
</tr>
</tbody>
</table>

CSTI-300 does not evoke emesis, alter the state of feces, or affect the general behavior of dogs after an oral dose of 1.0 mg/kg. The free plasma concentration taken 6 h after oral dosing was between 10 and 70× the Kᵢ for the h5-HT₃A receptor (dog plasma protein binding 30% ± 3%; unpublished observations). Data from six dogs. Impact of CSTI-300 on the behavior and gastrointestinal activity in dog.
serious side effects limits their utility. Thus, constipation can be a common side effect and can be severe in some cases (Chang et al., 2006; Andresen et al., 2008; Ford et al., 2009; Garsed et al., 2014; Fukudo et al., 2016; for systematic review see Zheng et al., 2017). In addition, rare instances of ischemic colitis have severely limited access of patients with IBS-d to these drugs. Although the pharmacological mechanism underlying the ischemic colitis is not understood, it likely arises from a combination of IBS-d and high levels of 5-HT3 receptor antagonism (see Introduction). The mechanism underlying constipation, however, is likely to be simply related to the level of 5-HT3 receptor inhibition. Hence a 5-HT3 receptor partial agonist such as CSTI-300 would be predicted to be less likely to cause constipation since some level of 5-HT3 receptor activity will be retained, even at high dosage. Furthermore, the more modest, competitive binding affinity of CSTI-300 for the 5-HT3 receptor relative to alosetron would allow the local extracellular 5-HT concentration to be more influential to promote receptor function.

The pharmacokinetics of CSTI-300 in rat and dog. In rat, after administration intravenously (1.0 mg/kg, top left) CSTI-300 had a half-life of 1.6 hours. With oral administration (1.0 mg/kg, middle left; 5.0 mg/kg, bottom left), CSTI-300 was metabolized with a half-life of approximately 2 hours for 1.0 mg/kg or 4 hours for 5.0 mg/kg. Data represent mean ± S.D., n = 3. In dog, after administration intravenously (1.0 mg/kg, top right), CSTI-300 had a half-life of 1.6 hours. Regarding oral administration (10 mg/kg, middle right; 30 mg/kg, bottom right), CSTI-300 was metabolized with a half-life of approximately 4 hours. Data represent mean ± S.D., n = 4.

### TABLE 2
Pharmacokinetic profile of CSTI-300 in rat and dog
Data represented as mean ± S.D. The pharmacokinetics of CSTI-300 in rat and dog.

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Dose (mg/kg)</th>
<th>AUC last a (h*ng/ml)</th>
<th>C max b (ng/ml)</th>
<th>T 1/2 c (h)</th>
<th>CL d (l/h per kilogram)</th>
<th>Vif e (l/kg)</th>
<th>%F g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (3)</td>
<td>1.0, i.v.</td>
<td>163 ± 27</td>
<td>235 ± 28</td>
<td>1.6 ± 0.2</td>
<td>6.2 ± 1.0</td>
<td>14.6 ± 4.3</td>
<td>24</td>
</tr>
<tr>
<td>Rat (3)</td>
<td>1.0, p.o.</td>
<td>39.8 ± 3.0</td>
<td>215 ± 30</td>
<td>2.3 ± 0.7</td>
<td>1.3 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>Rat (3)</td>
<td>5.0, p.o.</td>
<td>331 ± 102</td>
<td>138 ± 68</td>
<td>4.4 ± 2.7</td>
<td>0.5 ± 0.1</td>
<td>1.6 ± 0.18</td>
<td>89</td>
</tr>
<tr>
<td>Dog (4)</td>
<td>1.0, i.v.</td>
<td>764 ± 135</td>
<td>497 ± 48</td>
<td>1.6 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>364 ± 157</td>
<td>100</td>
</tr>
<tr>
<td>Dog (3)</td>
<td>0.3, p.o.</td>
<td>205 ± 105</td>
<td>66.1 ± 34.4</td>
<td>1.6 ± 0.18</td>
<td>0.5 ± 0.1</td>
<td>397 ± 68</td>
<td>100</td>
</tr>
<tr>
<td>Dog (3)</td>
<td>1.0, p.o.</td>
<td>1010 ± 712</td>
<td>3498 ± 725</td>
<td>3.7 ± 0.8</td>
<td>0.4 ± 0.1</td>
<td>3498 ± 725</td>
<td>100</td>
</tr>
<tr>
<td>Dog (4)</td>
<td>1.0, p.o.</td>
<td>12,098 ± 1076</td>
<td>9223 ± 1464</td>
<td>3.4 ± 0.6</td>
<td>0.25 ± 0.1</td>
<td>39,115 ± 13,049</td>
<td>100</td>
</tr>
<tr>
<td>Dog (4)</td>
<td>30, p.o.</td>
<td>39,115 ± 13,049</td>
<td>9223 ± 1464</td>
<td>3.4 ± 0.6</td>
<td>0.25 ± 0.1</td>
<td>39,115 ± 13,049</td>
<td>100</td>
</tr>
</tbody>
</table>

aArea under the plasma concentration vs. time curve from 0 to the last time point CSTI-300 was quantifiable in plasma.
bTime of maximum observed concentration of CSTI-300 in plasma.
cMaximum observed concentration of compound in plasma.
dApparent half-life of the terminal phase of elimination of CSTI-300 from plasma.
eTotal body clearance of CSTI-300.
fVolume of distribution.
gOral bioavailability; %F = (AUC last p.o. × Dose p.o.) / (AUC last i.v. × Dose i.v.).
Interestingly, there is a previous example of a 5-HT₃ receptor partial agonist being evaluated in patients with IBS, but it occurs in IBS-c rather than IBS-d. The rationale was that the intrinsic activity of pumosetrag (DDP733; MKC-733) for the 5-HT₃ receptor would promote motility within the gastrointestinal tract to reverse the symptoms, as had been predicted from rodent studies (Chetty et al., 2008). In human trials, although pumosetrag increased small intestinal transit and improved bowel habits of patients with IBS-c (Coleman et al., 2003; Fujita et al., 2005), mild nausea and abdominal discomfort were also reported, along with flushing (Evangelista, 2007), consistent with too much 5-HT₃ receptor activation by the drug. Nausea has also been associated with administration of another 5-HT₃ receptor agonist (Staner et al., 2001), and hence a potential concern would be that CSTI-300 may manifest similar emetic episodes in patients that would limit the potential clinical utility of the drug. In the present study, the potential of CSTI-300 to evoke emesis (along with monitoring for other potential adverse events) was directly assessed by oral administration of CSTI-300 to dogs at a dose resulting in relatively high plasma drug concentrations predicted to saturate the 5-HT₃ receptor. In these studies, CSTI-300 failed to induce vomiting or to change the behavior of the dog experiencing nausea. Furthermore, there was no evidence of increased gastric motility in these animals. It is acknowledged that no positive-control emetogen was included in these studies (e.g., cisplatin) although it is generally considered that dogs are relatively sensitive to emetogens, hence the inability of CSTI-300 to induce emesis or other abnormal behaviors in dogs is considered noteworthy by the authors. Additional studies of CSTI-300 in dogs and mini-pigs conducted primarily to further assess the pharmacokinetics of the drug, failed to evoke emetic episodes at doses of CSTI-300 up to 30 mg/kg p.o. (Guzzo et al., unpublished observations). Such findings support that CSTI-300 may be able to deliver efficacy to patients with IBS-d and carcinoid syndrome without side effects of nausea/vomiting or diarrhea from too much stimulation of the 5-HT₃ receptor. Indeed, Phase I clinical studies with CSTI-300 are now planned that will include monitoring of potential to modify gastrointestinal motility as well as the potential to evoke emesis.

TABLE 3
Summary of additional pharmacological and pharmaceutical characteristics of CSTI-300

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>High affinity and selectivity for 5-HT₃ receptor</td>
<td>No significant off-target activity when screened at 1.0 μM (see Supplemental Table 1 for list of targets)</td>
</tr>
<tr>
<td>Low probability of drug/drug interactions</td>
<td>CYPs (1A2, 2B6, 2C9, 2C19, 2D6, 3A4) IC₅₀ &gt; 100 μM</td>
</tr>
<tr>
<td>Good oral PK in rodent and dog</td>
<td>Fₚₘₑₜ = 42%, t½ = 4.2 h</td>
</tr>
<tr>
<td>Large margin of safety</td>
<td>NOAEL (no observed adverse effect level) = 300 mg/kg per day (7-day dosing in rat); 300 × ED₉₀ dose</td>
</tr>
<tr>
<td>Excellent pharmaceutical/biophysical properties</td>
<td>Good tolerability in dog; no emesis or loose stool at 1 mg/kg (&gt;10 × ED₉₀ concentration)</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>Nonmutagenic in micro-Ames Test</td>
</tr>
</tbody>
</table>

**ED₉₀**, 90% effective dose; **MM**, molecular mass; NOAEL, no observed adverse effect level; PK, pharmacokinetics.
are used for this indication (Kiesewetter and Raderer, 2013).

In summary, CSTI-300 is a high-affinity 5-HT3 receptor partial agonist that displays efficacy in a rodent model of IBS-d with some relevance to carcinoid syndrome. The drug displays good oral pharmacokinetics as well as desirable pharmacological characteristics, making it a promising candidate for subsequent clinical trial to evaluate the potential to deliver symptomatic relief to patients with for example IBS-d and carcinoid syndrome.

Authorship Contributions
Participated in research design: Manning, Moore, Sargent, Guzzo, Barnes.
Conducted experiments: Roberts, Grafon, Powell, Chen, Xie, Huang, Liu, Brady, Qureshi, Stamatakis, Manning, Moore, Sargent, Guzzo, Barnes.
Performed data analysis: Brock, Cooper, Manning, Moore, Sargent, Guzzo, Barnes.
Wrote or contributed to the writing of the manuscript with input from all authors.

References


