Inhibition of Heat-Stable Toxin–Induced Intestinal Salt and Water Secretion by a Novel Class of Guanylyl Cyclase C Inhibitors

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Background. Many enterotoxigenic *Escherichia coli* strains produce the heat-stable toxin, STa, which, by activation of the intestinal receptor-enzyme guanylyl cyclase (GC) C, triggers an acute, watery diarrhea. We set out to identify GCC inhibitors that may be of benefit for the treatment of infectious diarrheal disease.

Methods. Compounds that inhibit STa-induced cyclic guanosine 3′,5′-monophosphate (cGMP) production were selected by performing cyclase assays on cells and membranes containing GCC, or the related GCA. The effect of leads on STa/GCC-dependent activation of the cystic fibrosis transmembrane conductance regulator anion channel was investigated in T84 cells, and in porcine and human intestinal tissue. Their effect on STa-provoked fluid transport was assessed in ligated intestinal loops in piglets.

Results. Four N-2-(propylamino)-6-phenylpyrimidin-4-one–substituted piperidines were shown to inhibit GCC-mediated cellular cGMP production. The half maximal inhibitory concentrations were $\leq 5 \times 10^{-7}$ mol/L, whereas they were >10 times higher for GCA. In T84 monolayers, these leads blocked STa/GCC-dependent, but not forskolin/adenylyl cyclase-dependent, cystic fibrosis transmembrane conductance regulator activity. GCC inhibition reduced STa-provoked anion secretion in pig jejunal tissue, and fluid retention and cGMP levels in STa-exposed loops. These GCC inhibitors blocked STa-provoked anion secretion in rectal biopsy specimens.

Conclusions. We have identified a novel class of GCC inhibitors that may form the basis for development of future therapeutics for (infectious) diarrheal disease.

Keywords. secretory diarrhea; CFTR; enterotoxigenic *E. coli* (ETEC); guanylyl cyclase C.

Specific strains of the usually benign gut bacterium *Escherichia coli* produce enterotoxins that cause acute watery diarrhea, commonly referred to as *secretory diarrhea* [1]. These enterotoxigenic *E. coli* are endemic to developing parts of the world and are a significant cause of morbidity and mortality in these regions, annually affecting several hundred million children <5 years of age and accounting for tens of thousands of deaths [2, 3]. These same pathogens also cause traveler's diarrhea [1, 3].

Studies on symptomatic patients indicate a high prevalence of enterotoxigenic *E. coli* strains producing the heat-stable toxin, STa [2, 3]. STa is a small, cysteine-rich peptide that binds to the extracellular receptor domain of guanylyl cyclase (GC) C (GCC), located at the luminal membrane of intestinal epithelial cells [4]. GCC is a member of the family of membrane-associated, particulate GCs. Like GCA and GCB, the receptors for the natriuretic peptides, GCC is composed of an N-terminal receptor domain, connected via a single transmembrane helix to an intracellular moiety, containing the catalytic domain [4].

GCC and its endogenous peptide ligands, guanylin and uroguanylin, play a key role in balancing water absorption and hydration of the intestinal lumen, as

exemplified by the finding that loss of GCC function causes severe dehydration of the intestinal lumen, culminating in intestinal obstruction [5]. STa binding activates the catalytic domain with a potency exceeding that of the guanylins [4]. The ensuing surge in cellular cyclic guanosine 3′,5′-monophosphate (cGMP) levels leads up to anomalous salt and water secretion through protein kinase–mediated activation of the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, which results in excessive secretion of chloride ion and bicarbonate and inhibition of the sodium-hydrogen (Na^+/H^+) exchanger isotype 3 (NHE3), reducing Na^+ absorption [1].

In developing countries, management of secretory diarrhea is mostly limited to oral rehydration therapy, aimed at replenishing the body with salt and water to prevent life-threatening dehydration. More specific therapeutic options are largely unavailable [6]. From a mechanistic viewpoint, reduction of GCC activity offers a straightforward approach to limit enterotoxigenic E. coli–provoked secretory diarrhea. Inhibition of GCC-mediated cGMP production would not only curtail anion secretion but would also restore NHE3 activity, resulting in a comprehensive antidiarrheal action. However, development of specific and effective GCC blockers has proved challenging [7–10].

In the present study, small molecule compounds that inhibit STa-induced cGMP production in the GCC-containing colonic cell line T84 were tested for their ability to block STa-induced CFTR activity in T84 cells and in animal and human intestinal mucosa and also for their ability to block STa-induced cGMP production and fluid transport in pig intestine, in vivo.

MATERIALS AND METHODS

Human Tissue Studies
Rectal biopsy specimens were obtained from healthy volunteers (aged 22–24 years), who agreed to participate by giving written informed consent. This study was approved by the Medical Ethical Committee of the University of Utrecht Medical Centre.

Animals
For the study of bioelectrics, tissue was obtained from crossbred Yorkshire-Landrace pigs, aged 10–16 weeks. Studies were approved by the Ethical Committee for Animal Experiments of the Erasmus MC University Medical Center. For the study of fluid transport and cGMP production, 2-week-old suckling piglets were purchased from a commercial piggery. Experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/135).

Cell Culture
T84 and HeLa cells were routinely cultured as described elsewhere [11, 12]. For assessment of bioelectrics, 4×10^5 T84 cells were seeded on permeable membrane supports (Millicell) and cultured for 18–24 days. HEK293 cells, stably transfected with human GCC (hGCC) or rat GCC (rGCC) were produced and cultured as described elsewhere [13].

Guanylyl Cyclase Assays in Cells and Cell Membrane Preparations
For assessing STa- and atrial natriuretic peptide–induced cGMP production, cells were seeded in 96-well plates (4×10^4 cells per well) and grown to confluency within 3 days. Cells were briefly washed with phosphate-buffered saline (PBS) and incubated in Meyler solution supplemented with glucose [14], 1.0 mmol/L isobutyl-methylxanthine, with or without STa (Bachem) or atrial natriuretic peptide (Sigma), and GCC inhibitor compound. After 1 hour, cells were lysed in ice-cold PBS, 2.5 mmol/L ethylenediaminetetraacetic acid, and 1% Triton X100, and the cGMP content was assessed by means of enzyme-linked immunosorbent assay (ADI-901-013; Enzo Life Sciences). The basal cGMP content of cells was typically <3% of the level in toxin/hormone-stimulated cells. GCC assays on membrane preparations were performed as described elsewhere [15].

Bioelectrics of Pig Intestine, T84 Monolayers, and Human Rectal Tissue
Pigs were anesthetized and euthanized as described elsewhere [16]. After excision, jejunal tissue was flushed with ice-cold PBS supplemented with 0.1 mmol/L calcium chloride and 1 mmol/L magnesium chloride, and the outer muscle layers were removed by blunt dissection. Human rectal biopsy specimens were obtained with a suction biopsy device (Medicon) and stored briefly (<15 minutes) in ice-cold PBS.

The procedure for assessing the bioelectrics of intestinal tissue and T84 monolayers was essentially as described elsewhere [14]. Before the start of an experiment, rectal biopsy specimens were incubated for 45 minutes in the presence of the cyclo-oxygenase inhibitor indomethacin (10 µmol/L) to wash out endogenous secretagogues [17]. The data shown represent the short-circuit current (Isc), reflecting electrogenic transepithelial ion transport.

Measurement of In Vivo Fluid Transport and cGMP Production in Pig Jejunum
Anesthesia and surgery in piglets were performed as described elsewhere [18]. Briefly, starting 1 m caudal to the ligament of Treitz, 10 ligated loops were prepared (10 cm long and 2 cm apart). Care was taken to place the ligatures between the mesenteric arcades, ensuring an intact blood supply to the epithelium.

STa (1 nmol in 2 mL of saline) was instilled in 6 loops, arranged in 3 pairs of consecutive loops. Per pair, one loop was treated with compound, and the other served as vehicle (dimethyl sulfoxide, 0.1%) control. Remaining loops received saline only. After 1 hour, loops were excised, and their fluid content was assessed gravimetrically.

For cGMP measurements, 5 separate piglets were used. Loops were treated with STa and compound as described above. Loop content was collected after 1 hour, and centrifuged at 9000g for 5 minutes, and cGMP concentration in the luminal fluid was

assessed by enzyme-linked immunosorbent assay (ADI-901-013; Enzo Life Sciences).

Data Analysis

Fluid transport in piglets was analyzed using repeated measures analysis of variance and the Bonferroni post-hoc test for selected pairs. The production of cGMP in piglets was analyzed using repeated-measures analysis of variance and the Tukey post-hoc test (GraphPad Prism 5.0; GraphPad Software).

For bioelectrics, statistical significance of differences between means was assessed using the Student t test (2 sided). The concentration dependence of GC inhibition and enzyme kinetics were analyzed using nonlinear regression (GraphPad Prism version 5).

RESULTS

Identification of Potent and Specific Inhibitors of GCC

The N-2-(propylamino)-6-phenylpyrimidin-4-one–substituted piperidines presented here (Figure 1) were found to strongly reduce STa-provoked cGMP accumulation in T84 cells (Table 1).

To determine whether these leads act as true receptor antagonists, we assessed their potency in the T84 cell assay at an STa concentration of 0.1 μmol/L, close to the observed half maximal effective concentration (EC$_{50}$) for STa (0.07 μmol/L), and at a high, near saturating concentration of 3 μmol/L. We found that the inhibition potency was unaffected by the STa concentration, suggesting that these compounds do not compete with STa for GCC binding (Table 1).

Compounds showed similar activity in HEK293 cells heterologously expressing hGCC (GUCY2C), and activity was also preserved in membrane preparations originating from these cells (Table 1). The potency of inhibition of rGCC was markedly lower than that of hGCC, despite the >80% amino acid sequence identity between both orthologues (Table 1).

Because these assays were performed in the presence of the phosphodiesterase inhibitor isobutyl-methylxanthine, we rule out the possibility that the compounds act through stimulation of cGMP degradation. Furthermore, their activity in membrane preparations demonstrates that a putative stimulation of cellular cGMP efflux does not play a significant role in their cGMP-
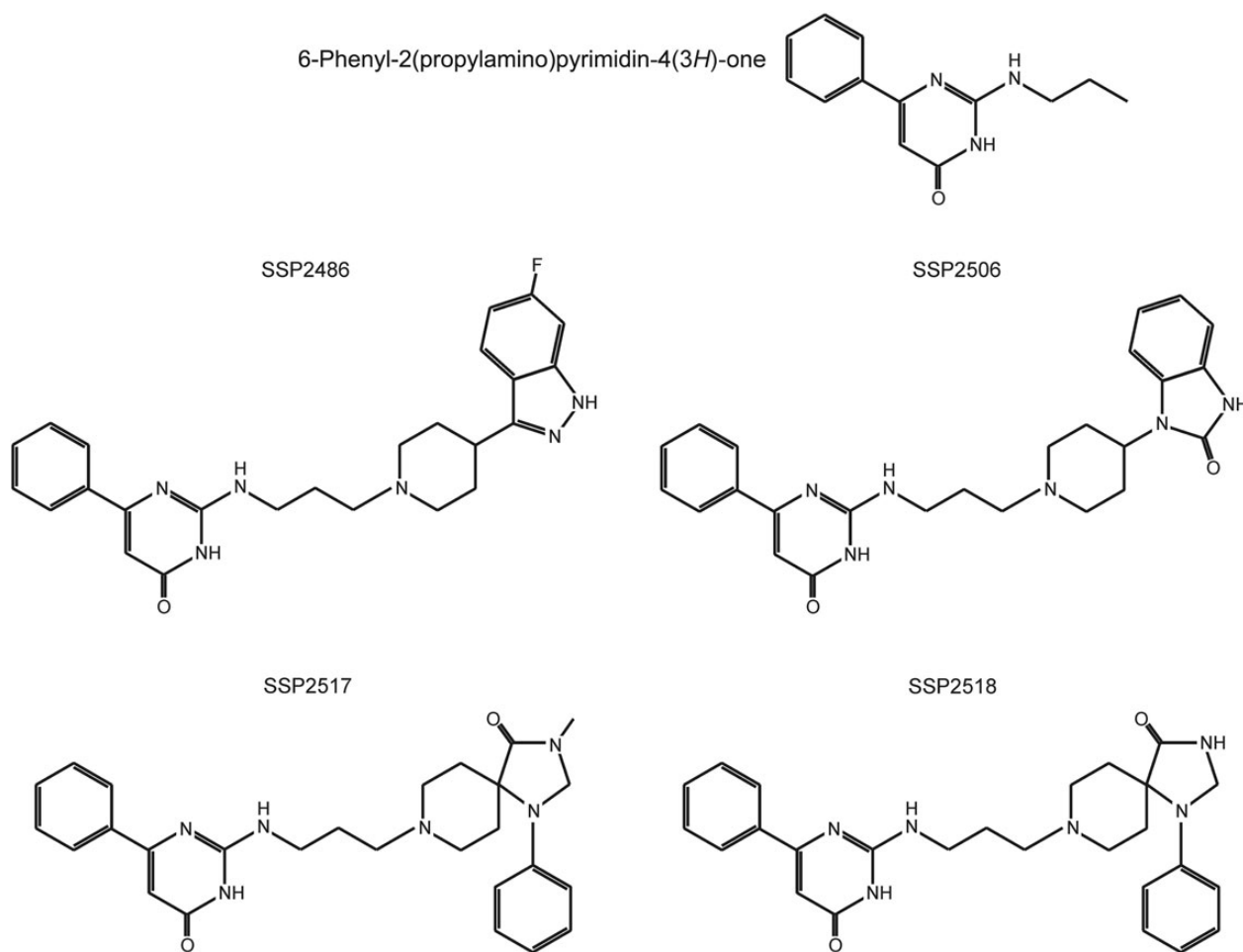
Figure 1. Chemical structure of the 4 N-2-(propylamino)-6-phenylpyrimidin-4-one–substituted piperidines.

Table 1. Potency of GC Activity Inhibition by 4 Lead Compounds[a]

Cells	$-Log_{10}$ of Half Maximal Inhibitory Concentration (pIC$_{50}$), Mean (SD), [No. of Independent Experiments]			
	SSP2486	SSP2506	SSP2517	SSP2518
T84 cells				
STa, 0.1 µmol/L	6.4 (0.2) [3]	6.6 (0.3) [10]	6.3 (0.2) [9]	6.6 (0.4) [13]
STa, 3 µmol/L	6.3 (0.3) [5]	6.7 (0.4) [9]	6.5 (0.3) [9]	6.7 (0.2) [10]
HEK293 (STa, 1 µmol/L)				
hGCC cells	6.3 (0.2) [5]	7.4 (0.6) [5]	7.0 (0.4) [4]	6.9 (0.4) [5]
hGCC membranes	6.4 [2]	6.1 [2]	6.5 [2]	7.2 (0.6) [3]
rGCC cells	5.1 (0.3) [3][b]	5.4 (0.3) [3][b]	5.1 (0.1) [3][b]	5.0 (0.5) [3][b]
HeLa cells (hGCA) (ANP, 0.1 µmol/L)	5.1 (0.3) [4][b]	5.2 (0.3) [4][b]	5.2 (0.3) [4][b]	5.2 (0.2) [4][b]

Abbreviations: ANP, atrial natriuretic peptide; GC, guanylyl cyclase; hGCA, human guanylyl cyclase A; hGCC, human guanylyl cyclase C (GCC); IC$_{50}$, half maximal inhibitory concentration; rGCC, rat GCC; SD, standard deviation; STa, heat-stable toxin.

[a] The production of cyclic guanosine 3′,5′-monophosphate was assayed in T84 cells, HeLa cells, and HEK293 cells expressing either hGCC or rGCC. Assays were also performed on membranes prepared from hGCC-HEK293 cells. GCC activity in T84 cells and HEK293 cells or membranes was assayed in the presence of STa, at the concentrations indicated. GCA activity in HeLa cells was stimulated by addition of ANP. Compounds were tested at concentrations ranging from 10^{-9} to 10^{-5} mol/L to derive −log IC$_{50}$ (pIC$_{50}$) values.

[b] Estimated value; only incomplete inhibition was attained in the concentration range tested.

lowering effect. Collectively, the data demonstrate that these compounds inhibit cellular cGMP accumulation by blocking GCC-mediated cGMP production.

Next, we tested the activity of these hGCC inhibitors toward the atrial natriuretic peptide–activated human GCA (hGCA), endogenously expressed by HeLa cells [12]. Although GCA is structurally closely related to GCC [19], we found that the compounds were considerably less potent as inhibitors of hGCA, (Table 1). Furthermore, we found that SSP2518 did not block nitric oxide–dependent cGMP production in HEK293 cells, which is mediated by the cytosolic soluble GC (Supplementary Figure 1).

Cyclase assays performed at varying concentrations of Mg^{2+}-guanosine triphosphate (GTP) showed that, at high concentrations, GTP inhibits enzyme activity, as reported elsewhere (Supplementary Figure 2) [20]. It seems that the regulation of hGCC by its substrate is complex, involving a separate GTP-dependent factor, possibly another nucleotide-binding sequence within hGCC (eg, the kinase homology domain) [19]. This inhibition by GTP somewhat confounds the analysis of the enzyme kinetics, which was limited to Mg^{2+}-GTP concentrations ≤3.3 mmol/L. In this range, positive cooperativity was observed, in line with the notion that 2 or 3 monomers have to align for formation of an active catalytic center [4, 19]. Compound SSP2518 did not affect cooperativity, but it increased the substrate concentration required for half-maximal activation, whereas it reduced the maximum rate of enzyme activity, suggesting an allosteric mode of action (mixed inhibition).

Blockage of CFTR-Mediated Secretory Responses to STa but Not Adenylyl Cyclase-Linked Stimuli by GCC Inhibitors

The effect of the 4 compounds on CFTR-mediated chloride-secretory responses was assessed in T84 monolayers, in an Ussing chamber setup. It was shown elsewhere that STa treatment does not affect cyclic adenosine monophosphate (cAMP) levels in T84 cells and that the cGMP-induced Isc response of T84 monolayers can be fully blocked by pharmacological CFTR inhibition [21, 22]. This indicates that cGMP-mediated Isc responses in T84 monolayers are fully CFTR dependent, and that STa-provoked CFTR activation in T84 cells does not require adenylyl cyclase (AC) activity.

STa provoked a prompt increase in the Isc in T84 monolayers, reflective of CFTR activation. The STa-provoked response was markedly attenuated by all 4 leads: inhibition was found both when STa was administered in the sustained presence of GCC inhibitor (Figure 2A and 2B) and when compound was administered after a full response had developed (Figure 2D and 2E).

The presence of a GCC inhibitor did not preclude CFTR activation by the AC activator forskolin, and the total Isc response elicited by combined STa/forskolin treatment was unaffected by GCC inhibition (Figure 2C). This not only demonstrated that these GCC blockers do not appreciably inhibit AC but also shows that the compounds do not have a direct action on the (shared) downstream components of these signaling routes, for example, the cAMP-dependent protein kinase (PKA) and CFTR.

SSP2518 Inhibition of STa-Provoked CFTR-Mediated Secretory Responses in Pig Jejunum

These GCC inhibitors exhibited an orthologue-specific inhibition profile, showing a low efficacy toward rGCC (Table 1). Accordingly, we found that they did not inhibit STa-provoked anion secretion in mouse intestine (not shown). However,
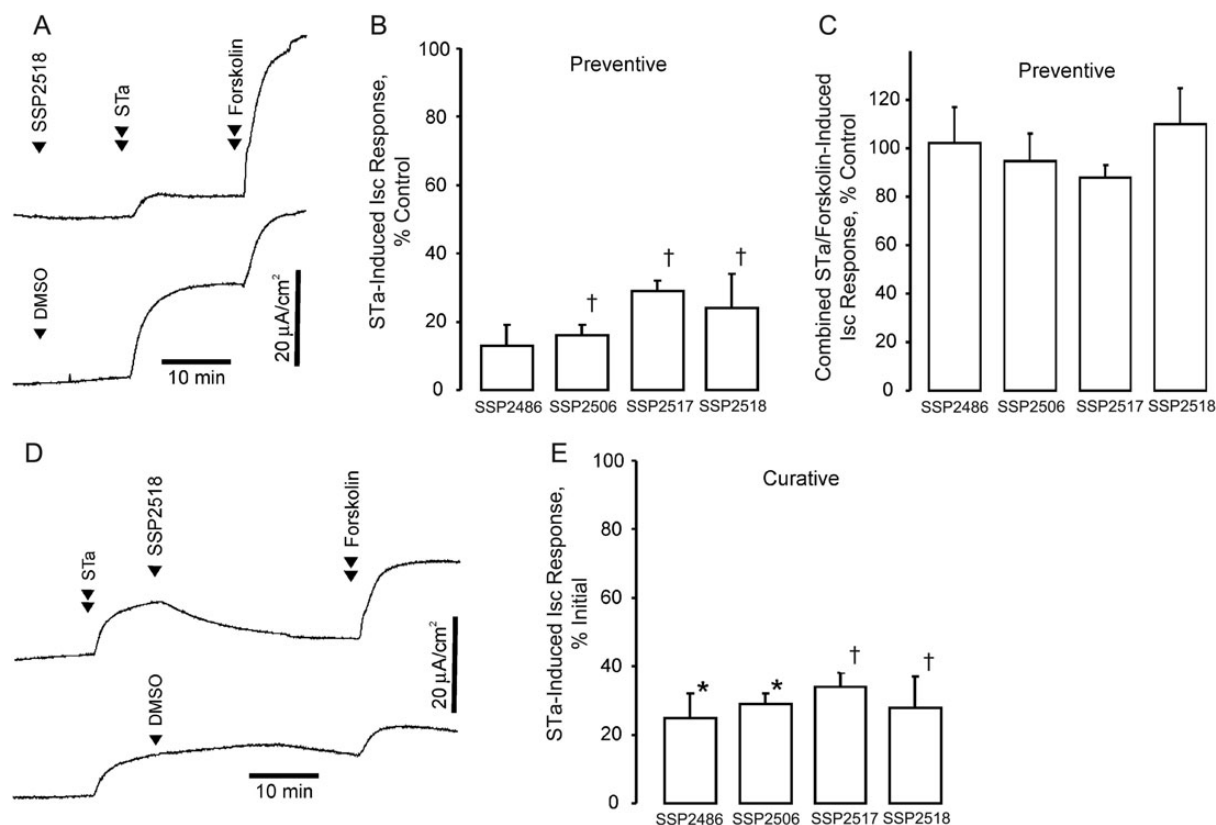
Figure 2. Guanylyl cyclase C (GCC) inhibitors block heat-stable toxin (STa)-induced cystic fibrosis transmembrane conductance regulator (CFTR) activation in T84 cells. *A*, Representative experiment illustrating the procedure for preventive testing of GCC inhibitors. Compound (1 μmol/L) or vehicle (dimethyl sulfoxide [DMSO], 0.1%) was added 15 minutes before the addition of STa (300 nmol/L). After the STa-induced short-circuit current (Isc) response had reached a peak level, forskolin (10 μmol/L) was added. *B*, Peak STa-induced Isc responses in the presence of compound, relative to a paired solvent control. The mean (standard deviation [SD]) peak Isc response in the presence of DMSO was 18.0 (1.2) μA/cm^2 (n = 16). *C*, Effect of GCC inhibitors on peak Isc responses after combined STa/forskolin treatment. The mean (SE) peak Isc response elicited by STa/forskolin was 35 (3) μA/cm^2 (n = 12). *D*, Representative experiment illustrating the procedure for curative testing of GCC inhibitors. Compound (1 μmol/L) or vehicle (DMSO, 0.1%) was added 10 minutes after STa (300 nmol/L). After another 20 minutes, forskolin (10 μmol/L) was added. *E*, Residual Isc responses 15 minutes after addition of GCC inhibitor, relative to the full Isc response, assessed just before addition of compound. For statistical analysis, values were compared with a paired solvent control (DMSO, 0.1%), which effected no apparent decrease in the Isc response over the 15-minute period (STa-induced Isc response as percentage of the initial response was mean [SE], 104% [11%]; n = 9). The number of experiments performed was 3–5 per group. *$P < .05$; †$P < .01$.

cyclase assays on intestinal plasma membrane preparations showed that porcine GCC was inhibited with similar efficacy as hGCC (not shown). Therefore, we used a pig model to assess the effect of GCC inhibition on STa-provoked intestinal ion and fluid secretion. In pig jejunum, in a curative setup, we observed inhibition of STa-provoked Isc responses by SSP2518 at concentrations of >5 μmol/L (Figure 3A). However, the transient nature of the STa response in pig jejunum, combined with a slow onset of the inhibition, confounded the interpretation of the data, and a clear effect of SSP2518 could be ascertained in only 5 of 10 experiments. When we tested SSP2518 in a preventive setup, essentially after the procedure as described for the T84 monolayers (Figure 2), we consistently found lowered STa-induced Isc responses at a concentration of 30 μmol/L (Figure 3B).

SSP2517 and SSP2518 Inhibition of STa-Provoked Fluid Secretion and cGMP Production in Pig Jejunum

After we had ascertained that SSP2518 reduced STa-induced CFTR activity in pig jejunum, in vitro, we investigated the effect of this compound and its analog SSP2517 on STa-induced fluid transport in pig jejunum, in vivo. STa treatment of ligated intestinal loops led to retention of luminal fluid (Figure 4A). Both SSP2517 and SSP2518 reduced STa-provoked fluid retention. STa treatment increased luminal cGMP levels (Figure 4B). This accumulation of cGMP in the lumen was abrogated by SSP2517.

SSP2518 Inhibition of CFTR-Mediated Secretory Responses to STa in Human Colon Tissue

SSP2518 was tested on suction biopsy specimens obtained from the distal colon of healthy volunteers. STa promptly induced a
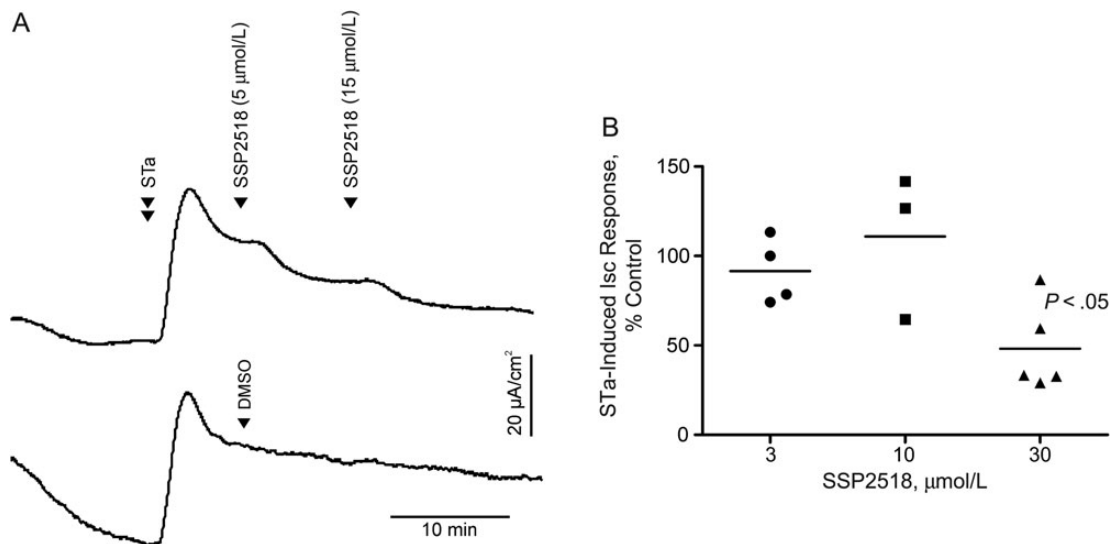
Figure 3. SSP2518 inhibits heat-stable toxin (STa)-induced chloride secretion in pig jejunum. *A*, STa induces a rapid increase in the short-circuit current (Isc) across pig jejunum, followed by a gradual run-down of the response. In this experiment, SSP2518 demonstrated concentration-dependent inhibition of the STa response, but such an effect was not always apparent. *B*, For preventive testing of guanylyl cyclase C (GCC) inhibitors, compound or vehicle (dimethyl sulfoxide [DMSO], 0.1%) was added 15 minutes before the addition of STa (100 nmol/L). The data depict peak STa-induced Isc responses in the presence of compound, relative to a paired solvent control. The mean (standard error) peak Isc response in the presence of DMSO was 10.4 (2.2) $\mu A/cm^2$ (n = 5).

robust Isc response in this tissue, which, in contrast to the response in pig jejunum (Figure 3), was sustained and even tended to gradually increase further after the initial phase of the response had leveled (Figure 5*A*). SSP2518, added after the initial phase of the Isc response had fully developed (10 minutes after STa addition), consistently induced a gradually progressing inhibition of the STa-provoked Isc response. Even after prolonged SSP2518 treatment (45 minutes), the tissue remained responsive to forskolin and to the Ca^{2+}-linked muscarinic receptor agonist carbachol.

However, when carbachol was added before forskolin but after STa, the response elicited by carbachol seemed to be lowered substantially by the presence of SSP2518, relative to a paired solvent control (Figure 5*C*). Closer examination of all 4 experiments performed according to this protocol showed that the carbachol-induced Isc response was correlated with the preceding, residual STa response that was maintained in the presence of SSP2518 (Figure 5*D*). We tentatively conclude that activation of an apically located cAMP/cGMP-regulated anion channel (ie, CFTR) is a prerequisite for Ca^{2+}-linked anion secretion in human distal colon. This implies that, in the absence of cAMP agonists, GCC inhibitors block STa- and carbachol-induced anion secretion to a similar extent.

DISCUSSION

We report the discovery of small molecule blockers of GCC that are active in vivo, in an animal model of STa-provoked fluid secretion, and block STa-induced chloride secretory responses in human intestinal biopsy specimens. In cell models, these N-2-(propylamino)-6-phenylpyrimidin-4-one–substituted piperidines blocked hGCC activity at submicromolar levels. In contrast to previously developed GCC blockers, these novel compounds showed little activity toward cyclases other than hGCC [7, 10, 23].

Because these hGCC inhibitors were active in membrane preparations originating from HEK293 cells heterologously expressing hGCC, it seems unlikely that they act on a soluble modulator of hGCC; instead, a direct action on hGCC, or a factor closely associated with hGCC and retained on membrane isolation, is indicated. That their activity is preserved in isolated membranes also shows that these novel GCC blockers have a mode of action different from the pyridopyrimidine-type GCC blockers described elsewhere [10].

Our results imply that the activity of these compounds depends on specific aspects of the structure and/or regulation of hGCC that sets it apart from other cyclases. However, these blockers did not display STa-competitive inhibition, suggesting that they do not interact with the STa-binding receptor domain. Congruent with this notion, Ussing chamber experiments on T84 monolayers showed that the compounds were also active when added solely to the basolateral bathing medium (whereas STa was administered solely to the apical bathing medium), supporting the explanation of an intracellular mechanism (Supplementary Figure 3). It follows that this class of inhibitors uncouples STa binding from formation of an active catalytic center. However, in view of the marked preference of this compound for hGCC (compared with rGCC, hGCA, and soluble
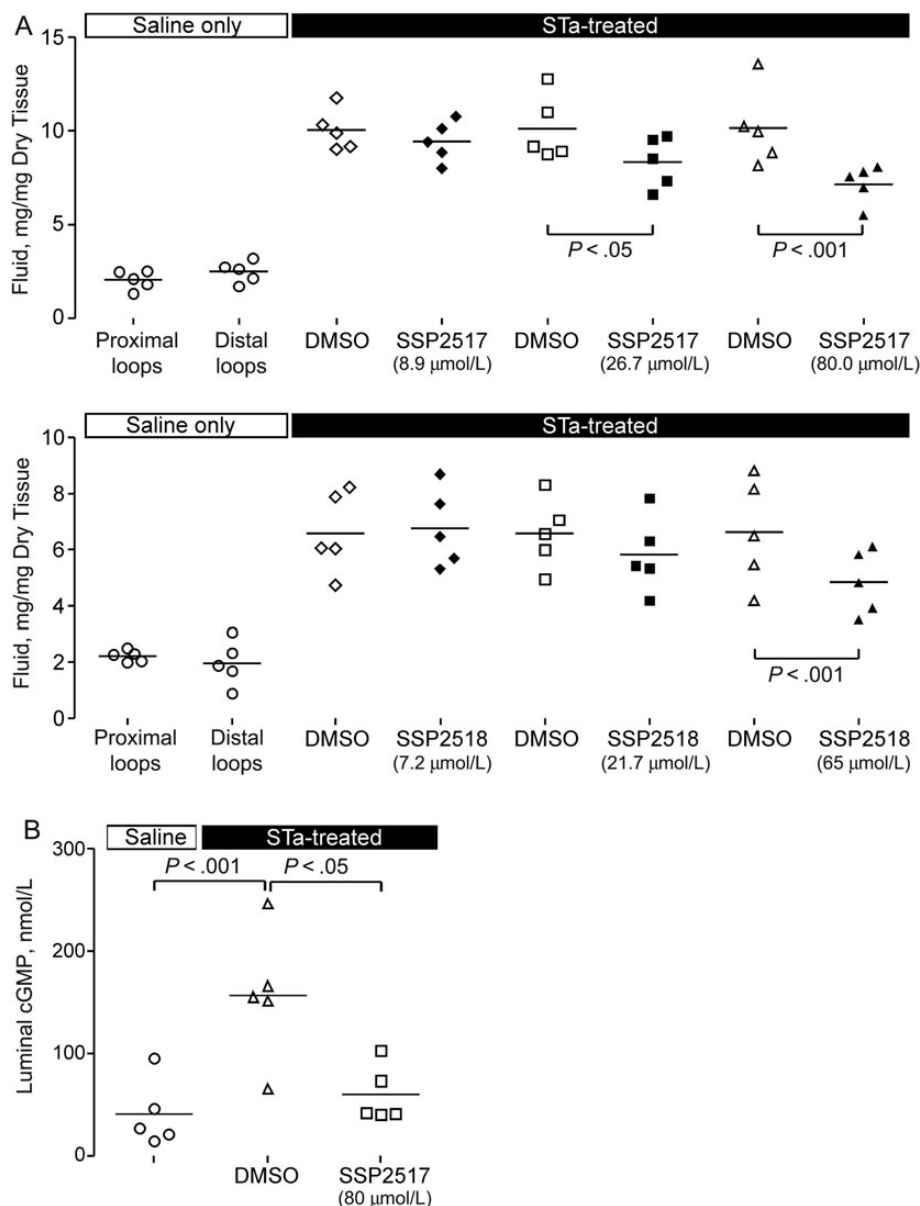
Figure 4. SSP2517 and SSP2518 inhibit the fluid secretory response elicited by the heat-stable toxin (STa) in pig jejunum, in vivo. *A*, Effect of SSP2517 and SSP2518 on fluid content of STa-treated ligated loops in pig jejunum. Each compound was tested in 5 animals, according to the procedure detailed in "Materials and Methods" section. Proximal and distal loops were treated with saline only. *B*, Effect of STa and SSP2517 treatment on the cyclic guanosine 3′,5′-monophosphate (cGMP) concentration in ligated jejunal loops. These experiments were performed in a separate group of 5 animals. Abbreviation: DMSO, dimethyl sulfoxide.

GC), a direct action on the catalytic domain is unlikely, considering that this domain in particular is highly conserved among GCs. This view is supported by the kinetic analysis of GCC inhibition by SSP2518, which suggests an allosteric mechanism.

The striking orthologue-specific inhibition profile of these leads may provide further insight into their mechanism of action. Sequence alignment shows that, relative to human and porcine GCC, the rodent orthologues contain only a limited number of amino acid substitutions. Although such subtle variations in the primary structure are found within all key

regulatory domains, it may be feasible to delimit the region that confers compound sensitivity by mutational analysis [24]. Interestingly, a few of these substitutions are found within the vicinity of a tyrosine residue just upstream of the catalytic domain. Phosphorylation of this residue by c-src suppresses catalytic activity, and inhibition of tyrosine phosphatases, by enhancing c-src phosphorylation/activation, effectively blocks STa-induced cGMP production in T84 cells [25]. Although we found that our compounds do not enhance c-src phosphorylation (Supplementary Figure 4), it is feasible that they mimic the
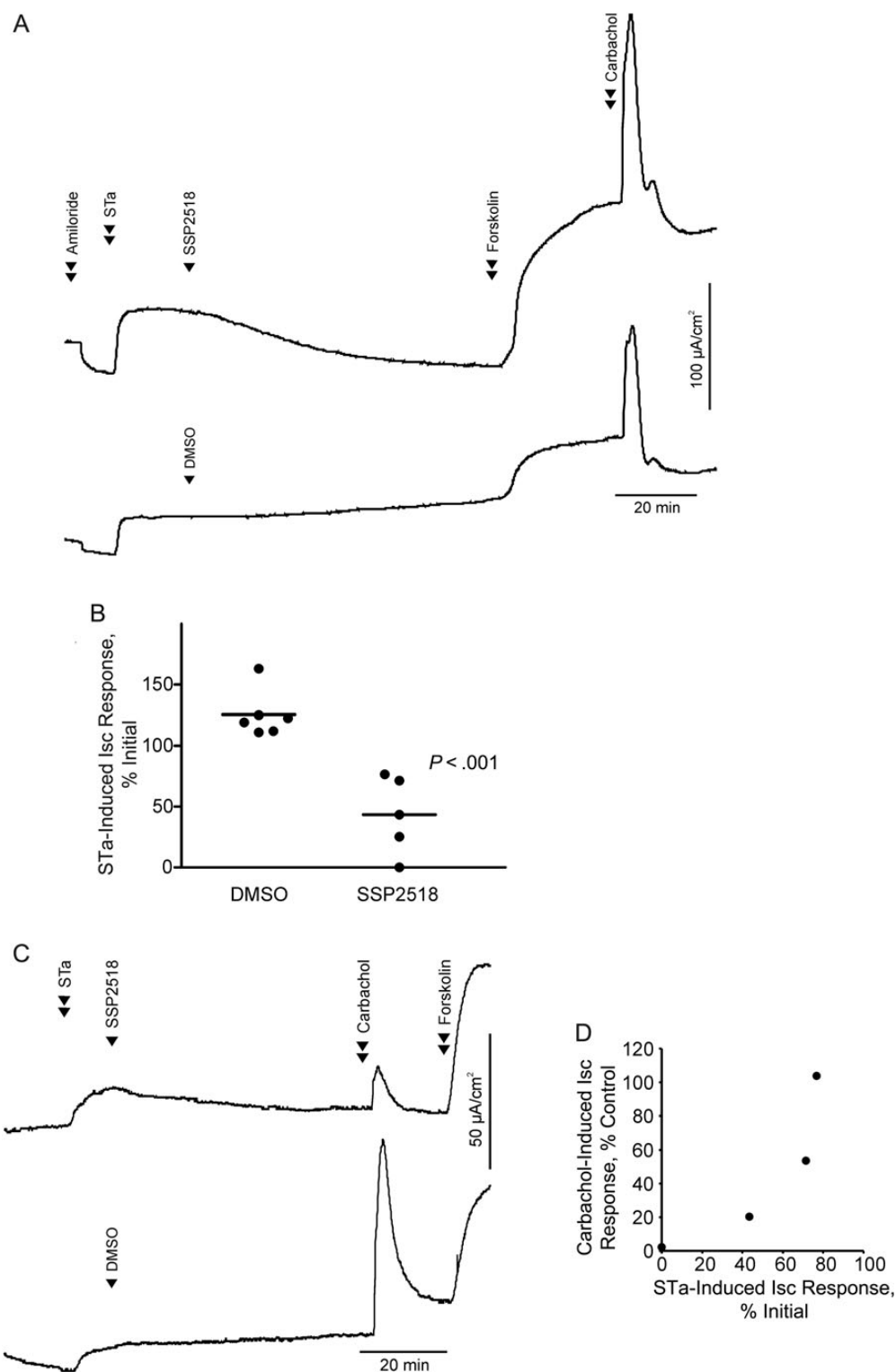
Figure 5. Guanylyl cyclase C inhibitors block the heat-stable toxin (STa)-induced chloride secretory response in human distal colon. *A*, One of 5 experiments demonstrating inhibition of the STa-induced short-circuit current (Isc) response by SSP2518. SSP2518 (30 µmol/L) or vehicle (dimethyl sulfoxide [DMSO], 0.1%) was added after the Isc response induced by STa (100 nmol/L) had reached a plateau. After 45 minutes, forskolin (10 µmol/L) was added, followed by carbachol (0.2 mmol/L). Amiloride (50 µmol/L) was added to block activity of the epithelial sodium channel (ENaC). *B*, Residual Isc responses 40 minutes after addition of SSP2518 or DMSO, relative to the Isc response, assessed just before addition of compound. *C*, When carbachol was administered before forskolin, SSP2518 inhibited the carbachol-induced as well as the STa-induced Isc response. SSP2518-treated tissue remained responsive to forskolin. *D*, Correlation between the size of the residual STa-induced Isc response in the presence of SSP2518 (data as shown in Figure 5*B*) and the size of the peak Isc response elicited by the subsequent addition of carbachol.

inhibitory effect of c-src–mediated phosphorylation by a direct interaction or enhance c-src activity by a mechanism different from phosphorylation.

Our experiments showed that the compound concentration required to attain significant inhibition of STa-provoked CFTR activity in intestinal tissue was considerably higher than in T84 cells. Conceivably, this reflects ineffective partitioning of these hydrophobic compounds in the epithelial compartment [26]. Furthermore, the putative downstream targets of cGMP in (porcine) intestine, a cGMP-dependent protein kinase (PRKG2) and a cGMP-inhibited phosphodiesterase (PDE3) that controls cAMP/PKA signaling, seem exquisitely sensitive to cellular cGMP levels [4, 27, 28]. Therefore, a considerable decrease in cGMP production may be required to reduce CFTR phosphorylation/activation.

Our experiments on pig intestinal loops show that even residual GCC activity in the presence of inhibitor may elevate intracellular cGMP levels enough to induce a substantial fluid secretory response, whereas cGMP efflux, mediated by a low-affinity transport system [29], is greatly reduced. In contrast to pig small intestine, in T84 cells, direct cross-activation of PKA by cGMP predominates [30]. Because PKA has a relatively low affinity for cGMP, a comparatively large increase in cGMP levels will be required to elicit full CFTR activation [27, 28, 30]. Congruently, in T84 cells the efficacy of CFTR inhibition (Figure 2B) matches the inhibition of cGMP production (Table 1), suggesting that cGMP production is the rate-limiting step in the Isc response.

The hGCC-specific inhibition profile of these compounds, particularly their low activity toward other cyclases, suggests that they could be valuable leads for the development of antidiarrheal drugs. Our experiments on T84 cells, pig jejunum, and human colon consistently show that these GCC blockers do not inhibit cGMP-independent AC- or Ca^{2+}-linked secretory responses. Although this is testament to their relatively specific mode of action, it also suggests that these compounds would be of limited use for treatment of secretory diarrhea caused by bacterial toxins that activate AC, for example, *Vibrio cholera* toxin (CT), or pathogens that exploit a Ca^{2+}-mediated secretory pathway.

However, Ca^{2+}-dependent secretagogues act in strong synergism with cAMP/cGMP signaling, probably because they do not activate CFTR directly but rather increase the driving force for anion extrusion [31]. In accordance, the procedure used to reduce the level of endogenous secretagogues in rectal biopsy specimens, prolonged incubation of the tissue in the presence of indomethacin and regular exchanges of the incubation medium, attenuates the response to carbachol [17]. Because of this synergism, endogenously produced Ca^{2+}-linked secretagogues may significantly amplify the ion and fluid secretory response elicited by STa, in vivo. Conversely, a reduction in cGMP levels through GCC inhibition, and a consequent reduction in CFTR phosphorylation/activation, as is effected by

SSP2518 in human distal colon, may also reduce ion and fluid secretion induced by Ca^{2+}-linked endogenous secretagogues and enterotoxins.

Furthermore, at present, we do not categorically exclude the possibility that GCC inhibition may also attenuate the secretory response provoked by enterotoxins that target AC/cAMP signaling. CT, probably by activating AC in enterochromaffin cells, causes the release of endogenous secretagogues and the activation of a local neuroendocrine signaling axis, which contributes significantly to fluid loss [1]. Conceivably, CT also stimulates (uro)guanylin release, indirectly provoking GCC activation. Congruent with this hypothesis, CT-provoked fluid secretion seems to be reduced in GCC-deficient mice [32].

Apart from management of enterotoxigenic *E. coli* infection, GCC blockers may also be of potential benefit for treating other syndromes characterized by aberrant intestinal fluid transport. It has been shown that gain-of-function mutations in GUCY2C not only cause chronic diarrhea but also predispose to the development of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [33]. Although genome-wide association studies do not link the GUCY2C locus to IBD (reviewed in [34]), and the occurrence of such mutations may therefore be restricted to a small number of families, it seems plausible that aberrant GCC signaling is more common—for example, because of an imbalance in the production of (uro)guanylin [35]. Furthermore, enterotoxigenic *E. coli* infection, though usually self-limited, may occasionally trigger prolonged intestinal distress, not unlike IBS [36].

This involvement of GCC signaling in the pathophysiology of IBD and IBS may not only reflect its well-established role in luminal hydration. In murine colitis models, GCC signaling was shown to regulate the release of proinflammatory cytokines [34, 37], and it has been proposed that GCC activity supports epithelial barrier function [38]. On this basis, GCC agonists are being considered as novel therapeutics for ulcerative colitis [39]. Collectively, these data indicate that intestinal GCC activity needs to be delicately balanced and that both protracted activation of GCC and its inappropriate silencing will lead to disease.

In conclusion, we report the development of potent and specific hGCC inhibitors, capable of blocking STa-provoked secretory responses in native intestine. Our data indicate that these compounds do not prevent STa binding but rather interrupt the formation of an active catalytic center. These lead compounds may serve the development of novel antisecretory/proabsorptive drugs, for management of infectious diarrheal diseases and, potentially, IBD and IBS.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary

data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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