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Chloride Channel-Targeted Therapy for Secretory Diarrheas

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Abstract

Secretory diarrheas caused by bacterial and viral enterotoxins remain a significant cause of morbidity and mortality. Enterocyte Cl^- channels represent an attractive class of targets for diarrhea therapy, as they are the final, rate-limiting step in enterotoxin-induced fluid secretion in the intestine. Activation of cyclic nucleotide and/or Ca^{2+} signalling pathways in secretory diarrheas increases the conductance of Cl^- channels at the enterocyte luminal membrane, which include the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated Cl^- channels (CaCCs). High-throughput screens have yielded several chemical classes of small molecule CFTR and CaCC inhibitors that show efficacy in animal models of diarrheas. Natural-product diarrhea remedies with Cl^- channel inhibition activity have also been identified, with one product recently receiving FDA approval for HIV-associated diarrhea.

Keywords

diarrhea; cholera; chloride channels; CFTR; CaCC; rotavirus

Introduction

Secretory diarrhea remains a major global health challenge, and represents the second leading cause of mortality globally in children under age 5 [1]. Repeated episodes of dehydration from diarrhea are also associated with impaired physical and mental development [2]. In developing countries major causes of secretory diarrheas include enterotoxin-producing bacteria such as *Vibrio cholerae* and enterotoxic *E coli*, viruses such as rotavirus, and enteroinvasive bacteria such as *Shigella* and *Salmonella* [1]. In developed countries secretory diarrheas are primarily caused by viruses such as rotavirus, although with the widespread use of rotavirus vaccines other pathogens such as norovirus have become increasingly prevalent [3].

Oral rehydration solution (ORS) to replace fluid losses and promote intestinal fluid absorption has been the primary therapy for secretory diarrhea, reducing mortality four-fold over the last 30 years [4]. However, there remains an unmet need for alternative and

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adjunctive antidiarrheal therapeutics, as ORS is not always effective, available or administered properly. Antisecretory drug therapy could have broad indications for infectious diarrheas in developing and developed countries, and potentially for diarrheas associated with certain cancer and HIV therapeutics [5]

Antisecretory Targets in the Intestinal Epithelium

The intestinal epithelium consists of villi and crypts, with absorption occurring mainly in villi and secretion in crypts. Fluid absorption in the small intestine is driven by the luminal Na^+/H^+ exchanger (NHE3), Na^+-glucose cotransporter (SGLT1), and Cl^-/HCO^-_3 exchanger (DRA) [6,7] (Figure 1). As in all epithelia the electrochemical driving force is established by a basolateral Na^+K^+-ATPase pump. The pro-absorptive solute transporters are constitutively active, though they can be modulated by second-messengers including cAMP and Ca^{2+} [8, 9]. NHE3, SGLT1 and DRA are thus potential membrane transporter targets to increase intestinal fluid absorption. In the colon, fluid absorption is also facilitated by the epithelial Na^+ channel (ENaC) and short-chain fatty acid (scfa) transporters (SMCT1) [10].

Intestinal fluid secretion is driven by active transepithelial Cl^- secretion, which creates the electrochemical force for paracellular Na^+ secretion and the osmotic driving force for transcellular water secretion (Figure 1). Cl^- is transported into the cell at the basolateral membrane by the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), which is driven by Na^+ and Cl^- concentration gradients produced by the Na^+K^+-ATPase and basolateral K^+ channels. The electrochemical gradient drives Cl^- secretion across the luminal membrane through CFTR and Ca^{2+}-activated Cl^- channels (CaCCs). NKCC1, CFTR, CaCCs and K^+ channels (KCNQ1/KNE3, KCNN4) are thus potential membrane transporter targets to reduce intestinal fluid secretion. The intestinal epithelium also expresses other chloride channels including ClC-2 and bestrophins [11, 12].

In addition to membrane transporters, a number of the cellular signalling molecules involved in mediating anion secretion represent potential pharmacological targets. Bacterial enterotoxins elevate cyclic nucleotides (cAMP and cGMP) [13, 14] (Figure 2A), and viral enterotoxins and some drugs elevate cytosolic Ca^{2+} [15, 16] (Figure 2B). There is thought to be significant cross-talk between cyclic nucleotide and Ca^{2+} signalling, with proposed mechanisms involving cAMP-induced Ca^{2+} elevation mediated by Epac [17], and compartmentalized Ca^{2+}-induced cAMP elevation mediated by membrane-associated Ca^{2+}-sensitive adenylyl cyclase-1 [18] (Figure 2C). Proof-of concept that these signalling pathways are potential antisecretory targets has been shown by the efficacy of a small molecule phosphodiesterase (PDE) activator, which reduces cAMP and cGMP, in a closed-loop model of intestinal fluid secretion [19]. Other potential targets include modulators of membrane macromolecular complexes such as agonists of lysophosphatidic acid (LPA) receptors, which inhibit CFTR function, or the more recently identified putative modulator MAST205 [20, 21]. Agonists of the Ca^{2+} sensing receptor CaSR have been shown to inhibit enterotoxin mediated fluid secretion [22] and act through a number of pathways including the enteric nervous system [23].

Involvement of CFTR and CaCC in Secretory Diarrhea

The involvement of the major apical Cl^- channels, CFTR and the intestinal CaCC, in secretory diarrheas is supported by numerous studies. There is strong evidence that CFTR is the Cl^- pathway in secretory diarrheas caused by the bacterial enterotoxins released in cholera and Traveler's diarrhea in both the small intestine and colon [24] (Figure 2A). Intestinal Cl^- and fluid secretion are absent in mice lacking CFTR and in CF patients [25], and colonic Cl^- transport in human tissue is effectively blocked by CFTR inhibitors [24].

The alternative, CaCC-mediated pathway, may be involved as well in these diarrheas, but likely represents the primary pathway for apical membrane Cl^- secretion in rotavirus (Figure 2B) and possibly in drug-induced secretory diarrheas [26].

Small-Molecule CFTR and CaCC Inhibitors

Three chemical classes of small-molecule CFTR inhibitors have emerged from high-throughput screening. The thiazolidinone $CFTR_{inh}$-172 [27] (Figure 3), which has been used widely in cystic fibrosis research, inhibits CFTR by binding at or near arginine-347 and stabilizing the channel closed-state [28]. The IC_{50} for inhibition of CFTR Cl^- current by $CFTR_{inh}$-172 ranges from ~300 nM to several μM depending on cell type and membrane potential. $CFTR_{inh}$-172 has low toxicity and is excreted with minimal metabolism [29]. Studies in mouse models of cholera and STa toxin-induced intestinal fluid secretion have demonstrated $CFTR_{inh}$-172 efficacy [24]. Structure–activity studies have identified thiazolidinones with greater water solubility than $CFTR_{inh}$-172 [30], including an analogue containing a 4-tetrazolophenyl in place of the 4-carboxyphenyl in $CFTR_{inh}$-172 that has shown efficacy in mouse models of polycystic kidney disease (PKD) [31].

PPQ/BPO compounds (Figure 3) are a second class of absorbable CFTR inhibitors with cytoplasmic site-of-action [32]. The IC_{50} is ~90 nM for PPQ-102 inhibition of CFTR Cl^- conductance. Structure-activity studies yielded BPO-27, which contains structural changes that greatly increase its metabolic stability, inhibition potency and aqueous solubility [33]. The IC_{50} for CFTR inhibition by (racemic) BPO-27 is ~8 nM. Chiral separation yielded an active R-enantiomer of BPO-27 with IC_{50} ~ 4 nM, with the S-enantiomer being inactive [32]. PPQ-102 and BPO-27 have shown efficacy in models of PKD, but have not been tested in diarrhea models.

Glycine hydrazides such as GlyH-101 (Figure 3) are a third class of CFTR inhibitors that target the CFTR pore on its extracellular surface [34]. Patch-clamp analysis showed a characteristic signature of an extracellular pore blocking inhibitor, including a linear current–voltage relationship that becomes inwardly rectifying following GlyH-101, with rapid single-channel flicker. CFTR inhibition by a membrane-impermeant PEG-hydrazide conjugate [35], and molecular modelling [36], further supported an extracellular site-of-action, which provides a unique opportunity to develop non-absorbable compounds for antisecretory therapy. The GlyH-101 analog iOWH032 (Figure 3), which weakly inhibits CFTR (IC_{50} ~ 8 μM), is in clinical trials [37]. However, it is theoretically unlikely that a low-affinity small-molecule glycine hydrazide will have antisecretory efficacy because of predicted rapid washout (by convection) of an externally targeted inhibitor (see below), and the poor inhibition potency of glycine hydrazides at interior-negative membrane potentials.

In an attempt to address the washout/potency liabilities, several non-absorbable macromolecular conjugates were synthesized containing a malonic acid hydrazide (MalH) CFTR-inhibiting moiety, including a MalH-lectin conjugate [38] (Figure 3). MalH-lectin conjugates had IC_{50} down to 50 nM and remained bound to CFTR for many hours, as compared to seconds for GlyH-101 or iOWH032. The improved potency of the MalH-lectin conjugate and its resistance to washout is likely due to trapping in the enterocyte glycocalyx. Multivalent MalH-PEG conjugates were also synthesized with nanomolar CFTR inhibition potency [35]. The development potential of these lectin and PEG conjugates is unclear.

Motivated by the potential efficacy of CaCC inhibitors for some secretory diarrheas, a phenotype-based small molecule screen was done using the human colonic cell line HT-29 [39]. Several classes of CaCC inhibitors were identified, the most potent being the 3-acyl-2-aminothiophene $CaCC_{inh}$-A01 (Figure 3). $CaCC_{inh}$-A01 fully inhibited CaCC-dependent halide flux in different intestinal cell lines and in response to different agonists, with IC_{50}

down to 1 μM, and was shown recently to prevent watery diarrhea in a neonatal mouse model of secretory diarrhea (unpublished observations). Subsequent target-based screening yielded TMEM16A-selective inhibitors [40], the most potent being $T16A_{inh}$-A01 (Figure 3). Though TMEM16A is not a major enterocyte CaCC, it is the principal CaCC in interstitial cells of Cajal and required for intestinal motility [41].

Convective Washout Reduces Efficacy of Surface-Targeted Cl^- Channel Inhibitors

As mentioned above, a concern for drugs with an extracellular target in intestinal crypts is convective drug washout, which reduces drug efficacy (Figure 4A). A convection-diffusion model was developed recently of drug washout in an anatomically accurate 3-dimensional model of the human intestine [42]. The model predicted greatly reduced inhibitor efficacy for rapid crypt fluid secretion as occurs in cholera. Figure 4B shows a single-crypt computation in which inhibitor efficacy in reducing fluid secretion is plotted as a function of inhibitor concentration (relative to its binding dissociation constant, K_d). Whereas 50% inhibition of fluid secretion occurs for inhibitor concentration ~ K_d when secretion rate is low, orders of magnitude greater inhibitor concentration is needed to prevent fluid secretion at high secretion rates as in cholera. It was concluded that the antisecretory efficacy of an oral, membrane-impermeant, surface-targeted inhibitor requires high inhibitor affinity (low nanomolar K_d) in order to obtain sufficiently high luminal inhibitor concentration (> 100-fold K_d), and sustained high luminal inhibitor concentration *or* slow inhibitor dissociation. Convective washout considerations are relevant to glycine hydrazide- and some natural-product-based Cl^- channel-targeted therapies.

Natural-Product Cl^- Channel Inhibitors

Natural products have been identified with antidiarrheal efficacy in humans and a putative mechanism of action involving Cl^- channel inhibition. Crofelemer, a heterogeneous proanthocyanidin oligomer extracted from the bark latex of South American tree *Croton lechleri*, was approved recently for HIV-associated diarrhea following clinical trials showing efficacy in reducing the number and severity of diarrhea episodes [43]. Investigation of the antisecretory mechanism of crofelemer revealed weak and partial (maximum ~60 %) inhibition of CFTR, though complete inhibition of CaCC with IC_{50} < 10 μM [44]. Whether CaCC inhibition by crofelemer can explain its efficacy in HIV-associated diarrhea is unclear. Crofelemer has not been tested in animal models having defined diarrheas.

Following a natural product screen that identified tannic acid as a general CaCC inhibitor, we found that red wines containing polyphenolic gallotannins fully inhibited intestinal CaCC without effect on CFTR [45]. In recent follow-up work, we generated an alcohol-free red wine extract with potent CaCC inhibition activity, and showed its efficacy in a neonatal mouse model of rotaviral diarrhea (unpublished data). The wine extract inhibited intestinal Ca^{2+}-activated Cl^- current and fluid secretion without affecting rotaviral infection of intestinal epithelial cells. CaCC inhibition may account for anecdotal reports of antidiarrheal action of red wines. Motivated by the possibility that known herbal antidiarrheal remedies might act by Cl^- channel inhibition, we recently screened a selection of diarrhea remedies from sources worldwide and identified a commonly used Thai herbal remedy that fully inhibited both CFTR and CaCC (unpublished observations). The herbal remedy showed efficacy in mouse models of cholera and rotaviral diarrhea. Chemical analysis of active ingredient(s) is in progress. Natural products thus represent a potentially inexpensive and immediately available therapy for secretory diarrheas with a defined mechanism of action.

Conclusions

Antisecretory drug therapy has considerable potential in reducing morbidity and mortality associated with infectious and some drug-induced and other diarrheas. Because severe secretory diarrhea is largely a concern in developing countries, challenges in drug development include the need for very low cost and high stability in a hot /humid environment, as well as obtaining funding to support commercial development of a new chemical entity with relatively low profit potential. The development or repurposing of existing natural products, such as wine or herbal extracts, is of particular interest based on their low cost and immediate availability for clinical testing against a variety of pathogens. The overall human and economic cost of diarrheal disease globally justifies a multi-factorial approach that includes pharmacological therapies as well as improvements in access to ORS, education, vaccination and sanitation.

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Highlights

cAMP (CFTR) and Ca^{2+}-activated (CaCC) Cl^- channels are expressed on enterocytes.

Enterocyte Cl^- channels are activated in major infectious secretory diarrheas.

Cl^- channel-targeted therapeutics for secretory diarrheas are emerging.

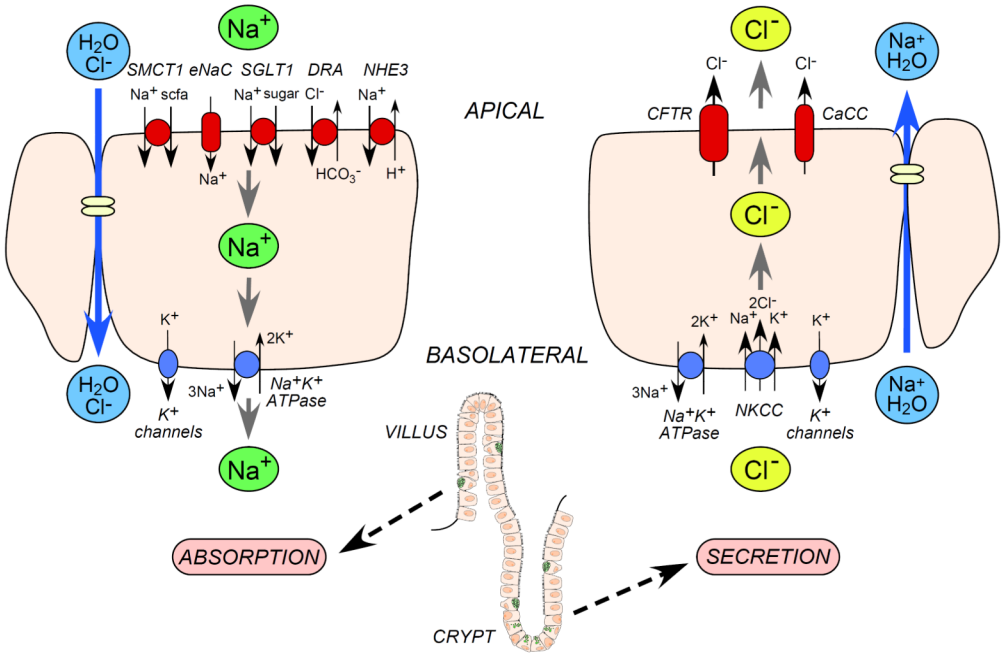

Figure 1.

Intestinal transport mechanisms. *Left*. Fluid absorption, which occurs primarily in villus epithelial cells, involves active transcellular Na^+ transport of sodium via apical membrane transporters and channels and the basolateral Na^+/K^+ ATPase, which drives passive Cl^- and water flux. *Right*. Fluid secretion, which occurs primarily crypt epithelial cells, involves active transcellular Cl^- transport from the basolateral side via the NKCC transporter and apical Cl^- transport channels, with corresponding passive Na^+ and water flux.

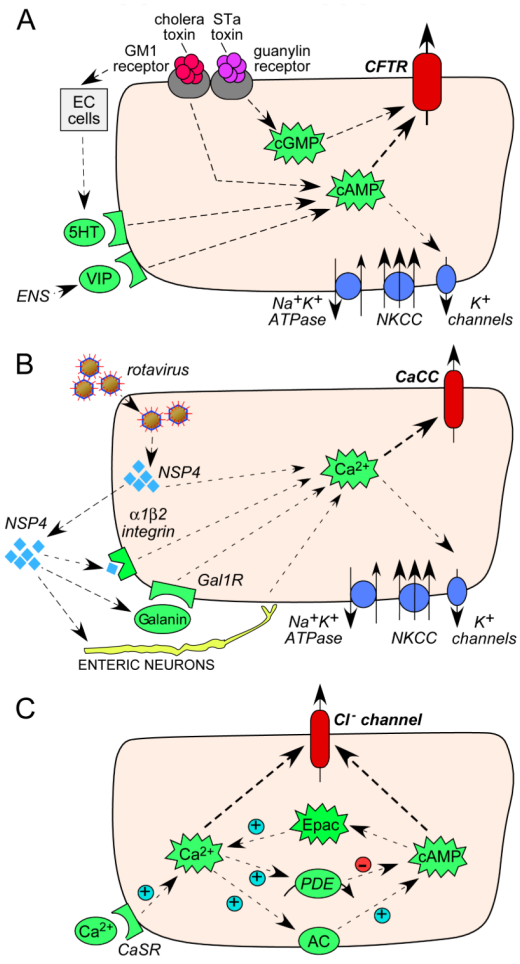

Figure 2.

Intestinal signal pathways controlling fluid secretion. A. Signaling pathways in CFTR activation by bacterial enterotoxins. Cholera toxin and heat stable enterotoxin (STa) bind to membrane receptors (GM1– ganglioside receptor, guanylin receptor) causing increases in cyclic nucleotides (cAMP, cGMP) and neurotransmitters, resulting in CFTR activation. EC – enterochromaffin cells, 5-HT – 5-hydroxytryptamine, VIP – vasoactive intestinal peptide, ENS – enteric nervous system. B. Signaling pathways in CaCC activation by rotavirus. Rotavirus releases NSP4 (non-structural protein 4), which causes elevation of cytoplasmic Ca^{2+} either: directly via binding to a membrane receptor (integrin $\alpha1\beta2$); via neuropeptide galanin; or through activation of enteric nerves. Gal1-R – galanin 1 receptor. **C.** Cross-talk between Ca^{2+} and cAMP pathways in intestinal epithelial cells. Epac – exchange protein directly activated by cAMP, PDE – phosphodiesterase, AC – adenylate cyclase, CaSR – calcium sensing receptor.

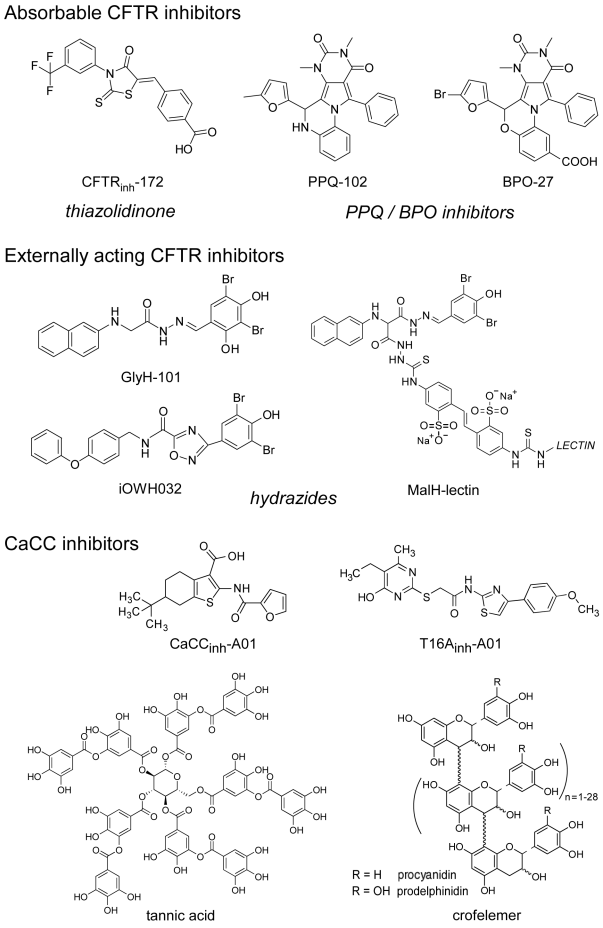

Figure 3.

Chemical structures of CFTR and CaCC inhibitors. Absorbable CFTR inhibitors include thiazolidiones and PPQ/BPO inhibitors. Externally acting CFTR inhibitors include hydrazide derivatives. Small-molecule and macromolecular CaCC inhibitors shown.

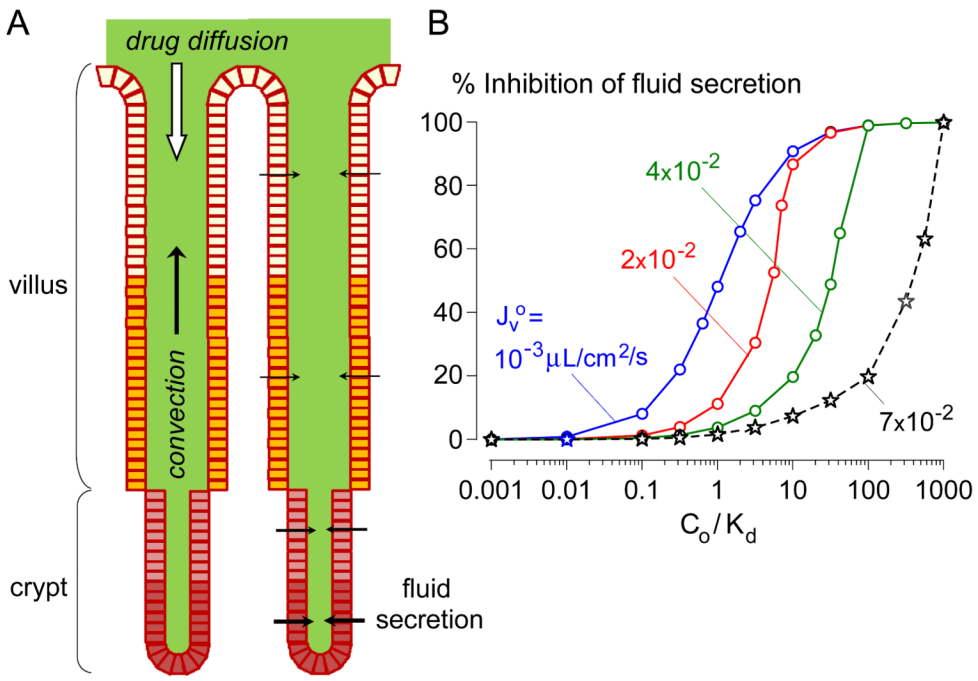

Figure 4.

Convective washout reduces the efficacy of enterocyte surface-targeted Cl^- channel inhibitors. A. Schematic of epithelial cell-lined crypt-villus units. Fluid secretion into the lumen produces convective (upward) solute transport opposing drug diffusion. B. Convective inhibitor washout requires a high concentration of a membrane-impermeant inhibitor in the intestinal lumen for antisecretory efficacy. Computations were done for a single crypt with human mid-jejunal anatomy. Percentage inhibition of net secreted fluid as a function of C_o/K_d (lumen inhibitor concentration / inhibitor dissociation constant) for indicated J^o_v (single-crypt fluid secretion in the absence of inhibitor). $J^o_v \sim 7 \times 10^{-2}$ v μL/cm^2/s is typical in cholera. Adapted from ref. 43.