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4,8-Dimethylcoumarin inhibitors of intestinal anion exchanger slc26a3 (down-regulated in adenoma, DRA) for anti-absorptive therapy of constipation

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KEYWORDS: Constipation, cystic fibrosis, SLC26A3, DRA

ABSTRACT: The chloride/bicarbonate exchanger SLC26A3 (down-regulated in adenoma, DRA) is expressed mainly in colonic epithelium where it dehydrates the stool by facilitating the final step of chloride and fluid absorption. SLC26A3 inhibition has predicted efficacy in various types of constipation including that associated with cystic fibrosis. We previously identified, by high-throughput screening, 4,8-dimethylcoumarin inhibitors of murine slc26a3 with IC_{50} down to ~150 nM. Here, we synthesized a focused library of forty-three 4,8-dimethylcoumarin analogs. Structure-activity studies revealed the requirement of 4,8-dimethylcoumarin-3-acetic acid for activity. The most potent inhibitors were produced by replacements at C7, including 3-iodo- (**4az**) and 3-trifluoromethyl- (**4be**), with IC_{50} of 40 nM and 25 nM, respectively. Pharmacokinetics in mice showed predicted therapeutic concentrations of **4az** for >72 h following a single 10 mg/kg oral dose. **4az** at 10 mg/kg fully normalized stool water content in a loperamide-induced mouse model of constipation. The favorable inhibition potency, selectivity within the SLC26 family and pharmacological properties of **4az** support its further preclinical development.

INTRODUCTION

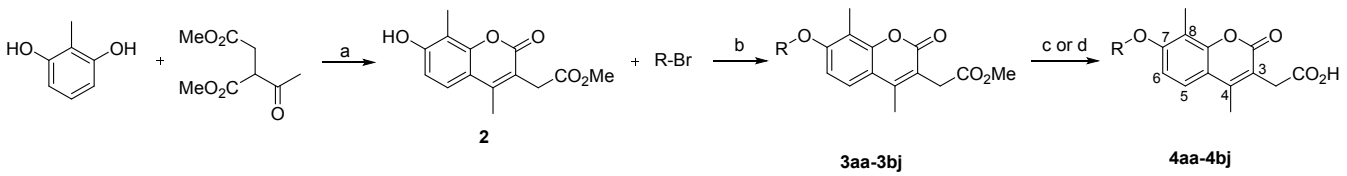
Constipation is a common clinical problem affecting ~15% of the US population,[1] with at least 3-fold greater prevalence in cystic fibrosis (CF) due to impaired function of the pro-secretory chloride channel CFTR in the intestine.[2] Currently available treatments for chronic constipation include dietary and lifestyle changes, over-the-counter medications such as osmotic and stimulant laxatives, and FDA-approved prescription drugs that stimulate intestinal fluid secretion.[3-4] The approved pro-secretory drugs lubiprostone,

linaclotide and plecanatide activate CFTR and perhaps apical membrane chloride channels indirectly, and showed limited efficacy in clinical trials.[4] These drugs are unlikely to be effective in CF because they primarily rely on functional CFTR. We recently identified activators of wild-type CFTR with pro-secretory action that showed greater efficacy than lubiprostone and linaclotide in mouse models of constipation.[5-6] The CFTR activators, as expected, were not effective in CF mice lacking functional CFTR. An alternative approach for increasing stool hydration in constipation is inhibition of intestinal fluid absorption. Tenapanor, an inhibitor of the sodium-hydrogen exchanger 3 (NHE3) in small intestine and proximal colon,[7] recently completed a Phase 3 clinical trial for constipation predominant irritable bowel syndrome (IBS-C), showing limited efficacy,[4] perhaps due to intact fluid absorption in more distal parts of the intestine. There remains an unmet need for more efficacious anti-constipation drugs with alternative mechanisms of action for the general population and particularly for CF subjects.

The SLC26A3 protein, originally named down-regulated in adenoma (DRA), is a chloride/anion exchanger expressed most strongly at the luminal plasma membrane of intestinal epithelial cells in colon.[8-10] On the basis of the finding that loss-of-function mutations in *SLC26A3* in humans cause chloride-losing diarrhea,[11] as does *slc26a3* knockout in mice,[12] SLC26A3 inhibition, by reducing colonic fluid absorption and thus blocking terminal step of stool dehydration, is predicted to be effective as an anti-absorptive therapy for all forms of constipation, including that associated with cystic fibrosis.

Using a cell-based high-throughput screen, we recently identified 4,8-dimethylcoumarin inhibitors of the slc26a3 anion exchanger.[13] The most potent compound was 4,8-dimethyl-7-(*m*-bromobenzyloxy)-coumarin-3-acetic acid **4ba**, which reversibly inhibited SLC26A3-mediated anion transport with IC_{50} ~150 nM and showed efficacy in a mouse model of constipation. Here, following an initial analysis of 176 commercially available analogs of the originally identified inhibitors, we synthesized a focused library of forty-three 4,8-dimethylcoumarin analogs, which included a compound that inhibited slc26a3 selectively with IC_{50} of ~25 nM and had favorable pharmacological properties and efficacy in an experimental mouse model of constipation.

Scheme 1. Synthesis of 4,8-dimethylcoumarin analogs[a]



[a]Reagents: (a) H_2SO_4, MeOH, 86%. (b) K_2CO_3, acetone, reflux, 62-99%. (c) NaOH, MeOH, 75 °C, 19-96%. (d) LiOH, MeCN, rt, 28% for **4aq**, 27% for **4ar**.

RESULTS

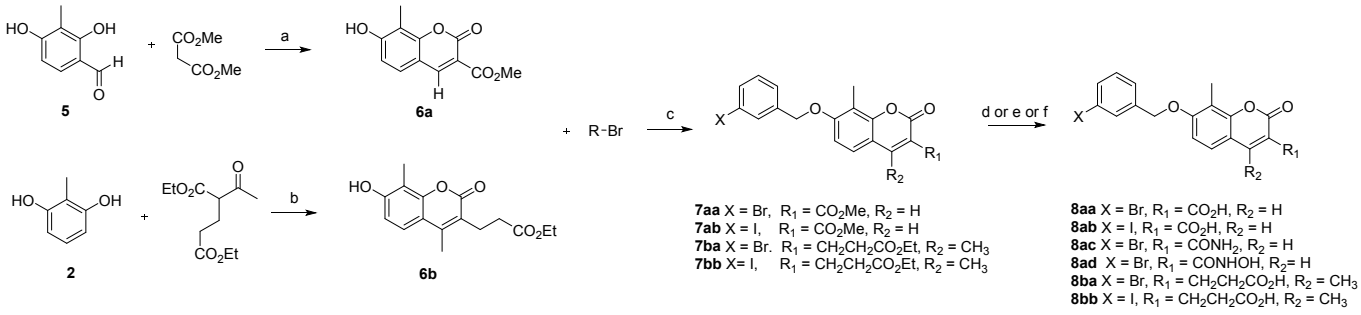
Preliminary structure-activity relationship (SAR) analysis. Initially, 176 commercially available coumarin analogs of our originally reported inhibitor **4ba** (DRA_{inh}-A250) were tested.[13] SAR revealed the requirement of methyl substitution at C4 and C8,

and an acetic acid moiety at C3, with C7 substituents producing a wide range of activity. Of 80 compounds purchased with different C7 substituents (representative compounds shown in Figure S1), 7-hydroxy- (**S1**), 7-methyl- (**S2**) and 7-methoxy-coumarin (**S3**) were inactive, as were styrenyloxy- (**S11**), naphthylmethyloxy- (**S14**), isoprenoxy- (**S13**), and several annulated coumarins and analogs containing an additional fused ring. Based on the initial SAR, compounds having different C7 substitutions were synthesized, as well as compounds with acetic acid replacements.

Synthesis. Scheme 1 shows the synthesis of **4aa-4bj**. Pechmann reaction of 2-methyl resorcinol with dimethyl acetylsuccinate in the presence of sulfuric acid afforded 4,8-dimethyl-7-hydroxycoumarin ester **2** in 86% yield. *O*-Alkylation of **2** with alkyl bromides (**3aa-3aj**) or aryl methylbromides (**3ak-3ap, 3at-3bj**) gave substituted benzyloxy-4,8-dimethylcoumarins **3aa-3bj** in 28-98% yields, which were hydrolyzed with 1N sodium hydroxide in methanol to give **4aa-4bj** in 52-96% yields. For the benzenesulfonyl analogs **3aq** and **3ar**, substitution of **2** with *m*-Cl-, or *m*-CH_3-benzenesulfonyl chloride with potassium carbonate give **3aq** and **3ar** in 68 and 71% yields, respectively. Hydrolysis of esters **3aq** and **3ar** was accomplished with lithium hydroxide in acetonitrile to afford **4aq** and **4ar**.

Scheme 2 shows the synthesis of formic acid derivatives **8aa-8ad**. Methyl 8-methyl-7-hydroxybenzopyranone-3-carboxyl-ate **6a** was prepared by condensation of 2,4-dihydroxy-3-methylbenzaldehyde **5** with dimethyl malonate under sulfuric acid condition. Alkylation of **6a** with substituted benzyl bromide afforded **7aa** and **7ab**, which were hydrolyzed to give **8aa** and **8ab**. Aminolysis of **7aa** with ammonia gas in THF gave amide analog **8ac**. Hydroxamic acid analog **8ad** was synthesized by reaction of ester **7aa** with hydroxylamine under basic conditions. For the propionic acid analogs, Pechmann type reaction with 2-methyl resorcinol and diethylacetyl glutarate under acidic conditions afforded **6b**. *O*-Alkylation of **6b** with bromo- or iodobenzyl bromide gave **7ba-7bb** which upon hydrolysis under basic conditions gave **8ba-8bb**.

Scheme 2. Synthesis of 8-methylcoumarin carboxylate analogs[a].



[a]Reagents: (a) H_2SO_4, MeOH, 57%. (b) H_2SO_4, EtOH, 81%. (c) K_2CO_3, acetone, reflux, 86-93%. (d) NaOH, MeOH, 75 °C, 44-94%, for **8aa-8bb**. (e) NH_3 in THF, 66% for **8ac**. (f) NH_2OH-H_2O, NaOMe, MeOH, 54% for **8ad**.

SAR analysis for inhibition of slc26a3-mediated chloride/iodide exchange. All synthesized analogs were tested for inhibition of slc26a3-mediated chloride/iodide exchange using a cell-based kinetic assay in which fluorescence was measured in Fischer rat thyroid cells expressing (murine) slc26a3 and a yellow fluorescent protein halide sensor (YFP) following extracellular addition of iodide.[8]

Table 1 summarizes slc26a3 inhibition data for compounds with different C7 substituents.

Slc26a3 inhibition was compared for analogs containing various alkyl, allyl, heterocyclic, benzenesulfonyl, and substituted benzyl groups at C7. Propyl (**4ad**) or allyl (**4aa**) analogs showed no inhibition at 5 μM. Inhibition with micromolar IC_{50} was seen with elongated alkyl chains, including butoxy-, pentoxy- and hexyloxy- analogs (**4ae-4ag**). Analogs containing cyclic alkyl chains (**4ah-4aj**) showed mildly reduced inhibition compared to linear alkyl analogs (**4ad-4ag**). To investigate heterocyclic analogs, substituted pyridines, pyrrol and 5-trifluoromethylfuran were studied. Pyridine (**4ak-4an**) and pyrrol (**4ap**) analogs were inactive at 5 μM, with only the 5-trifluoromethylfuran analog (**4ao**) showing strong activity with IC_{50} of 0.3 μM, similar to that of original compound DRA_{inh}-A250 (**4ba**). To investigate benzyl analogs, *o*-, *m*- and *p*-halobenzyl bromides were studied (**4at-4bc**). *p*-Substituted halobenzyl analogs (**4at-4av**) showed no inhibition at 5 μM, whereas *o*-substituted halobenzyl analogs (**4aw-4ay**) showed weak inhibition, with IC_{50} of 4.5 μM for *o*-iodobenzyl (**4aw**), 10 μM for *o*-bromobenzyl (**4ax**), and 18 μM for *o*-chlorobenzyl (**4ay**) analogs. Notably, *m*-substituted halobenzyl analogs showed strong inhibition, with IC_{50} of 0.04 μM for iodobenzyl **4az**. With the knowledge that meta-substitution increased inhibition activity, we studied *m*-nitro **4bd**, *m*-trifluoromethyl **4be**, and *m*-cyclopropyl **4bf** analogs. *m*-Nitrobenzyl **4bd** was less potent (IC_{50} 0.5 μM) than **4bb**, and *m*-cyclopropylbenzyl **4bf** was more potent (0.07 μM), with *m*-trifluoromethylbenzyl **4be** having the greatest potency with IC_{50} of 0.025 μM. For comparison, the commercially available *m*-methylbenzyl analog **4bk** had IC_{50} of 0.5 μM. The 3,5-dimethylbenzyl analog **4bg** had much reduced potency compared to its mono-substituted analog **4bk**, and 3,5-bistrifluoromethylbenzyl analog **4bh** (IC_{50} 0.3 μM) was much less potent than monosubstituted **4az**. Additional F-substitutions (**4bi, 4bj**) containing *m*-trifluoromethylbenzyl mildly reduced inhibition compared to **4az**, with additional *m*-substitution (**4bj**) showing greater inhibition than *o*-substitution (**4bi**). *m*-Trifluoromethylphenethyl analog **4as** showed no inhibition at 5 μM. Benzenesulfonyl analogs **4aq** and **4ar** showed no inhibition at 5 μM.

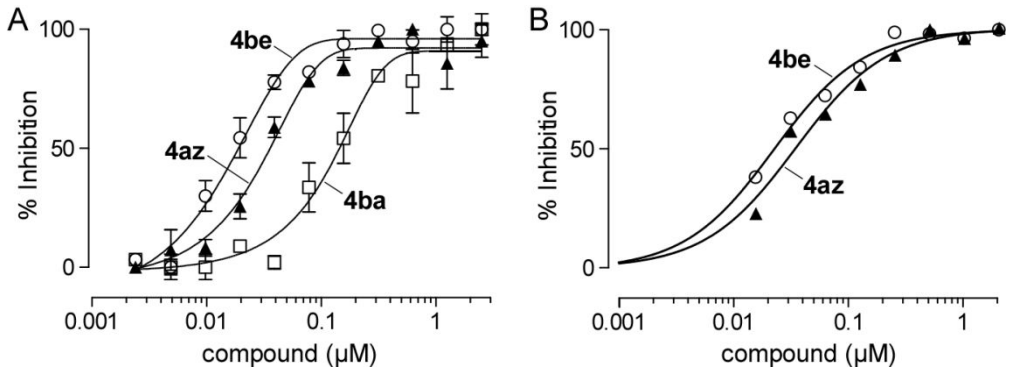
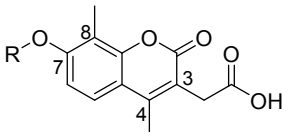
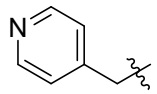
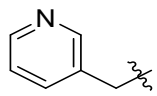
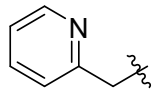
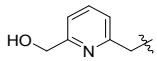
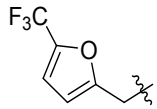
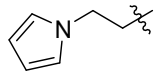
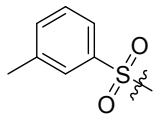
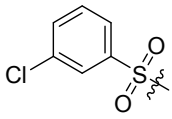
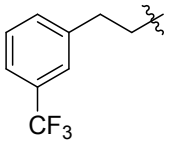
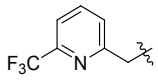


Figure 1. Concentration dependent slc26a3-mediated anion exchange inhibition data. (A) Inhibition of slc26a3-mediated Cl^- / I^- exchange by indicated compounds (mean ± S.E.M., n = 3). (B) Inhibition of slc26a3-mediated Cl^- / HCO_3^- exchange (mean ± S.E.M., n = 12–28 individual cell regions analyzed from 3 or more separate replicates). Curves are data fits to single-site inhibition model.

Table 1. Inhibition of DRA (slc26a3-mediated Cl^-/I^- exchange) by **4aa-4bl**.



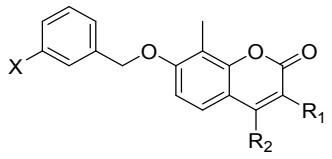
Compound	R	Isolated Yield (%)	IC_{50} (μM)[a]	Compound	R	Isolated Yield (%)	IC_{50} (μM)[a]
4aa	Allyl-	60	>5	**4at**	4-Iodobenzyl	84	>5
4ab	$CH_3OCH_2CH_2$-	19	>5	**4au**	4-Bromobenzyl	75	>5
4ac	$HO_2CCH_2CH_2$-	66	>5	**4av**	4-Chlorobenzyl	79	>5
4ad	$CH_3CH_2CH_2$-	76	>5	**4aw**	2-Iodobenzyl	68	4.5±1.3
4ae	$CH_3(CH_2)_2CH_2$-	76	1.5±0.3	**4ax**	2-Bromobenzyl	59	10±2.5
4af	$CH_3(CH_2)_3CH_2$-	96	1.0±0.4	**4ay**	2-Chlorobenzyl	63	18±3
4ag	$CH_3(CH_2)_4CH_2$-	94	1.5±0.4	**4az**	3-Iodobenzyl	56	0.04±0.003
4ah	$(CH_2)_4CH$-	85	4.5±2.0	**4ba**	3-Bromobenzyl	60	0.15±0.02
4ai	$(CH_2)_3CHCH_2$-	76	3.4±0.8	**4bb**	3-Chlorobenyzl	73	1.0±0.5
4aj	$(CH_2)_4CHCH_2$-	77	1.8±0.5	**4bc**	3-Fluorobenzyl	62	0.9±0.4
4ak		23	>5	**4bd**	3-Nitrobenzyl	52	0.5±0.1
4al		28	>5	**4be**	3-(CF_3)-benzyl	90	0.025±0.01

4am		31	>5	**4bf**	3-cyclopropyl-benzyl	88	0.07±0.003
4an		45	>5	**4bg**	3,5-dimethyl-benzyl	84	15±3
4ao		59	0.3±0.05	**4bh**	3,5-bis(CF3)-benzyl	79	0.3±0.1
4ap		79	>5	**4bi**	2-F, 5-(CF_3)-benzyl	78	0.1±0.1
4aq		28	>5	**4bj**	3-F, 5-(CF_3)-benzyl	68	0.06±0.05
4ar		27	>5	**4bk**[b]	3-CH_3-benzyl	-	0.5±0.2
4as		88	>5	**4bl**		34	>5

a) Data representative of 3 replicates. b) Purchased from ChemDiv.

Next, the acetic acid portion on C3 was investigated. Preliminary data showed that acetic acid had much greater inhibition potency than its corresponding ester, and that benzyl or alkyl in place of acetic acid reduced inhibition. To determine the best acid substituents on C3, we tested formic acid and propionic acid analogs with Br- or I-substituted 7-oxybenzyl-8-dimethylcoumarin. As listed in Table 2, replacement of acetic acid on C3 with formic acid (**8aa-8ab**) or propionic acid (**8ba-8bb**) reduced inhibition. Interestingly, hydroxamic acid **8ad** had IC_{50} of 0.45 μM, whereas its corresponding acid **8aa** was inactive. To compare with **4ba**, the corresponding amide of **8aa**, namely **8ae**, showed much reduced inhibition, supporting the importance of the acetic acid moiety for inhibition. To investigate inhibitor selectivity, **4be** and **4az** were chosen for further studies.

Table 2. Inhibition of DRA (slc26a3-mediated Cl^-/I^- exchange) by **8aa-8ae**, **8ba-8bb**.



Compound	X	R_1	R_2	Isolated Yield (%)	IC_{50} (μM)[a]
4ba	Br	CH_2CO_2H	Me	60	0.15±0.02
8aa	Br	CO_2H	H	44	>5
8ab	I	CO_2H	H	62	>5
8ac	Br	$CONH_2$	H	66	>5
8ad	Br	CONHOH	H	34	0.45±0.1
8ae	Br	CH_2CONH_2	Me	57	3.3±0.4
8ba	Br	$CH_2CH_2CO_2H$	Me	89	>5
8bb	I	$CH_2CH_2CO_2H$	Me	94	>5

a) Data representative of 3 replicates.

Inhibition of DRA (slc26a3)-mediated chloride / bicarbonate exchange. The concentration-dependence measurements reported in Table 1 and 2 were done for inhibition of slc26a3-mediated chloride/iodide exchange. Fig. 1A shows representative data obtained using the YFP quenching assay, which fitted well to a single-site inhibition model with IC_{50} of 0.040 and 0.025 μM for **4az** and **4be**, respectively. For chloride / bicarbonate exchange, which is the physiologically relevant transport process in colon, cytoplasmic pH was measured in slc26a3-expressing FRT cells using the pH-sensitive fluorescent indicator BCECF.[9] BCECF-labeled cells were incubated with a chloride- and bicarbonate containing solution, after which a chloride-free, bicarbonate-containing solution was added. The rate of cytoplasmic alkalinization was inhibited by **4az** and **4be** with IC_{50} of 0.035 μM and 0.023 μM, respectively (Fig.

1B). In control experiments, no change in cytoplasmic pH was observed in cells not expressing slc26a3, or in experiments where bicarbonate was replaced with chloride in exchange solutions (not shown).

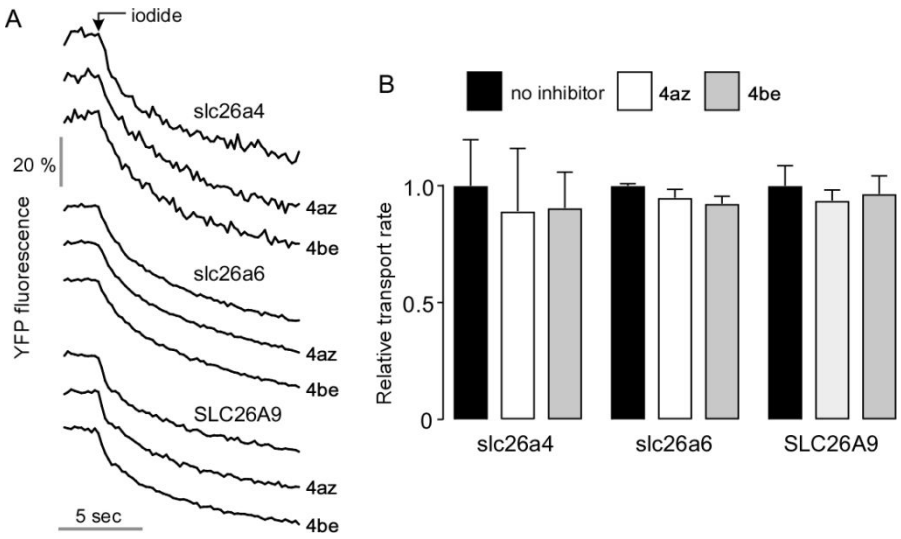


Figure 2. Selectivity of slc26a3 inhibitors. Original data curves (A) and summary (B) of relative inhibition of slc26a3 homologs by **4az** and **4be** (mean ± S.E.M., n = 3). Differences not significant.

DRA inhibition selectivity of 4az and 4be. To study selectivity, anion exchange assays were done on related solute carrier 26 family members, including (murine) slc26a4 (pendrin; ~48 % amino acid identity to slc26a3), (murine) slc26a6 (PAT-1; ~38 % amino acid identity), and (human) SLC26A9 (~39 % amino acid identity). Chloride/iodide exchange was not significantly inhibited by 10 μM **4az** or **4be** in cells expressing each of these solute carrier family 26 members, with original fluorescence time course data shown in Fig. 2A and summary data in Fig. 2B. Based on the inhibition activity, aqueous solubility and preliminary in vitro microsomal stability test (data not shown), **4be** and **4az** were chosen for further studies including in vivo efficacy.

Pharmacokinetics in mice. Fig. 3 shows the serum concentrations of **4ba**, **4be** and **4az** after single dose (5 mg/kg for **4ba** and **4be**, 10 mg/kg for **4az**) oral or intraperitoneal administration at zero time. **4ba** had levels well above the IC_{50} determined in vitro for at least 24 h after single dose oral or intraperitoneal administration. **4be** had high serum levels initially, but disappeared relatively rapidly with little compound remaining at 16 h. **4az** had predicted therapeutic concentrations for several days after single dose oral or intraperitoneal administration, with serum concentration of **4az** of approximately 1 μM even at 72 h. The deduced pharmacokinetic parameters for **4ba**, **4be** and **4az** are given in Table 3. Computed oral bioavailability values were (mean ± S.E.M.): 54 ± 6.5% (**4ba**), 16 ± 7% (**4be**) and 39 ± 4% (**4az**).

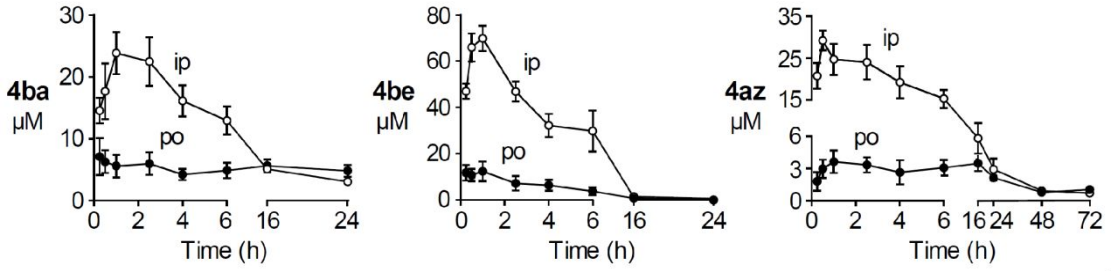


Figure 3. Pharmacokinetics. Serum concentrations of **4ba**, **4be** and **4az** in mice after single dose intraperitoneal or oral administration at 5 mg/kg (**4ba** and **4be**) or 10 mg/kg (**4az**). Mean ± S.E.M., 3 mice per group. ip: intraperitoneal, po: per oral.

Table 3. Pharmacokinetic parameters of slc26a3 inhibitors.

Compound	Route	C_{max} (μM)	T_{max} (h)	AUC (μmol*h/L)
4ba	Oral	9.4 ± 1.8	5.8 ± 5.1	124 ± 15
	IP	24.3 ± 3.1	1.5 ± 0.5	231 ± 33
4be	Oral	12.9 ± 3.7	0.8 ± 0.2	69 ± 28
	IP	70.2 ± 5.0	0.8 ± 0.2	418 ± 78
4az	Oral	4.7 ± 0.5	6.5 ± 4.8	130 ± 12
	IP	29.1 ± 2.3	0.5 ± 0	331 ± 42

Efficacy in a mouse model of constipation. Opioids such as loperamide slow gut motility[14] and are commonly used to induce constipation in animal models as they have direct relevance to opioid-induced constipation, one of the most common types of constipation. Fig. 4A shows the experimental model in which slc26a3 inhibitor was administered orally 1 hour prior to intraperitoneal injection of loperamide, with stool collected over the following 3 hours. As shown in Fig. 4B, **4be** administration partially prevented constipation in loperamide-treated mice as quantified by improved 3-hour stool weight, pellet number and water content. The efficacy of **4be** was similar to that found previously for original inhibitor **4ba**.[13] Notably, **4az** administration fully normalized stool water content in loperamide-treated mice (Fig. 4C), even at a low dose of 3 mg/kg. In control mice not treated with loperamide, **4az** had no significant effect on stool parameters compared to vehicle treatment.

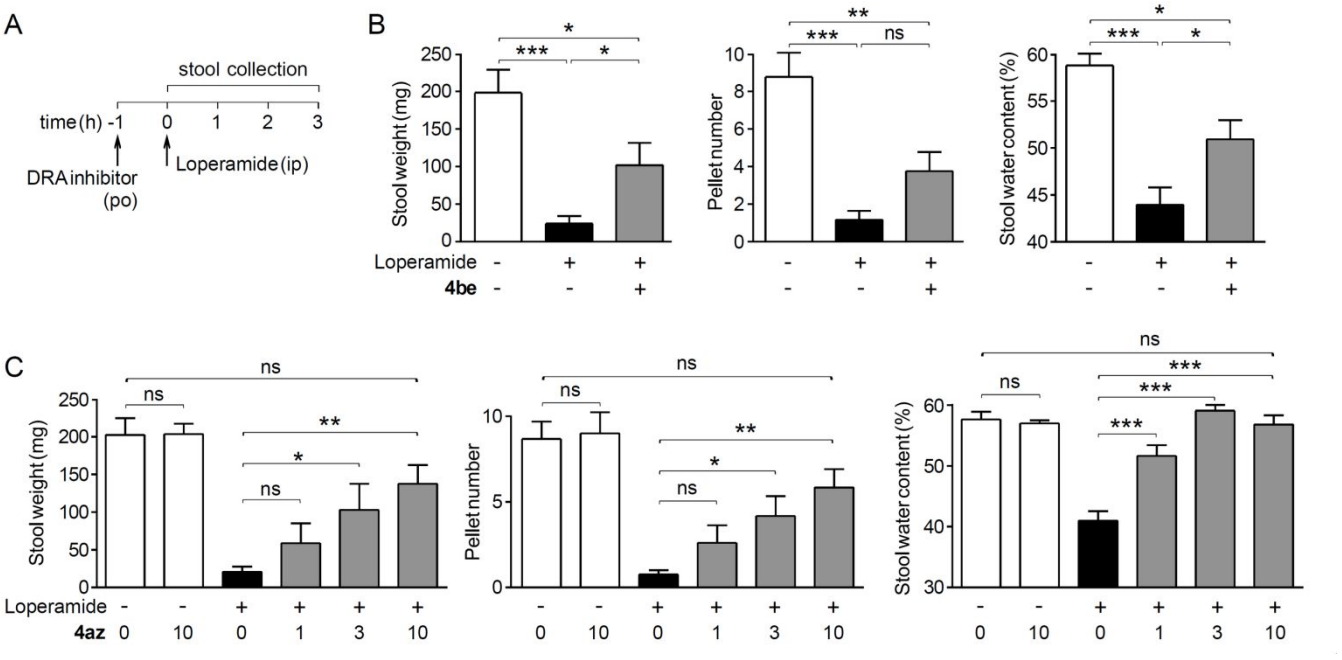


Figure 4. Inhibitor efficacy in a loperamide-induced constipation model in mice. **A.** Experimental protocol. **B. 4be** at 10 mg/kg improved 3-hour stool weight, pellet number and water content in loperamide-treated mice (mean ± S.E.M., 4-6 mice per group). **C.** Dose-dependence of **4az** (in mg/kg) effect on 3-hour stool weight, pellet number and water content in loperamide-treated mice (5-8 mice per group, comparisons made with one-way analysis of variance with post-hoc Newman Keuls multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant ($p \geq 0.05$). ip: intraperitoneal, po: per oral).

DISCUSSION AND CONCLUSION

The goal of this study was to advance 4,8-dimethylcoumarin inhibitors of the slc26a3 anion exchange protein for anti-absorptive therapy of constipation. SAR analysis of commercially available 4,8-dimethylcoumarin analogs as well as a focused library of synthesized compounds identified compounds with ~10-fold improved potency compared with reference compound **4ba**, with good pharmacokinetic properties and efficacy in a mouse model of constipation when administered orally. A low oral dose (1 mg/kg) of **4az** produced greater than 50% normalization of stool hydration in loperamide-treated mice, suggesting effective compound accumulation in enterocytes in colon. It is not possible, however, to directly relate serum concentrations to efficacy in constipation because we do not know the concentration of free compound in serum, the intracellular compound concentration, and whether the compound accumulates in enterocytes by delivery from the luminal or basolateral surfaces of the colonic epithelium. We speculate, though do not know with certainty, that the 4,8-dimethylcoumarins act from the cytoplasmic surface of slc26a3 because they were effective when administered intraperitoneally (data not shown) and their onset of action was not immediate in cell cultures in vitro.[13]

Depending on their physicochemical properties, slc26a3 inhibitors could accumulate at their target site, the colonic epithelium, by delivery from the luminal membrane with minimal systemic absorption, or by absorption in small intestine and delivery from the basolateral membrane.

Compounds **4be**, **4az** and **4ba** have favorable drug-like properties according to the Lipinski's rule of 5,[15] with molecular mass less than 500 Da (406, 464, and 416, respectively), tPSA of 72.8, pKa of 3.70, cLogP of 4.6-4.8, and multiple hydrogen bond acceptors and donors. In general, coumarins are polyphenolic derivatives found in plants that contain a benzopyranone moiety. They are considered as phytoalexins with diverse cytoprotective and modulatory activities including antimicrobial, antiviral, anticancer, anti-inflammatory, antioxidant and central nervous system activities.[16] Prior studies have provided information on the coumarin substituents (C3, 4, 7 and 8) involved in the various biological activities. For example, 3-acetic acid coumarin analogs have MRSA antibacterial activity,[17] DNA gyrase inhibition,[18] ATPase inhibition,[19] antituberculosis activity,[20] and inhibition of NF-kB/DNA interaction.[21] 3-carboxamide coumarin analogs inhibit monoamine oxidase.[22, 23] The most widely used coumarin-containing drug is warfarin.[24] Warfarin and its analogs contain a hydroxyl group at C4, in which its replacement by sulfhydryl, halogen or hydrogen abolished anticoagulant activity.[25] The compounds reported herein are not expected to have anticoagulant activity because they do not contain a 4-hydroxy group. For the C7 substituent on coumarin, 6,7-annulated coumarins were reported as inhibitors of NF-kB/DNA interaction,[26, 27] and C7 biaryl coumarin analogs as antibacterials.[28]

This study builds on our original identification, by high-throughput screening, of the first selective inhibitors of anion exchanger SLC26A3.[13] Compounds **4be** and **4az** synthesized and characterized herein fully inhibited slc26a3-mediated anion exchange with IC_{50} down to 25 nM. **4az** gave sustained therapeutic concentration in serum for over 72 h after single-dose oral or intraperitoneal administration, and fully normalized stool hydration in a loperamide mouse model of constipation. Having favorable inhibition potency in vitro and in vivo, selectivity within the SLC26 family, and pharmacokinetics, **4az** is suitable candidate for further preclinical development.

EXPERIMENTAL DETAILS

General synthesis procedure.

All chemicals and solvents were used as purchased and used without further purification. Reaction progress was monitored by TLC using pre-coated silica gel plates with fluorescence at 254 nm and ethylacetate/*n*-hexane solvent system. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance 300 instrument using $CDCl_3$, CD_3OD, trifluoroacetic acid-*d*, acetic acid-d_4, or DMSO-d_6 as solvent. Chemical shifts are given in parts per million (ppm). HRMS was performed using a hybrid quadrupole Orbitrap mass analyzer, QExactive (Thermo, Bremen, Germany) with an electrospray ionization (ESI) source. The mass resolution was set as 70000 at m/z 200, and mass accuracy >3 ppm. Purity of all final compounds was 95% or higher as determined by high performance liquid

chromatography (HPLC) using UV absorbance at 254 nm. HPLC was done on an Xterra MS C18 column (2.1 mm × 100 mm, 3.5 μm) with 0.2 mL/min water/acetonitrile (containing 0.1% formic acid), 25 min linear gradient, 5−95% acetonitrile.

Methyl 2-(7-hydroxy-4,8-dimethyl-2-oxo-2H-chromen-3-yl) acetate (2).

To a solution of 2-methylresorcinol (5.0 g, 40 mmol) and dimethyl 2-acetylsuccinate (7.6 g, 40 mmol) in absolute methanol (40 mL) was added 20 mL of sulfuric acid at 0 °C for 1 h. The reaction mixture was stirred at room temperature for 24 h and then the mixture was poured into water. The resulting precipitate was collected by filtration, washed with water, and dried at 50 °C under vacuum overnight to give an off-white solid (9.0 g, 86% yield) of the desired product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.14 (d, 1H, J = 8.2 Hz), 6.65 (d, 1H, J = 8.2 Hz), 3.81 (s, 3H), 3.74 (s, 2H), 2.32 (s, 3H), 2.21 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 162.3, 157.3, 151.7, 150.1, 122.5, 114.9, 113.1, 111.8, 111.5, 52.6, 32.7, 15.3, 7.9; LRMS: 263 ($M + H^+$).

Methyl 2-(7-((3-bromobenzyl)oxy)-4,8-dimethyl-2-oxo-2H-chromen-3-yl)acetate (3ba)

A mixture of **2** (200 mg, 0.8 mmol), bromobenzyl bromide (270 mg, 1.1 mmol), and potassium carbonate (210 mg, 1.5 mmol) in acetone (10 mL) was heated at reflux overnight. The reaction mixture was cooled to rt and poured into ice water. The resulting precipitate was collected by filtration, washed with methanol, and dried to give 170 mg (96%) of the product as white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.61 (brs, 1H), 7.51-7.48 (m, 1H), 7.46 (d, 1H, J = 8.9 Hz), 7.39 (d, 1H, J = 8.0 Hz), 7.29 (t, 1H, J = 7.8 Hz), 6.87 (d, 1H, J = 8.9 Hz), 3.75 (brs, 2H), 3.73 (s, 3H), 2.39 (brs, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 161.8, 158.6, 151.6, 149.0, 138.8, 131.2, 130.2, 130.0, 125.5, 122.76, 122.72, 116.5, 114.5, 114.4, 108.1, 69.5, 52.2, 32.7, 15.3, 8.4; LRMS: 431 ($M + H^+$).

2-(7-((3-Bromobenzyl)oxy)-4,8-dimethyl-2-oxo-2H-chromen-3-yl)acetic acid (4ba)

To a solution of methyl ester **3ba** (160 mg, 0.4 mmol) in methanol (8 mL) was added NaOH (25 mg, 0.6 mmol) in water (2 mL) and refluxed for 1 h. The mixture was cooled to room temperature, diluted with water, and acidified with 1N HCl. The resulting precipitate was collected by filtration, washed with water and dried. Final product was recrystallized in methanol to give the product as a white powder (105 mg, 68%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.70 (brs, 1H), 7.65 (d, 1H, J = 9.0 Hz), 7.55 (d, 1H, J = 8.4 Hz), 7.51 (d, 1H, J = 7.9 Hz), 7.41 (d, 1H, J = 7.9 Hz), 7.12 (d, 1H, J = 9.0 Hz), 5.29 (s, 2H), 3.51 (s, 2H), 2.34 (s, 3H), 2.26 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.0, 161.4, 158.4, 151.1, 149.1, 140.1, 131.2, 130.4, 126.7, 124.0, 122.2, 117.8, 114.4, 112.9, 109.2, 69.3, 52.2, 15.5, 8.5; HRMS for $C_{20}H_{17}BrO_5$: calcd, 417.0332; found, 417.0330 ($M + H^+$).

Mice

Mice were bred at the University of California San Francisco (UCSF) Animal Facility. Animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Pharmacokinetics

Pharmacokinetics of **4ba, 4be** and **4az** were determined in mice. Following single-dose intraperitoneal or oral administration of **4ba** (5 mg/kg, in saline with 5% DMSO and 10% Kolliphor HS 15), **4be** (5 mg/kg, same vehicle as **4ba**) or **4az** (10 mg/kg, in PBS with 5% DMSO and 20% (2- hydroxypropyl)-β-cyclodextrin), blood samples were collected via retroorbital puncture at specified time points and serum was separated by centrifugation at 4000 *g* for 20 min at 24 °C. Compound concentrations were measured in serum using LC/MS. Oral bioavailability was calculated by using area under the curve (AUC) for oral and ip routes as AUC (oral) / AUC (ip).

Loperamide constipation model

Female CD1 mice (age 8–10 weeks) were administered loperamide (0.3 mg/kg, intraperitoneally, in 5% ethanol in PBS, 0.1 mg/mL final concentration) to produce constipation or vehicle in control mice. **4be** (5 mg/kg), **4az** (1, 3 or 10 mg/kg, in PBS with 5% DMSO and 20% (2-hydroxypropyl)-β-cyclodextrin) or vehicle were administered by oral gavage 1 h before loperamide. After loperamide injection, mice were placed individually in metabolic cages with free access to food and water. Stool samples were collected for 3 h, and total stool weight and number of fecal pellets were quantified. To measure stool water content, stool samples were dried at 80° C for 24 h and water content was calculated as [wet weight-dry weight]/wet weight. [29, 30]

Anion exchange assays

Assay of (murine) slc26a3-mediated chloride/iodide exchange was done as described[8] in which FRT rat thyroid epithelial cells expressing slc26a3 and halide-sensitive EYFP-H148Q/I152L/F46L were incubated in phosphate buffered saline (PBS) prior to addition of iodide-substituted PBS (140 mM NaCl substituted with NaI). The rate of fluorescence quenching was determined as a quantitative measure of chloride/iodide exchange. Measurement of chloride/bicarbonate exchange was done as described[14] in which intracellular pH in FRT cells expressing slc26a3 was measured using the pH-sensitive probe BCECF in a bicarbonate-containing buffer following chloride replacement by gluconate. Transporter selectivity was studied as described[13] in which FRT cells containing slc26a transporters were preincubated with 0 or 10 μM **4az** or **4be** for 10 min prior to anion exchange assay.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Structures of commercial slc26a3 inhibitors, and synthesis details and spectroscopic characterization of new molecules (PDF)

Molecular strings and data for all final inhibitor candidates (CSV).

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Author Contributions

SL performed synthesis and compound analysis, some transport assays, and LC/MS for pharmacokinetic analysis. OC performed murine constipation and pharmacokinetic studies. PMH performed some transport assays. All authors designed experiments, analyzed data and wrote the manuscript. OC, PMH and ASV developed the original concept for this study.

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ABBREVIATIONS

DRA, down-regulated in adenoma; SAR, structure-activity relationship; AUC, area under the curve; ip, interperitoneal; po, per oral; slc26a3, solute carrier 26 family member a3; YFP, yellow fluorescent protein halide sensor; BCECF, 2',7'-Bis(carboxy ethyl)-5(6)-carboxyfluorescein; FRT, Fischer rat thyroid epithelial cells.

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