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Synthesis of phenylpyrimidinones as guanylyl cyclase C inhibitors

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Diarrhea is one of the most important causes of mortality in the developing world, being responsible for 2.5 million deaths each year. Many of these deaths are caused by enterotoxigenic strains of bacteria, like *Escherichia coli,* that produce enterotoxins that cause acute watery diarrhea, commonly defined as secretory diarrhea. Studies on symptomatic patients indicate a high prevalence of enterotoxigenic *E. coli* strains producing the heat-stable toxin, STa. STa is a small, cysteine-rich peptide that binds to the extracellular receptor domain of guanylyl cyclase C (GCC), located at the luminal membrane of intestinal epithelial cells. 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. From a mechanistic viewpoint, reduction of GCC activity offers an efficient approach to limit enterotoxigenic *E. coli*– provoked secretory diarrhea. Inhibition of GCC-mediated cGMP production would not only reduce anion secretion, but would also restore NHE3 activity, resulting in a comprehensive antidiarrheal action. In the present study, two novel phenylpyrimidinone derivatives were simultaneously synthesized and tested for their ability to block STa-induced CFTR activity in T84 cells.

1. Introduction

Diarrhea is one of the principal causes of mortality in children in the developing world, being responsible for 2.5 million deaths per year (Guerrant et al. 2002; Kosek et al. 2003), and about 60% of these deaths are caused by enterotoxigenic strains of *Escherichia coli* (Qadri et al. 2005) producing the heat-stable toxin STa. STa, a small cysteine-rich peptide, which binds to intestinal epithelial cell membrane receptor, guanylyl cyclase type C (GCC), and activates this enzyme in order to increase the synthesis of cyclic guanosine 3',5'-monophosphate (cGMP) (Field et al. 1978; Hughes et al. 1978; Vaandrager 2002). GCC is a transmembrane protein characterized by an extracellular receptor domain, a short membrane span and an intracellular catalytic domain (Schulz et al. 1990). Natural endogenous ligands of GCC are peptide hormones guanylin and uroguanylin, which regulate secretion of salts and fluids by the intestine (Forte et al. 2004). Upon stimulation of GCC, elevated levels of cGMP induce activation of a cGMP-dependent protein kinase and of a chloride-ion channel, cystic fibrosis transmembrane conductance regulator (CFTR). Activation of CFTR increases transport of chloride into the intestinal lumen and accumulation of water and sodium ions, thus causing diarrhea (Vaandrager 2002). 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 (Donowitz et al. 2012). 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 (Jaleel et al 2004; Tian et al. 2008; Zhang et al. 1999; Kots et al. 2008). Among the most studied chemical classes, already known as GCC inhibitors some 6-phenylpirimidinone derivatives (SSP2517, SSP2518 and SSP2506) (Fig. 1) were found to strongly reduce STa-provoked cGMP accumulation in T84 cells (Bijvelds et al. 2015).

With the purpose to obtain synthetic analogues of SSP2506 (Bijvelds et al. 2015), we focused our attention on the variation of the biological activity arising from the modification of the anchoring position of the propyl chain between the piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one residue and the pyrimidinone ring.

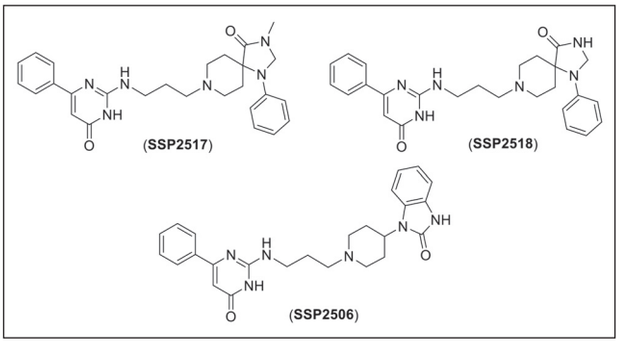


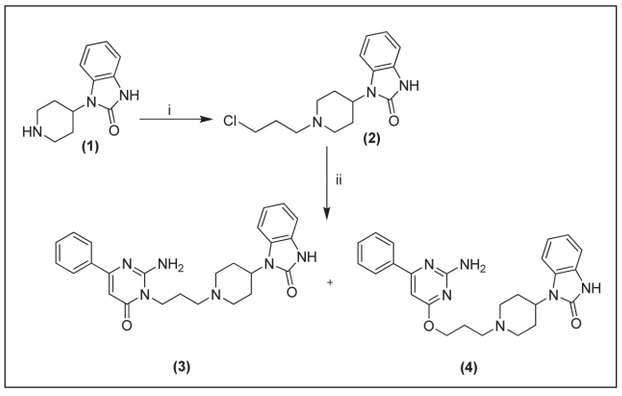
Fig. 1: Chemical structures of SSP2517, SSP2518 and SSP2506.

2. Investigations, results and discussion

2.1. Chemistry

We developed a convenient and efficient synthetic strategy reported in the Scheme. Surprisingly, the designed synthetic procedure led to the simultaneous preparation of 1-(1-(3-(2-amino-6-oxo-4-phenylpyrimidin-1(6*H*)-yl)propyl)piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one (**3**) and its *O*-alkylated derivative, 1-(1-(3-(2-amino-6-phenylpyrimidin-4-yloxy)propyl)piperidin-4-yl)-1*H*-benzo[d]imidazol-2(3*H*)-one

(**4**). Briefly, the intermediate 1-(1-(3-chloropropyl)piperidin-4-yl)-1*H*-benzo[d]imidazol-2(3*H*)-one (**2**) was prepared starting from 1-(piperidin-4-yl)-1*H*-benzo[d]imidazol-2(3H)-one (**1**) by reaction with 1-bromo-3chloropropane in anhydrous tetrahydrofuran under N_2 atmosphere at 60 °C. Subsequent condensation of compound **2** with the 6-phenylisocitosine, performed in CH_3CN in the presence of K_2CO_3 and NaI, under reflux, provided a 50:50 mixture of the two regioisomers. Compounds **3** and **4** were separated by chromatography on silica gel column and further purified by crystallization from the appropriate solvent.



Scheme: Reagents and conditions: (i) $Br(CH_2)_3Cl$, THF, N_2, 80°C, 6h (ii) 6-Phenylisocitosine, K_2CO_3, NaI, CH_3CN, 80 °C, 12h.

The newly obtained compounds gave satisfactory elemental analyses and were characterized by ^1H NMR, ^{13}C NMR and mass spectrometry (API 2000 Applied Biosystem). NMR and MS data were consistent with the proposed structures. Moreover, interpretation of ^1H and ^{13}C 1D NMR, COSY, HSQC and HMBC 2D NMR experiments allowed the assignment of all resonances of the protons and carbons for compounds **3** and **4**.

2.2. Structural analysis

More in detail, distinction between the structures of the compounds **3** and **4** was primarily made on the basis of their ^1H and ^{13}C NMR spectra. Taking the isomeric structures **3** and **4**, the N-CH_2 protons of **3** resonated at δ H 3.93, whereas the O-CH_2 protons signal of **4** appeared downfield at δ 4.35, due to the greater electronegativity of oxygen. Significant changes in chemical shift were also seen for the pyrimidinic protons. In compound **3**, the 5-H resonates at

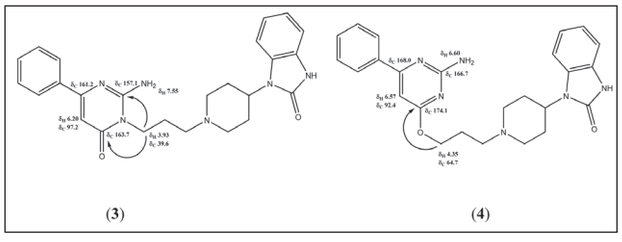


Fig. 2: Key HMBC correlations (arrows) for the compounds **3** and **4**.

δ 6.20; in **4**, the corresponding signal was at δ 6.57 reflecting the greater aromatic character of the alkoxyisocytosine. The chemical shifts in the ^{13}C NMR spectra showed analogous trends. In fact, in compound **3** the CH_2 from the propyl chain linked to N-isocytosine resonates at δ 39.6; whereas, in the O-linked isocytosine **4** the corresponding peak was at δ 64.7. Furthermore, the HMBC correlations of CH_2 to δ H 3.93 of compound **3** with C-2 and C-4 of the isocitosine residue indicated the binding of methylene to N-3 of isocitosine, while the HMBC correlations of CH_2 to δ H 4.35 of compound **4** with C-4 to d_C 174.1 confirmed the structure of compound **4** (Fig. 2).

2.3. In vitro inhibitory activity in cGMP accumulation stimulated by STa in T84 cells

Both the compounds were then dissolved in DPBS and *in vitro* tested on human colorectal carcinoma cells (T84 cells) in order to verify their ability to block STa-induced CFTR activity. In particular, cells were treated with vehicle (DPBS with 0.1% DMSO) or compounds in DPBS for 10 minutes. Then, cGMP accumulation was stimulated with *E. coli* enterotoxin (STa, 1 mM) for 10 min and successively cGMP was extracted with 0.1 M HCl and measured by ELISA.
For these experiments, we previously ruled out the possibility that the compounds acted through stimulation of cGMP degradation, evaluating the effect of these compounds at 50 mM in T84 cells stimulated with STa in the presence of the phosphodiesterase inhibitor isobutyl-methylxanthine (data not shown).
Our results shows that the *O*-alkylated derivative **4**, compared to the N-alkylated derivative **3**, was characterized by a higher inhibitory activity in cGMP accumulation stimulated by STa in T84 cells. In fact, the IC_{50} observed in our study were 0.7 mM (log IC_{50} 6.1) and 0.5 mM (log IC_{50}: 6.2) for compounds **3** and **4**, respectively (Figs. 3 and 4). These values are similar to that reported for the reference compound SSP2506 with an IC_{50} of 0.4 mM (log IC_{50}: 6.7) (Bijvelds et al. 2015).

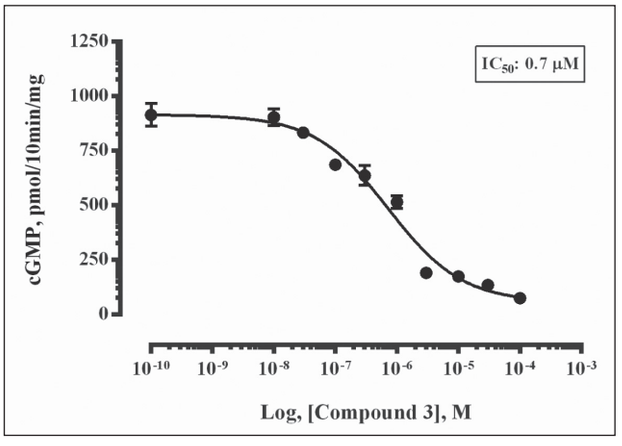


Fig. 3: Concentration effect curve of compound **3** on cGMP accumulation in cells. T84 cells were pre-treated with 0.1-100 µM of compound or vehicle (0.1% DMSO) for 10 min and then treated with 1 µM of STa for 10 min. Intracellular cGMP was assayed in the extract. Results are presented as mean±SEM. *n*= 3.

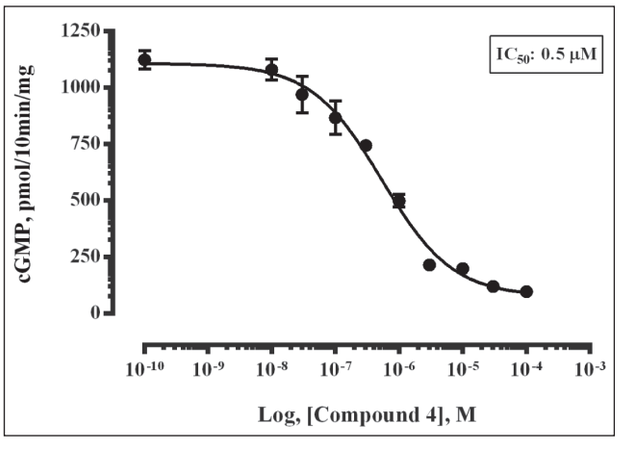


Fig. 4: Concentration effect curve of compound **4** on cGMP accumulation in cells. T84 cells were pre-treated with 0.1-100 µM of compound or vehicle (0.1% DMSO) for 10 min and then treated with 1 µM of STa for 10 min. Intracellular cGMP was assayed in the extract. Results are presented as mean±SEM. *n*= 3.

3. Discussion

The data obtained allow us to outline some structural considerations useful in the development of new lead compounds for this target. In particular, it is evident that modifying the anchoring posi-

tion of the propyl chain the inhibitory activity is maintained. Moreover, as previously shown (Stewart-Ruiz et al. 2002; Parkinson et al. 1997), adenine nucleotides are not required for ligand activation suggesting that these novel phenylpyrimidinones could act through an allosteric mechanism of GCC inhibition. Furthermore, in these derivatives, differently from previously synthesized phenylpyrimidinones (Bijvelds et al. 2015), a primary amine is present, in analogy with 2-chloroadenosine, a purine derivative which is also reported in literature as GCC inhibitor. Finally these molecules can represent another valid tool to better elucidate the molecular mechanism regulating GCC and intestinal fluid homeostasis.

4. Experimental

4.1. Chemistry

4.1.1. Material and methods

All reagents were commercial products purchased from Aldrich. Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Agilent INOVA spectrometer; chemical shifts were referenced to the residual solvent signal (DMSO-d_6: δH = 2.49, δC = 39.0). Homonuclear ^{1}H connectivities were determined by COSY experiments. Two and three bond ^{1}H-^{13}C connectivities were determined by gradient 2D HMBC experiments optimized for a ^{2,3}J of 8 Hz.
Chemical shifts are reported in ppm using Me_4Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet), q (quartet), qt (quintet), dd (double doublet), bs (broad singlet), m (multiplet). Mass spectra of the final products were performed on API 2000 Applied Biosystem mass spectrometer. Where analyses are indicated only by the symbols of the elements, results obtained are within ± 0.4% of the theoretical values. All reactions were followed by TLC, carried out on Merck silica gel 60 F_{254} plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Solutions were dried over Na_2SO_4 and concentrated with Buchi rotary evaporator at low pressure.

*4.1.2. Synthesis of 1-(1-(3-chloropropyl)piperidin-4-yl)-1H-benzo[d] imidazol-2(3H)-one (**2**)*

A mixture of 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one (**1**) (4.36 g, 0,02 mol) and 1-bromo-3chloropropane (1.05 g, 0.00667 mol) in anhydrous tetrahydrofuran (70 ml) was stirred for 6 h at 60 °C under N_2 atmosphere. After evaporation, the residue was dissolved in CH_2Cl_2. The solution was washed with water, dried on anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography using dichloromethane/methanol 9:1 (v/v) as eluent. The combined product fractions were evaporated, yielding 1.750 g (90 %) of the desired product as a oil; mp 230-232 °C; ^{1}H NMR (500 MHz, DMSO-d_6, *J* in Hz) δ 1.43 (m, 2H); 1.88 (quint, 2H); 2.26 (m , 2H); 2.43 (t, 2H, *J=6.2*); 2.59 (m, 2H); 2.94 (m, 2H); 3.46 (m, 1H); 3.70 (t, 2H, *J=6.2*); 6.94 (m, 2H); 7.10 (m, 2H); 8.2 (s, 1H).
ESI-MS: 294.3 $[M+H]^+$.
Anal. ($C_{15}H_{20}ClN_3O$), C, H, N.

*4.1.3. Synthesis of 1-(1-(3-(1,6-dihydro-6-oxo-4-phenylpyrimidin-2-ylamino)propyl)piperidin-4-yl)-1H benzo[d]imidazol-2(3H)-one (**3**) and 1-(1-(3-(2-amino-6-phenylpyrimidin-4-yloxy)propyl)piperidin-4-yl)-1H-benzo[d]imidazol-2(3H)-one (**4**)*

A mixture of 1-(1-(3-chloropropyl)piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3H)-one (**2**) (0.006 mol), and NaI (0.0066 mol) in acetonitrile was stirred under reflux for 30 min. Then 6-phenylisocitosine (0.06 mol) and anhydrous K_2CO_3 (0.0066 mol) were added. The reaction mixture was stirred under reflux for 12 h. After cooling, the mixture was filtered, concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and the solvent removed *in vacuo*. The crude mixtures were purified by silica gel column chromatography using diethyl ether/methanol 9:1 (v/v) as eluent. The crude products **3** and **4** were recrystallized from diethyl ether.

1-(1-(3-(1,6-dihydro-6-oxo-4-phenylpyrimidin-2-ylamino)propyl)piperidin-4-yl)-1H-benzo[d]imidazol-2(3H)-one (**3**): Yield: 35 %; mp 228-230 °C; ^{1}H NMR (500 MHz, DMSO-d_6, *J* in Hz) δ 1.64 (m, 1H), 1.83 (m, 2H), 2.05 (m, 1H), 2.36 (overlapped, 1H), 2.38 (overlapped, 2H), 3.03 (m, 1H), 3.93 (t, 2H), 4.14 (m, 1H), 6.20 (s, 1H), 6.90 (overlapped, 1H), 6.95 (overlapped, 2H), 7.21 (d, 7.4, 1H), 7.41 (overlapped, 2H), 7.43 (overlapped, 1H), 7.55 (s, 2H, NH_2), 7.95 (m, 1H), 10.80 (s, 1H, NH).^{13}C NMR (125 MHz, DMSO-d_6, signals assigned from HSQC data) δ 24.9, 29.3, 39.6, 50.6, 52.9, 54.4, 97.2, 109.4, 121.1, 127.1, 128.8, 129.5, 130.5, 138.0, 154.9, 157.1, 161.2, 163.7.
ESI-MS: 445.4 $[M+H]^+$.
Anal. ($C_{25}H_{28}N_6O_2$), C, H, N.

1-(1-(3-(2-amino-6-phenylpyrimidin-4-yloxy)propyl)piperidin-4-yl)-1H-benzo[d] imidazol-2(3H)-one (**4**): Yield: 33 %; mp 148-150 °C; ^{1}H NMR (500 MHz, DMSO-d_6, *J* in Hz) δ 1.66 (m, 1H), 1.93 (m, 2H), 2.04 (m, 1H), 2.36 (m, 1H), 2.48 (m, 2H), 3.00 (m, 1H), 4.12 (m, 1H), 4.35 (t, 6.5, 2H), 6.57 (s, 1H), 6.60 (s, 2H, NH_2), 6.90 (overlapped, 1H), 6.95 (overlapped, 2H), 7.21 (d, 7.4, 1H), 7.41 (overlapped, 2H), 7.43 (overlapped, 1H), 7.95 (m, 1H), 10.80 (s, 1H, NH).^{13}C NMR (125 MHz, DMSO-d_6, signals assigned from HSQC data) δ 26.9, 29.2, 50.9, 53.4, 54.9, 64.7, 92.4, 109.4, 121.1, 127.4, 128.9, 130.7, 131.8, 140.5, 156.9, 166.7, 168.0, 174.1.
ESI-MS: 445.4 $[M+H]^+$.
Anal. ($C_{25}H_{28}N_6O_2$), C, H, N.

4.2. In vitro assays

4.2.1. Cell culture

Human colorectal carcinoma cells, or T84 cells, were from the American Type Culture Collection (CCL248) and were grown in Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (Sigma-Aldrich, #D8900) supplemented with 10% FBS (Gibco, #12657029) and penicillin-streptomycin (Gibco, #15140122) in a humidified atmosphere of 95 % air/5 % CO_2 at 37 °C.

4.2.2. cGMP accumulation in cells

T84 cells were grown to confluence in 12-well plates (Santa Cruz Biotechnology, #sc-204444) and washed three times with DPBS (pH 7.4). Cells were treated with vehicle (DPBS with DMSO [Sigma-Aldrich, #D2650], 0.1%, 0.5 mL per well) or compound in DPBS for 10 minutes. Then, cGMP accumulation was stimulated with enterotoxin of *E. coli* (STa, 1 µM [Bachem, #404429]) for 10 min. Medium was aspirated and cGMP was extracted with 0.1 M HCl (0.3 mL per well) and measured by ELISA (acetylation protocol; Cayman Chemical, #581021).

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Conflicts of interest: None reported.

Supplementary data: Supplementary data associated with this article are available from the authors.

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