PRP Pharmacology Research & Perspectives Open Access

ORIGINAL ARTICLE

Therapeutically targeting guanylate cyclase-C: computational modeling of plecanatide, a uroguanylin analog

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Keywords
guanylate Cyclase C, linaclotide, molecular dynamics, plecanatide, uroguanylin

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Funding Information
Financial Support for this study provided by Synergy Pharmaceuticals Inc. We also acknowledge the support of the Life Science Research Network Wales grant # NRNPGSep14008, an initiative funded through the Welsh Government's Ser Cymru program.

Received: 15 August 2016; Revised: 23 November 2016; Accepted: 30 November 2016

Pharma Res Per, 5(2), 2017, e00295, doi: 10.1002/prp2.295

doi: 10.1002/prp2.295

Abstract

Plecanatide is a recently developed guanylate cyclase C (GC C) agonist and the first uroguanylin analog designed to treat chronic idiopathic constipation (CIC) and irritable bowel syndrome with constipation (IBS C). GC C receptors are found across the length of the intestines and are thought to play a key role in fluid regulation and electrolyte balance. Ligands of the GC C receptor include endogenous agonists, uroguanylin and guanylin, as well as diarrheagenic, Escherichia coli heat stable enterotoxins (ST). Plecanatide mimics uroguanylin in its 2 disulfide bond structure and in its ability to activate GC Cs in a pH dependent manner, a feature associated with the presence of acid sensing resi dues (Asp2 and Glu3). Linaclotide, a synthetic analog of STh (a 19 amino acid member of ST family), contains the enterotoxin's key structural elements, including the presence of three disulfide bonds. Linaclotide, like STh, activates GC Cs in a pH independent manner due to the absence of pH sensing residues. In this study, molecular dynamics simulations compared the stability of pleca natide and linaclotide to STh. Three dimensional structures of plecanatide at various protonation states (pH 2.0, 5.0, and 7.0) were simulated with GRO MACS software. Deviations from ideal binding conformations were quantified using root mean square deviation values. Simulations of linaclotide revealed a rigid conformer most similar to STh. Plecanatide simulations retained the flexi ble, pH dependent structure of uroguanylin. The most active conformers of ple canatide were found at pH 5.0, which is the pH found in the proximal small intestine. GC C receptor activation in this region would stimulate intraluminal fluid secretion, potentially relieving symptoms associated with CIC and IBS C.

Abbreviations

CIC, chronic idiopathic constipation; FGID, functional gastrointestinal disorder; GC C, guanylate cyclase C; GI tract, gastrointestinal tract; IBS C, irritable bowel syndrome with constipation; RMSD, root mean square deviation; ST, family of heat stable enterotoxin produced by enterotoxigenic *Escherichia coli* that include STh and STp; STh, 19 amino acid member of ST family.

Introduction

Chronic idiopathic constipation (CIC) and irritable bowel syndrome with constipation (IBS C) are two of the most common conditions affecting the gastrointestinal (GI) tract, creating a burden on healthcare resources and lead ing to significant negative impact on quality of life (Hei delbaugh et al. 2015). These disorders are characterized by diminished stool frequency, straining and abdominal pain (IBS C) or discomfort (CIC). CIC alone affects 14%

of the North American population, is challenging to treat and poses a significant burden on health resources (Suares and Ford 2011).

Guanylate cyclase C (GC C) receptors play a crucial role in the maintenance of normal bowel function and thus have potential as a target for pharmaceutical inter vention in to treat numerous functional gastrointestinal disorders. The GC C receptor is a membrane spanning protein uniformly expressed along the epithelial brush border throughout the intestine (Forte 1999). Recently plecanatide and linaclotide have been developed for the treatment of two of these disorders, CIC and IBS C (Shailubhai et al. 2013).

The GC C receptor is activated by its endogenous pep tides uroguanylin and guanylin that differentially bind to the receptor in the varying pH environments found along the GI tract (Fan et al. 1997). Uroguanylin is primarily expressed and preferentially binds GC C receptors in the slightly acidic (pH 5 6) regions of the duodenum and jejunum (Forte 1999). Guanylin is primarily expressed in the ileum and colon, activating GC C receptors under more basic conditions (pH 7 8) (Kita et al. 1994). Bind ing of uroguanylin or guanylin to the GC C receptor ini tiates a signaling cascade leading to accumulation of intracellular cyclic guanosine monophosphate (cGMP) (Vaandrager et al. 1997), which helps maintain fluid and electrolyte balance, promotes visceral analgesia, and reduces inflammation in the GI tract (Hughes et al. 1978; Pitari 2013; Shailubhai et al. 2015; Hanning et al. 2014).

The overlapping yet distinct activities of guanylin and uroguanylin suggest that the tight and tunable regulation of GC C receptors is essential for proper GI function, a feature that becomes readily apparent by the conse quences of dysfunctional GC C activity (Whitaker et al. 1997). Overactivation of GC C receptors by the *E. coli* enterotoxin (STh) triggers an uncontrolled release of elec trolytes and water into the intestinal lumen resulting in diarrhea (Brierley 2012).

Studies have shown STh to be 10 times more potent than uroguanylin and 100 times more potent than guanylin in binding to GC C receptors (Hamra et al. 1993). The X ray structure of STh reveals that the molecule is locked into a constitutively active, right handed spiral formation stabi lized by three intrachain disulfide bridges in a 1 4/2 5/3 6 pattern (Gariepy et al. 1987; Ozaki et al. 1991; Shimonishi et al. 1987). Unlike STh, the endogenous peptides have only two disulfide bridges which likely results in their improved flexibility; Klodt et al. (1997). The flexibility afforded by absence of a third disulfide bridge allows uroguanylin and guanylin to adopt two topological isoforms (A and B) of which only the A form is biologically active (Marx et al. 1998; Skelton et al. 1994). The pH dependent activity of uroguanylin is linked to two charged acid sensing aspartic acid residues on its N terminus (Hamra et al. 1997). The absence of these residues within STh allows it to bypass pH checkpoints governing GC C receptor activation, allowing for supraphysiological activation of GC C along the length of the small intestine and colon (Hamra et al. 1997).

Pharmacologic agonists that mimic the activity of known GC C agonists have been developed to treat patients with CIC and IBS C. Linaclotide, a synthetic ana log of STh, is available for the treatment of CIC and IBS C (Lembo et al. 2011). Like STh, linaclotide has three disulfide bonds and demonstrates pH independent activa tion of GC C receptors (Fig. 1) (Busby et al. 2010).

Plecanatide is a recently developed GC C agonist and uroguanylin analog that is currently in Phase 3 trials for CIC and IBS C (Synergy Pharmaceuticals Inc 2015a,b). Plecanatide is an orally administered, pH dependent ago nist of the GC C receptor that shares the structural and physiological characteristics of uroguanylin. Plecanatide has two disulfide bonds, similar to uroguanylin, and con tains two acidic N terminal amino acids allowing the two molecules to maintain the same pH dependent binding

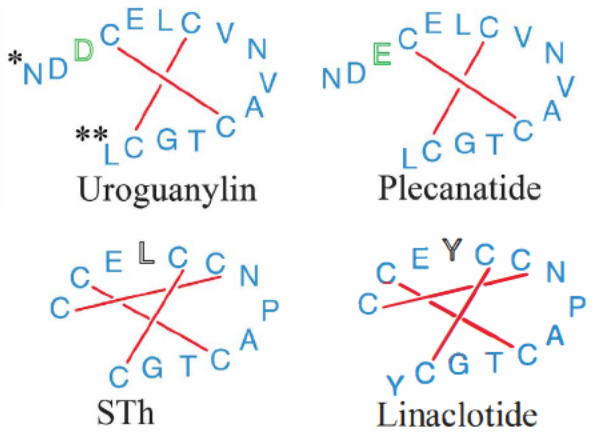

Figure 1. Amino acid structures of guanylate cyclase C receptor agonists examined in this study Synthetic analogs linaclotide and plecanatide share similar amino acid sequences with GC C agonists STh and uroguanylin, respectively. Plecanatide, like uroguanylin contains two pH sensing residues on its N terminus The pH sensing residue (aspartatic acid, D) of uroguanylin is replaced with another pH sensing residue (glutamic acid, E) in plecanatide (green). In this study, the pH sensing aspartic acid and glutamic acid residues of plecanatide were differentially protonated to reflect pH values 2.0 (Asp2, Glu3), 5.0 (Asp2 ,Glu3; Asp2, Glu3) & ≥7 0 (Asp2 , Glu3). Simulations of the crystal structure of STh used a truncated version of the full toxin, comprised of residues 5 17 of the full heat stable enterotoxin protein representing the core bioactive pharmacophore of peptide. (A). STh and linaclotide both lack pH sensing residues on their N terminal ends and are stabilized into a constitutively active conformer by the presence of 3 disulfide bonds. Structurally, the peptides differs by the replacement of leucine (L) in STh with tyrosine (Y*) in linaclotide.

characteristics (Fig. 1) (Shailubhai et al. 2013). Given the experimental challenges of studying the unique character istics of its pH sensitive structure, computational methods were employed to characterize behavior of plecanatide at various pH states.

Molecular dynamics (MD) simulations compared the flexibility and conformation of plecanatide and linaclotide. As expected, linaclotide was shown to adopt rigid confor mations, which do not deviate significantly from the struc ture of STh. In contrast, plecanatide showed greater flexibility and an ability to adopt several conformations which vary in response to pH values (2.0, 5.0 and 7.0). Active plecanatide conformations were more similar to uroguanylin than to the STh peptide, suggesting that pleca natide has a similar pH dependent activity profile as uro guanylin and that plecanatide's activity can be differentially regulated in the GI tract, with higher activity in the more acidic proximal small intestine and lower activity in the more basic distal small intestine and colon.

Materials and Methods

Peptide preparation

The NMR structures of uroguanylin (PDB ID: 1UYA) and the crystal structure of STa (PDB ID: 1ETN) (Fig. S1) were used as starting points for the MD simula tions (Ozaki et al. 1991). The STa family of heat stable peptides includes STh and STp (Nataro and Kaper 1998). The series of experiments used in this study used the STh sequence as reference.

Structural models of plecanatide, linaclotide, and STh were built by appropriately modifying the uroguanylin and STa sequences, respectively, using the builder tools in molec ular operating environment [(MOE 2015.4) www.chemc omp.com]. (Molecular Operating Environment 2015).

Simulations and root mean standard deviation (RMSD) values of linaclotide and each configuration of plecanatide were compared to the enteric pathogen, STh, which was selected as the reference peptide because of its enhanced affinity for the GC C receptor compared to other ago nists. The structural rigidity of STh confers one main conformation that would bind the receptor. Similarities to this one conformer would reflect a drugs ability to activate GC C.

MD simulations

All MD simulations of plecanatide, STh, and linaclotide were performed and analyzed using the GROMACS 4.5 simulation package (Hess et al. 2008). MD simulations of the structure of STh used residues 6 18 of the full heat stable enterotoxin protein (C C E L C C N P A C T G C), considered to be the pharmacophore of the molecule required for maximum biological activity (Ozaki et al. 1991; Yoshimura et al. 1985).

Four protonation states of plecanatide were modeled by placing the appropriate charge on Asp2 and Glu3, reflecting the most abundant species at three pH environ ments: pH 2.0 (Asp2, Glu3), pH 5.0 (Asp2 , Glu3; Asp2, Glu3), and pH 7.0 (Asp2 , Glu3) (Fig. 1 circle). The ini tial structure of each peptide was placed in a cubic box with TIP 3P water and energy minimized using a Steepest Descent Minimization Algorithm. The system was equili brated via a 50 ps MD simulation at 310 K in a NVT canonical environment followed by an additional 50 ps simulation at constant pressure of 1 atm (NPT). After the equilibration phases, a 500 ns MD simulations were per formed at constant temperature (310 K) and pressure with a time step of 2 fs. The system energy and peptide spatial coordinates (trajectory file) were stored every 300 ps for further studies. All MD simulations were run in triplicate for each peptide.

After removing the first 100 ns, considered as system stabilization time, the remaining 400 ns of the MD trajec tory of every single run for each group of triplicate exper iments were combined using the tricat function. The combined trajectories (3999 frames) were examined using the g cluster function, setting gromos as the clustering method with an RMSD cut off of 0.1 nm. The different structural cluster groups were obtained as a pdb file. Cluster groups representing at least 10% of the total pop ulation for each peptide were selected as the most repre sentative structure for that peptide.

RMSD comparisons

The representative cluster conformations of the different peptides were used for the RMSD comparison against the main conformation of the STh cluster.

RMSD comparisons were performed using MOE 2015.4 with the major STh cluster structure serving as a reference for the superimposition of other peptide conformations.

Results

MD clustering

Table 1 shows the results obtained from the structural cluster calculations. From this data it is possible to appre ciate how flexible plecanatide is, compared to STh and Linaclotide. The latter peptides generated more populated cluster than any of the plecanatide forms. RMSD analysis of each MD simulations calculated against the most rep resentative clusters also confirm this observation (Fig. S1).

Table 1. Representative cluster analysis.

Compound and test condition	Cluster size[1]	% On the total frames[2]	Average RMSD (Å) ± SD[3]
STh	2273	56	0.1252 ± 0.0821
Linaclotide	3592	89	0.0715 ± 0.0421
Plecanatide pH>7.0	1108	28	0.1827 ± 0.0862
Plecanatide	524	13	0.3208 ± 0.1325
pH 5.0 (Asp /Glu)[4]	497	12	0.2425 ± 0.1044
	446	11	0.1252 ± 0.1107
Plecanatide pH 5.0 (Asp/Glu)	610	15	0.2885 ± 0.1363
Plecanatide pH<2.0	709	18	0.2115 ± 0.0825

[1]The cluster size refers to the number of frames that are forming the cluster. The total number of frames is 3999.
[2]The % is calculated on the total number of frames.
[3]The average RMSD is calculated against the most representative cluster conformation for each peptide.
[4]Three representative clusters were obtained for this peptide.

MD simulations of STh and linaclotide

In addition to providing information on structure, the simulations also provided information on the degree of internal rigidity and flexibility within the peptide. This can be observed in the MD simulations shown in Figure 2A, in which overlapping snapshots of STh show very little deviation. The fact that the snapshots show a high degree of overlap across all rounds of simulations indicates that STh is a fairly rigid molecule with little internal flexibility. This constrained geometry is thought to be established by the three disulfide bonds of the peptide leading to a structural rigidity and high binding affinity of STh for the GC C receptor (Ozaki et al. 1991).

MD simulations of linaclotide, which differs from the STh peptide used in the simulations by one amino acid substitution and an additional residue at the C terminus, also reveal a rigid molecule that adopts a similar conformation as STh (Fig. 2B). The rigidity of this peptide was also confirmed by a RSM fluctuation analysis of the MD trajectory (Supplemental Information, Fig. S2). Some variability can be seen within the C termini of the peptides, likely due to the extra amino acid in linaclotide compared to STh. Moreover, the conformation of the interaction loops (regions binding the GC C receptor) is homologous between the two molecules (Fig. 2B *) (Ozaki et al. 1991).

MD simulations of plecanatide

MD simulations of plecanatide were conducted by altering the amino acid sequence of the NMR structure of uroguanylin (Marx et al. 1998). Because pH has been shown to alter the ability of uroguanylin to activate GC C receptors, simulations were conducted on the four ionization states of plecanatide's structure, reflecting three different pH values, by altering the protonation states of Asp2 and Glu3 residues. It should be noted that plecanatide contains an additional pH sensitive residue, Glu5. However, unlike Glu3, its side chain is oriented away from the interaction loop and, given its position between the two disulfide bonds, Glu5 does not have the conformational freedom to affect the orientation of the loop itself. Furthermore, this specific residue is highly conserved across the whole range of GC C binding peptides, and includes STh and linaclotide which are not affected by pH variations (Busby et al. 2010). For these reasons, the protonation state of Glu5 should not affect the activity of uroguanylin and plecanatide.

To represent plecanatide at pH 5.0, which corresponds to the pH of the duodenum and proximal jejunum, two

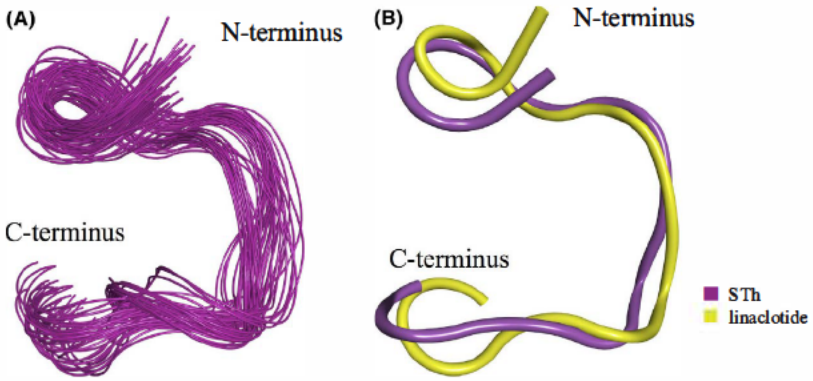

Figure 2. MD simulations of STh and linaclotide. (A) Overlapping snapshots of STh from MD simulations reveal that the peptide adopts a single stable structure with little flexibility. (B) Superimposition of representative structures from the STh and linaclotide. Variations between structures at the C terminus reflects changes in conformation induced by the additional tyrosine of linaclotide. Simulations reveal that linaclotide adopts a similar conformation as STh especially so within the region of the GC C interaction loop (*). MD, Molecular dynamics.

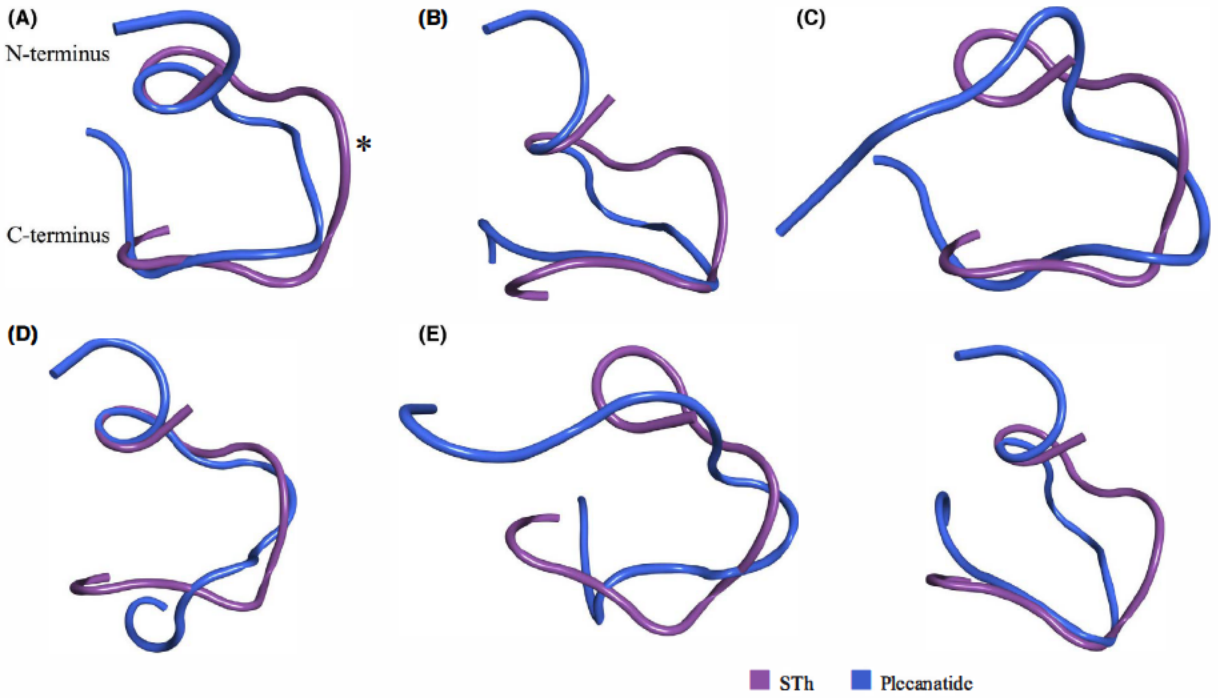

Figure 3. Overlapping snapshots of STh and plecanatide at pH values 5.0 (A D), 2.0 (E) and >7.0 (F) using MD simulations. (A–C) Overlay of the three predominant Asp /Glu conformations of plecanatide and STh at pH 5.0 (RMSD values A: 2.38 Å; B:2.48 Å; C:3 44 Å) (D) The predominant Asp/Glu conformation of plecanatide and STh at pH 5.0 (RMSD value 1.93 Å). The overlapping conformation of the interaction loops (*) in A and D suggest these are active forms of plecanatide able to bind to and stimulate GC C receptors. (E) Plecanatide conformations at pH 2.0 Asp/Glu differ from those of STh with no overlap of interaction loop (RMSD value 3.45 Å). (F) Simulations of the double negative form of plecanatide, Asp-/Glu , representing the protonation state at pH > 7.0, reveal a single plecanatide structure that has a minimal overlap of the interaction loop (RMSD value 2.54 Å). MD, Molecular dynamics

protonation configurations were analyzed. In one, Asp2 is protonated (Asp/Glu), whereas in the other, Glu3 is pro tonated (Asp /Glu). These ionization states were based on the consideration that, as these residues are on the flexible N terminus and exposed to solvent, their pKa values would be between 3.5 and 4.5 (values dependent on the input peptide conformation as calculated on http://bio physics.cs.vt.edu/H++, version 3.2) (Anandakrishnan et al. 2012); hence, the monoprotonated states would likely be present at pH 5.0. Simulations of the two protonation states indicate that plecanatide is flexible at this pH and can adopt several conformations. Figures 3A–C show an overlay of the three predominant Asp /Glu conformations of plecanatide and STh, and Figure 3D shows the pre dominant Asp/Glu conformation of plecanatide and STh. In two of these structures (Fig. 3A and D), the interaction loop (*) of plecanatide overlays well with that of STh, indicating that these two conformations of plecanatide are capable of binding to and subsequently activating GC C receptors.

Simulations of the double protonated form of pleca natide (Asp/Glu) were conducted to assess the structure and dynamics of the peptide at pH 2.0. The plecanatide conformations observed at this pH differ from those of STh (Fig. 3E). Based on these results, plecanatide is unli kely to adopt a conformation capable of binding to GC C at this highly acidic pH level, a feature which would mimic uroguanylin's inability to activate GC C receptors at this pH.

Simulations of the double negative form of plecanatide (Asp /Glu), which represent the protonation state observed at pH > 7.0, reveal a single predominant pleca natide structure (Fig. 3F). The portion of the peptide that interacts with the GC C receptor adopts a different con formation in plecanatide than in STh indicating dimin ished activity of the molecule at this pH value.

Interestingly, in the Asp/Glu ionization state of pleca natide at pH 5, an interaction occurred between the negatively charged acidic side chain of Glu3 residue in the N terminus and the positively charged side chain of Asn9 in the interaction loop (Fig. 4). This interaction between the Glu3 residue of the N terminus and the Asn9 residue of the interaction loop seems to stabilize pleca natide in its most active conformation at pH 5. This

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